POLYTECHNIC INSTITUTE OF PORTO
SCHOOL OF ALLIED HEALTH SCIENCES

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Proteomic Analysis of the Influence of the Adipocyte Secretome on Glioma Gl261 Cells

Master in Biochemical Technology in Health
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Polytechnic Institute of Porto

School of Allied Health Sciences

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A dissertation for the obtention of the degree of Master in Biochemistry in Health Technology, under the supervision of Prof. Dr. Rúben Fernandes and co-supervision of Dr. Carlo Sala and MSc. Joana Almeida. The outcomes of the present work were partially submitted for scientific divulgation as a paper.

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Abstract

Glioma is the most frequent form of malignant brain tumor in the adults and childhood. There is a global tendency toward a higher incidence of gliomas in highly developed and industrialized countries. Simultaneously obesity is reaching epidemic proportions in such developed countries. It has been highly accepted that obesity may play an important role in the biology of several types of cancer. We have developed an *in vitro* method for the understanding of the influence of obesity on glioma mouse cells (Gl261).

3T3-L1 mouse pre-adipocytes were induced to the maturity. The conditioned medium was harvested and used into the Gl261 cultures. Using two-dimension electrophoresis it was analyzed the proteome content of Gl261 in the presence of conditioned medium (CGl) and in its absence (NCGl). The differently expressed spots were collected and analyzed by means of mass spectroscopy (MALDI-TOF-MS).

Significantly expression pattern changes were observed in eleven proteins and enzymes. RFC1, KIF5C, ANXA2, N-RAP, RACK1 and citrate synthase were overexpressed or only present in the CGl. Contrariwise, STI1, hnRNPs and phosphoglycerate kinase 1 were significantly underexpressed in CGl. Aldose reductase and carbonic anhydrase were expressed only in NCGl.

Our results show that obesity remodels the physiological and metabolic behavior of glioma cancer cells. Also, proteins found differently expressed are implicated in several signaling pathways that control matrix remodeling, proliferation, progression, migration and invasion. In general our results support the idea that obesity may increase glioma malignancy, however, some interesting paradox finding were also reported and discussed.

Key words: Glioma, Cancer, Adipose tissue, Obesity, Proteomics, 2D, Mass spectroscopy

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Abbreviation Index

2D-PAGE – Two-dimensional polyacrylamide gel electrophoresis

AKT – also known as Protein Kinase B (PKB)

ANXA2 – Annexin A2

ATP – Adenosine triphosphate

BMI – Body Mass Index

CGl – Conditioned glioma cells

CNS – Central Nervous System

DCM – Dilated cardiomyopathy

DMEM – Dulbecco´s Modified Eagle´s Medium

DNA – Deoxyribonucleic acid

DTT – Dithiothreitol

ERK – Extracellular signal-regulated kinases

FBS – Fetal bovine serum

HCC – Hepatocellular carcinoma

HIF-1α – Hypoxia inducible factor-1α

hnRNPs – Heterogeneous nuclear ribonucleoprotein complexes

HOP – Hsp70-Hsp90 organizing Protein

HPV – Human papillomavirus

Hsp – Heat shock proteins

IAA – Iodoacetamide

IARC – International Agency for Research into Cancer

IL – Interleukin

KIF5C – Kinesin heavy chain isoform 5C

MALDI-TOF-MS – Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry

Mr – Relative molecular masses

mRNA – Messenger Ribonucleic Acid

N-RAP – Nebulin-related-anchoring protein

NADPH – Nicotinamide adenine dinucleotide phosphate-oxidase

NCGl – Non-conditioned glioma cells

PCNA – Proliferating Cell Nuclear Antigen

PGK1 – Phosphoglycerate Kinase 1

pI – Isoelectric point

RACK1 – Receptor for activated C kinase 1

RFC1 – Replication factor C subunit 1

ROS – Reactive oxygen species

STI1 – Stress-induced phosphoprotein 1

TCA – Tricarboxylic acid

TFA – Trifluoroacetic

TNF-α – Tumor Necrosis Factor α

VEGF – Vascular Endothelial Growth Factor

WAT – White Adipose Tissue

WCRF – World Cancer Research Fund

WHO – World Health Organization

INTRODUCTION

INTRODUCTION

CENTRAL NERVOUS SYSTEM TUMORS

A central nervous system (CNS) tumor begins when normal cells in the brain or the spinal cord change and grow uncontrollably, forming a mass. Several different types of tumors, benign and malignant, have been identified in the CNS. They often form in different areas, develop from different cell types, and may have a different outlook and treatment. The prognoses for these tumors are related to several factors, such as the age of the patient, the location and histology of the tumor.

Brain and spinal cord tumors are different in adults and children. In adults, about half of all CNS tumors are malignant, whereas in pediatric patients, more than 75% are malignant. Malignant primary brain tumors are the leading cause of death from solid tumors in children and the third leading cause of death from cancer in adolescents and adults aged 15 to 34 years.

Common presenting symptoms include headache, seizures, nausea, vomiting, neurocognitive symptoms, personality changes and altered mental status. For most benign CNS tumors that require treatment, neurosurgeons can offer curative resections or at least provide significant relief from mass effect. There are not known environmental factors associated with brain tumors however mutations and deletions of so-called tumor suppressor genes are thought to be the cause of some forms of CNS tumors.

The lack of effective treatments for most primary and secondary malignant CNS tumors is still a reality. However, the past decade has witnessed an explosion in the understanding of the early molecular events in malignant primary CNS tumors, and for the first time in history, oncologists are seeing that a plethora of new therapies targeting these molecular events are being tested in clinical trials. There is hope on the horizon for the fight against these deadly tumors (1–3).

GLIOMA

Glioma is a broad category of brain and spinal cord tumors and it is called a glioma because it comes from glial cells. Malignant glioma is the most common subtype of brain cancer in the adult and pediatric populations and has been subject of increasingly research over the past two decades that led to considerable advances in the understanding of their basic biology and pathogenesis (4,5).

Malignant gliomas are histologically heterogeneous and invasive. A number of studies have investigated molecular subclasses in Gliomas. There are different grading systems in use, the most common is the World Health Organization (WHO) grading system that distinguishes glioma according to their constitutive cells, such as astrocytomas (glioblastoma), oligodendroglioma and oligoastrocytoma. The astrocytomas can be classified according to histological features in four different grades of gliomas based on the pathologic evaluation of the tumor. According to this grading system, tumours are graded from I (least advanced disease - best prognosis) to IV (most advanced disease - worst prognosis). Low-grade gliomas, WHO grade II, are well-differentiated (these are not benign but still portend a better prognosis for the patient). High-grade, WHO grade III-IV, gliomas are undifferentiated (these are malignant and carry a worse prognosis – glioblastoma). Grade I tumors are biologically benign and can be cured if they can be surgically resected; grade II tumors are low-grade malignancies that may follow long clinical courses, but early diffuse infiltration of the surrounding brain renders them incurable by surgery; grade III tumors exhibit increased anaplasia and proliferation over grade II tumours and are more rapidly fatal; grade IV tumours exhibit more advanced features of malignancy, including vascular proliferation and necrosis, and as they are recalcitrant to radio/chemotherapy they are generally lethal within 12 month (4,6–8).

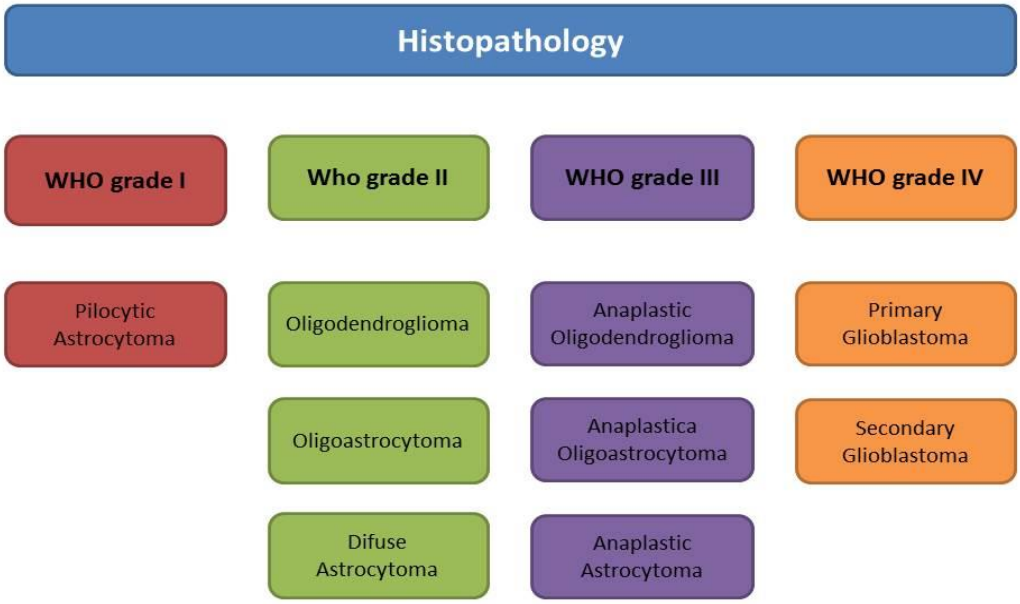


Figure 1 – World Health Organization (WHO) grading system for histological features.

Prognosis is extremely poor, with a median survival time of approximately 12 to 15 months and is almost invariably fatal (6). This tumor represents about 12-15% of all primary brain tumors and about 60-75% of all astrocytomas. Gliomas increase in frequency with age, and affect more men than women. Only 3% of childhood brain tumors are glioblastomas (6,8,9).

Glioblastoma is the primary type brain tumor and is the most common and aggressive malignant glioma in humans. Representing up to 50% of all primary brain gliomas, accounts for approximately 12-15% of all brain tumors and median survival is less than 15 months and overall survival is less than 10% at 5 years. These tumors are usually highly malignant, fatal and extremely invasive because of the fast cellular reproduction supported by a large network of blood vessels, contributing to the poorer prognosis of these patients. Glioblastoma contain so many different types of cells, usually penetrates deep into the brain, making the tumor difficult to remove for surgery. Some cells may respond well to certain therapies, while others may not be affected at all. This is why the treatment plan for glioblastoma may combine several approaches. These tumors also can affect parts of the brain that control speech, vision or motor functions. Treatment can involve chemotherapy, radiation, radiosurgery, corticosteroids,

antiangiogenic therapy, surgery and experimental approaches such as gene transfer (9–12).

Nowadays, the management of patients with glioblastoma continues to harbor significant challenges, and comprehensive genetic screens of tumor tissues and signaling pathways have been explored to develop molecular based targeted therapies. Expression profiling studies have revealed that molecular classification of gliomas may be of significant prognostic value.

LINKING OBESITY AND CANCER

In 2001, different studies from the International Agency for Research into Cancer (IARC) and the World Cancer Research Fund (WCRF) have reported a relationship and established a link between obesity and cancer risk (13,14). Excess adiposity is related with an increase the incidence and/or death rates from a wide variety of human cancers, being the most commons colon, rectum, esophagus, kidney, pancreas, gallbladder, ovary, cervix, liver, prostate and certain hematopoietic cancers (13–17). Excess adiposity is related with an increase the incidence and/or death rates from a wide variety of human cancers (18,19). It is important to understand the pathophysiological mechanisms that play a role in the link between obesity and cancer focusing on future preventive and therapeutic strategies for cancer (18).

The relationship between the increase and dysfunction of adipose tissue might thus be a possible cause of cancer. Obesity and accelerated weight gain, in adults, are associated with increasing incidence of all central nervous system tumors, mostly glioma. At the present time, it is not clear which factors might be involved in this relationship but energy balance during childhood and/or adolescence may play a role in the etiology of adult-onset glioma. Genes that influence obesity are highly expressed in the brain and could also mediate glioma susceptibility (5,20).

Adipose tissue is also recognized by its endocrine function as producer of biological mediators important to the body's physiology (18,19). Thus, its dysfunction results in altered serum levels of adipokines, and some of these hormones and growth factors, secreted by adipocytes, appear to be associated with carcinogenesis (13,18). The suggested mechanisms underlying these associations namely include biological pathways linking energy balance and cancer risk, should be more closely investigated.

OBESITY AS A DISEASE RISK FACTOR

The concept of energy homeostasis consists of the exact equilibrium between caloric intake and energy utilization. Energy expenditure takes the form of physical activity, which refers to all voluntary movement, basal metabolism which includes the biochemical processes necessary in life, and adaptive thermogenesis (15). The development of obesity occurs when caloric intake routinely exceeds caloric expenditure over a prolonged time leading to the increased body mass including the accumulation of subcutaneous and visceral fat, promotes weight gain (15–17). Obesity is defined as an abnormal or excessive fat accumulation, this is, an increased storage of fatty acids in an expanded adipose tissue mass (15,21).

During the past several decades, the excess adiposity in the population is an unintentional consequence of the economic, social, and technological advances. Foods low in cost, abundant and palatable with high caloric density are readily available in prepackaged forms and in fast-food restaurants. Labor-saving technologies and widespread availability of electronic devices in homes have greatly reduced the amount of physical activity that used to be part of daily life and promoted a sedentary life style. Considering genetic and environmental factors to the etiology of obesity, studies have concluded that about 30% to 40% of the variance in body mass index (BMI) can be attributed to the genetics factor and 60% to 70% to the environment. The interaction between genetics and environment is also important being that some people already genetically predisposed to develop obesity only express this genotype if exposed to certain adverse environmental conditions, such as high-fat diets and sedentary lifestyle (16,17). Obesity is a complex disease caused by different factors such as genetic, diet, lifestyle and environmental factors (15).

The World Health Organization (WHO) estimates that worldwide there are approximately 1.6 billion individuals aged 15 years and older are overweight (BMI $\geq$ 25 kg/m2), 400 million of whom are obese (BMI $\geq$ 30kg/m2) (13,15). In the United States 24.2% of men and 23.5% of women were obese in 2005, and 21.9% of men and 24.4% of women were obese in the United Kingdom in 2007 (13).

Obesity is a major cause of morbidity and mortality and is associated with an increased risk for many disorders including hypertension, dyslipidemia, insulin resistance and type II diabetes mellitus, atherosclerosis, described altogether as metabolic syndrome, as well as different kinds of cancer (13,15–17,21).

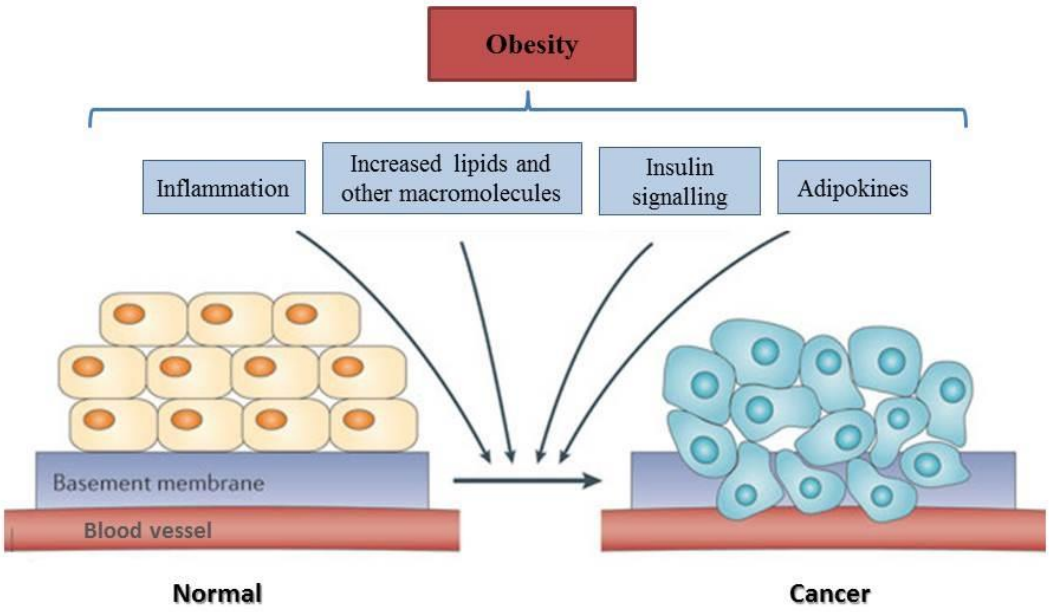


Figure 2 – Summary of pathways that may link obesity to cancer development. A convergence of increased inflammation, insulin signalling, increased availability of lipids and other macromolecules, and changes in adipokine signalling may contribute to the conversion of normal epithelial cells to an invasive tumour. Although all of these pathways can contribute to cancer in certain circumstances, it remains unclear whether these pathways are predominantly required for cancer in obese humans. Adapted from Khandekar *et al*. 2011.

Different studies from the International Agency for Research into Cancer (IARC) in 2001 and the World Cancer Research Fund (WCRF) have observed a relationship and established a link between obesity and cancer risk (13). The risk of endometrial cancer and breast cancer after menopause is higher in obese women. Excessive adiposity may increase the incidence and/or death rates from a wide variety of human cancers, being the most common colon and rectum, esophagus, kidney, pancreas, gallbladder, ovary, cervix, liver, prostate, and certain hematopoietic cancers (13,15–17).

While public health policies aim the reduction of obesity epidemy and implementation of strategies for this problem, there is a simultaneous need to better understand the biological processes linking obesity and cancer as a precondition to the development of new approaches to prevention and treatment (13).

ADIPOSE TISSUE – A MAJOR PLAYER IN METABOLISM

In most mammalian, adipose tissue is classified according molecular and histological characteristics in white adipose tissue (WAT) and brown adipose tissue. The first is most abundant and the second plays an important role in thermogenesis, only found in humans during childhood when participating in maintaining body heat. The WAT acts as a reserve of energy, states that during caloric deprivation can be mobilized by releasing free fatty acids will be oxidized in other tissues and organs. This tissue promotes thermal isolation and plays an important role in the regulation of glucose homeostasis (22–25).

The adipose organ is made up of adipocytes, vascular tissue and immune cells. Adipose tissue beyond the adipocytes (most abundant cells) also has several other cell types such preadipocytes, stromal cells, fibroblasts, leucocytes and macrophages (22–27). Adipocytes vesicles have more than 90% of fat occupation of the total cell volume, which in addition to functioning as energy deposits also show high amounts lipolytic activity releasing free fatty acids into plasma as needed (24).

Contrary to what was thought for many years, adipose tissue is not just an "energy storage" of triglycerides but also an endocrine organ affecting immunological processes and metabolism of the body. Adipose tissue secrets a number of bioactive mediators referred to as adipocytokines, which may be hormones, enzymes and growth factors. Such substances have an auto and paracrynic effect on adipocytes production, although they also have an endocrynic effect on other tissues and organs. They regulate energy homeostasis within the organism by directing both energy intake and expenditure (22–27).

However, it was only in 1994 with the discovery of leptin, the first hormone produced by adipocytes to be identified that led to the acceptance of adipose tissue as an endocrine organ (23). After the discovery of leptin several other hormones produced by adipocytes were found such as adiponectin, angiotensin, resistin and visfatin and addition of various cytokines such as Tumor Necrosis Factor α (TNF α), interleukin 1 (IL-1), IL-6, IL-8 and growth factors such as Vascular Endothelial Growth Factor (VEGF), which participate in inflammation (24–26). Adipose tissues dysfunction results

in altered serum levels of adipokines, and some of these hormones and growth factors secreted by adipocytes appear to be associated with carcinogenesis (13,18). The inflammation, insulin resistance and angiogenesis may be a better qualitative indicator of the potential carcinogenesis of obesity (18,19). Angiogenesis is a critical step during carcinogenesis and VEGF represents a good biomarker in cancer studies. The major causes of therapeutic resistance in glioma cancer it seems to be a formation of abnormal tumor vasculature and glioma cells invasion (28,29).

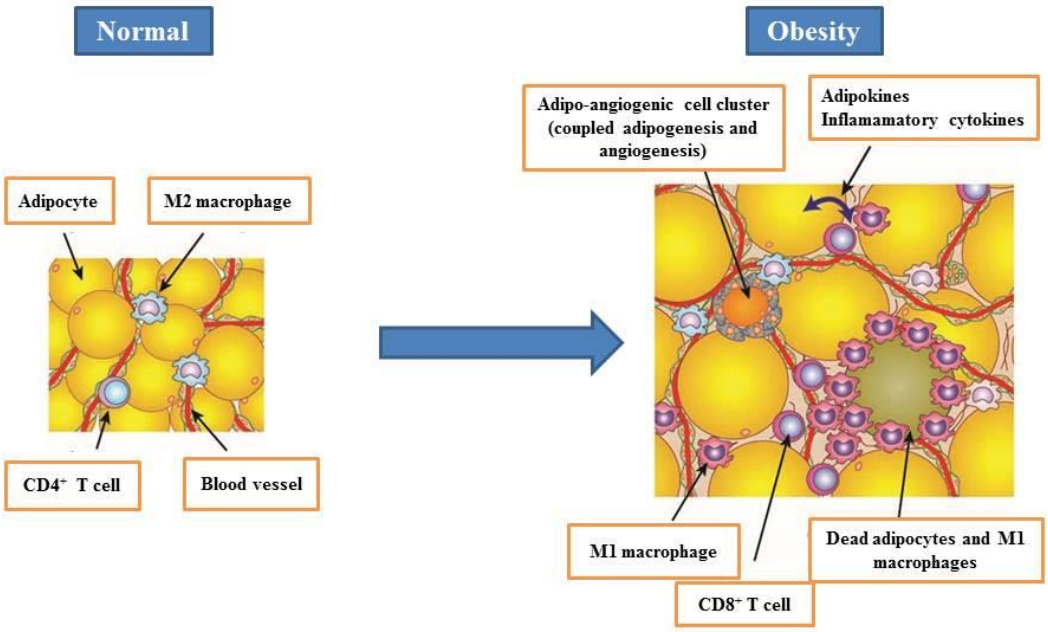


Figure 3 – Adipose tissue composition. Adipose tissue obesity is a chronic inflammatory disease and induces inflammatory cascades, including accumulation of immune cells, activation of leukocyte-endothelial interaction, coupled angiogenesis and adipogenesis, and adipocyte cell death. Adapted from Nishimura *et al.* 2011.

ADIPOSE TISSUE AS AN ENDOCRINE ORGAN

LEPTIN

Leptin was the first adipokine to be discovered. Leptin is encoded by the *ob* gene and acts at the level of the hypothalamus which regulates energy homeostasis and body weight, producing satiety signals, thereby suppressing food intake and stimulating energy expenditure. Obese individuals have higher leptin levels. In common forms of obesity are observed an increase in leptin levels associated with leptin resistance, particularly the impact anorexigenics core, for reasons not yet clarified (18,22–24,30–32).

This hormone has several other functions including regulation hematopoietic, and angiogenic also acts as a mediator in immune and inflammatory processes. Leptin is a pleiotrophic hormone in relation to the link between obesity and cancer. Being mitogenic for various cell types and it is antiapoptotic and proangiogenic in itself, also in synergy with vascular endothelial growth factor (VEGF) and a potent proinflammatory agent. Leptin belongs to the family of adipokines since the molecular structure is homologous to the family of cytokines (13,18,23,24,26,30).

ADIPONECTIN

Adiponectin is the most abundant adipokine and is exclusively produced in the adipose tissue. Is a polypeptide hormone with a molecular weight 30kDa consisting of 244 amino acids containing an amine group (N) following signal terminal. Adiponectin is found in plasma in two forms, long form (t-adiponectin) and short form or globular fragment (g-adiponectin) (13,18,30–32).

Adiponectin works differently from the other adipokines in almost all of its biological properties and effects. This hormone plays an important role in the regulation of energy balance and glucose homeostasis, working as antidiabetic, anti-inflammatory,

antiatherogenic, proapoptotic and antiangiogenic, by increasing insulin sensitivity. Adiponectin provides indirect protection against carcinogenesis. The levels of plasma adiponectin are decreased in some pathological conditions such as obesity, insulin resistance and cancer (13,18,23,26,30–32).

RESISTIN

Resistin is a protein composed of 114 amino acids, with 12.5 kDa being encoded by the gene RETN. The hormone is synthesized by adipocytes, in mice, and in adipocytes, myocytes, particularly in pancreatic cells and mononuclear cells (macrophages) in humans. The plasma levels are highly correlated with the adipose tissue mass (26,30,31).

Several studies have shown that levels of resistin are increased in obesity in humans and rodents. Like other adipokines, resistin plays a significant role in energy homeostasis, for thermogenesis and insulin resistance. The human resistin appears functions to perform pro-inflammatory since it promotes the synthesis of pro-inflammatory cytokines such as TNF, IL-1, IL-6 and IL-12 (30,31).

INTERLEUKIN 6

Adipose tissue is the primary site of production of Interleukin 6 (IL-6), cytokine which can directly cause inflammation (21,26,30,32). The plasma concentration of IL-6 correlates positively with the amount of adipose tissue, obesity and insulin resistance, while it is inversely related to insulin sensitivity (21,31,32).

Participates in immune regulation, the regulation of cellular functions such as proliferation, apoptosis, angiogenesis and cell differentiation, acts as a regulator of lipid and protein metabolism in adipose tissue and modulates the hypothalamic-pituitary-adrenal (18,21,30).

TNF-α

The tumor necrosis factor α (TNF-α) is a pro-inflammatory cytokine which has been described with capacity to promote tumor necrosis. This factor is produced by adipose tissue, mostly by resident macrophages. This factor is secreted by monocytes and macrophages by binding to receptors on neutrophils promoting response chemotactic. TNF-α is also expressed in adipocytes of adipose tissue, predominantly in subcutaneous adipose tissue and is related to the mass total fat. Circulating levels of this factor are increased in obesity and decrease significantly after weight loss.

VEGF AND ANGIOGENESIS

The vascular endothelial growth factor (VEGF) is a mediator that has molecular weight of 46 kDa and their plasma concentrations are positively correlated with the amount of visceral adipose tissue (33). Also performs functions mitogenic stimulating cell migration, angiogenesis and vascular permeability. It has been shown that VEGF plays an important role in vascularization of malignant solid tumors facilitating cell invasion and the formation of distant metastases (18,33).

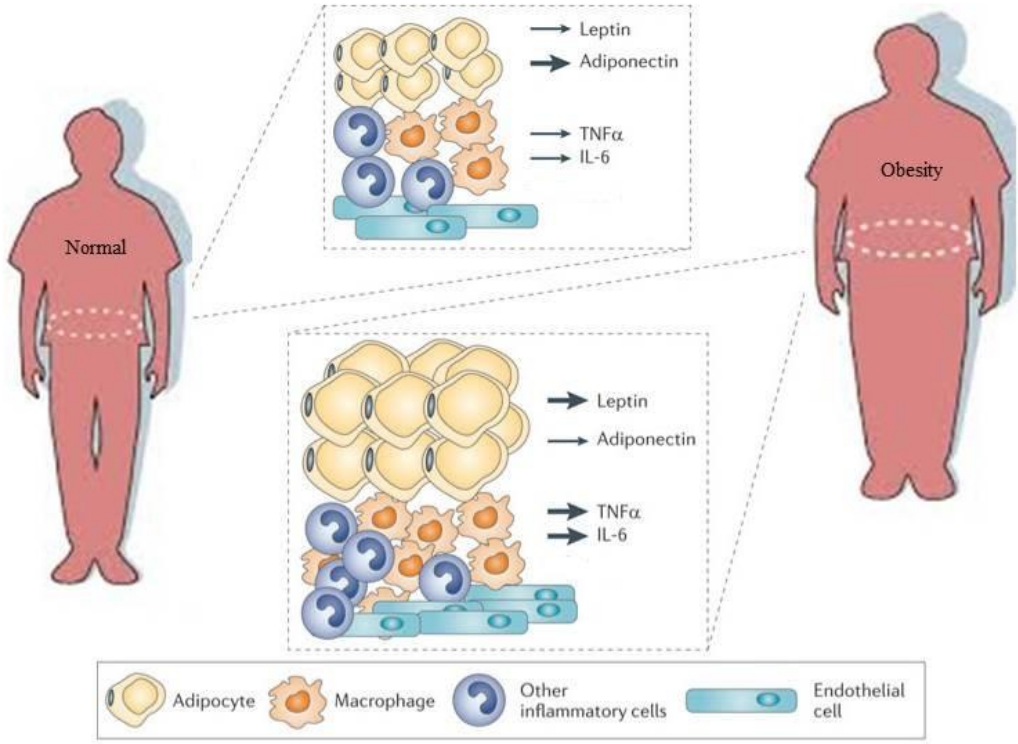


Figure 4 – Changes in adipose tissue in obesity. In the obese state, there is an increase in the size and number of adipocytes, as well as increases in the inflammatory and endothelial compartments of the stromal-vascular fraction. The release of most adipokines is elevated in the state of obesity. This is also true for the metabolically beneficial hormone leptin. However, leptin signaling is concomitantly impaired, making it an unattractive target for the treatment of obesity. One exception from generally elevated adipokine levels is adiponectin, whose secretion is diminished in obesity. Inflammatory cytokines (IL-6 and TNFα, are highly upregulated in adipose tissue of obese subjects. Adapted from Khandekar et al. 2011.

PROTEOMICS

Proteomics is a large-scale comprehensive study of a specific proteome, including information on protein abundances, their variations and modifications, along with their interacting partners and networks, in order to understand cellular processes.

The greatest promise for the detection and treatment of cancer lies in the deep understanding of molecular basis for disease initiation, progression and efficacious treatment based on the discovery of unique biomarkers. Although progress in cancer genomics has been rapid during the past few years, it only provides us with a glimpse of what may occur as dictated by the genetic code. In reality, we still need to measure what is happening in a patient in real time, which means finding tell-tale proteins that provide insight into the biological processes of cancer development. This is because genes are only the "recipes" of the cell, while the proteins encoded by the genes are ultimately the functional players that drive both normal and disease physiology (7,34,35).

POTENTIAL BENEFIT OF GLIOMA PROTEOMICS

The accessibility of cancer-related proteins in malignant glioma has triggered extensive protein-focused research for the hunt of biomarkers. In addition to the urgent need for novel efficient drug targets against malignant gliomas, the identification of glioma biomarkers will provide a much needed contribution to diagnosis, treatment decision, prognosis and assessment of treatment response. Current diagnosis is based on classical histopathological examination, a challenging task considering the heterogeneity of the disease and the inherent subjective nature of histopathological grading. Proteomics has the ability to interrogate a variety of biospecimens for their protein contents and accurately measure the concentrations of these proteins. This can provide scientists and clinicians with a powerful tool to understand the different processes involved in cancer development and progression in hope to identify biomarkers specific for these cellular processes along with those indicating efficacious therapeutic intervention (7,34,35).

OBJECTIVES

The present study, aims to develop an *in vitro* rodent model for the study of the influence of obesity role in glioma. We propose a model in which cell line GL261 a mouse glioma is cultured in the presence or absence of a conditioned medium of 3T3-L1 mature adipocytes. The 3T3-L1 pre-adipocytes were differentiated under controlled experiments. We have used the mature adipocytes secreted adipokines (secretome) and enriched the GL261 medium, followed by analysis of the proteins on GL261 on a 2-dimensional proteomic gel approach, so different spots could be studied by means of mass spectroscopy. The major findings will be explored in the present work.

Specific objectives

- Preparing 3T3-L1 conditioned medium on Gl-261 growth.
- Perform bidimensional electrophoresis (2D) in the whole Gl-261 cell protein content with and without 3T3-L1 conditioned medium.
- Analysis of the 2D gels.
- Understand the differences between the glioma cells Gl-261 under the effect of the conditioned medium in relation to those who grown without that influence.

MATERIALS AND METHODS

MATERIALS AND METHODS

CHEMICALS

The reagents acetonitrile, iodoacetamide (IAA), dl-dithiothreitol (DTT) (99% w/w) and trypsin from porcine pancreas (proteomics grade) were purchased from Sigma (Steinheim, Germany). Formic acid puriss for mass spectrometry (≥98%), ammonium bicarbonate (>99.5% w/w) and the matrix assisted laser desorption ionization MALDI matrix α-Cyano-4-hydroxycinnamic acid (α-CHCA) puriss for MALDI-MS were from Fluka (Buchs, Switzerland). Trifluoroacetic acid (TFA, 99% v/v) was from Riedel-de-Haën (Seelze, Germany). ProteoMass Peptide MALDI-MS Calibration Kit (MSCAL2) from Sigma was used as mass calibration standard for MALDI-TOF-MS.

CELL CULTURE

For the current work it were used the mouse cell lines 3T3-L1 (purchased from American Type Culture Collection) and GL-261 (gently given by Prof. Pedroso Lima, CNC, Coimbra, Portugal).

The Mouse pre-adipocyte cell line 3T3-L1 and glioma cell line GL-261 were cultured in DMEM (Dulbecco´s Modified Eagle´s Medium with 4.5g/L Glucose & L-Glutamine, without Sodium Pyruvate. Santa Cruz Biotechnology, Inc.), supplemented with 10% FBS (Fetal bovine serum), and 1000 units/mL gentamycin solution, maintained in T-25 tissue culture flasks in 5% CO_2/95% air at 37^{o}C in a humidified incubator.

ADIPOCYTE DIFFERENTIATION AND CONDITIONED MEDIUM COLLECTION

3T3-L1 pre-adipocytes were propagated and allowed to reach confluence. After 2 days (day 0), the differentiation was initiated by addition of a hormonal mixture composed of 2 µM insulin, 1 µM dexamethasone and 0.25 mM isobutylmethylxanthine. Three days later (day 3), the induction medium was replaced by complete medium supplemented with insulin only. At day 6 cultures were washed twice in phosphate buffered saline and incubated in serum-free medium. After 24h (day 7), medium was harvested from the adipocytes cultures, spun for 3000 g for 5 minutes and the supernatant (mature adipocytes conditioned medium) was stored at -80ºC for the subsequent treatments.

Afterwards, glioma cells were divided in two distinct groups. On one hand, glioma cells GL-261 were grown under the influence of the mature adipocytes secretome were designated conditioned glioma cells (CGl). On the other hand, the control group was designated as non-conditioned glioma cells (NCGl).

SAMPLE PREPARATION FOR 2D-PAGE

Sample preparation for 2D-PAGE was performed according to published procedures with minor modifications (36). The cells were manual detached from the flask and were mechanically lysed at 4^{o}C in lysis solution [CHAPS 4%, 5 mM Tris, pH 8.8, 0.05% protease inhibitor cocktail (Sigma)] using a glass potter, and the samples were centrifuged at 2000 g for 15 minutes at 4^{o}C in order to eliminate aggregates. The protein concentrations in each sample were measured by means of a DC Bio-Rad assay. A total of 900 mg of protein from each sample was precipitated with cold acetone and resuspended in thiourea buffer (7M urea, 2M thiourea 2% CHAPS, 2% ASB14, 5% glycerol, 40 mM DTT, 4 mM TCEP, 1% 3–10 IPG buffer, Amersham), and the samples were mixed overnight at 4^{o}C in the dark and clarified by centrifugation at 16000 g for 15 minutes at 4^{o}C. The supernatant was first separated by isoelectric focusing over a pH range of 3–10 using precast first-dimension drystrip 3–10 NL 11 cm (Bio-Rad)

following a multi-step protocol for 90,000 Vht (Protean IEF cell, Biorad). The first dimension strip was equilibrated in 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 2% SDS plus 16 mM DTT for 20 minutes, and then plus 25 mM iodioacetamide for 15 minutes, and loaded on a midi format (11 cm) 9–16% acrylamide gel to separate the proteins by molecular weight. Second-dimension runs were performed using Biorad midi cells at 30 V 1 h, 300 V 4 h at a constant temperature of 18^{o}C. Protein spots were revealed using home-made blue Coomassie staining, and the gel images were acquired by means of an Image scanner at 300 DPI resolution and analyzed using Image2D Master Platinum software (both from Amersham).

At least three gels per condition were included in the analysis. Normalized spot volume values were studied using SPSS software version 13.0 for statistical analysis (SPSS Inc.). In brief, the data for each spot match set were analyzed using a box-plot test in order to eliminate outliers, a K-S test to check normal distribution, an F test to analyze the variance, and finally Student's t test to compare the mean values, which were considered significant when p 0.01.

IN-GEL PROTEIN DIGESTION

In-gel digestion of the excised proteins with trypsin was performed according to published procedures with minor modifications (37–39). Protein spots were destained for 10 min with a solution of acetonitrile 50% (v/v) in 25nM NH_4HCO_3 and mixed vigorously using a vortex. After removal of the destaining solution, gel pieces were washed with water (100 µL) for 10 min using vigorous vortexing. This step was repeated twice. Afterwards, the gel pieces were dehydrated in acetonitrile (100 µL) for 10 min, which was then removed and the gel pieces were placed to dry in a vacuum centrifuge. Reduction of protein disulfide bonds was performed with a solution 10mM of dithiotreitol (DTT) in 25mM NH_4HCO_3 (25µL), for 15 min at 60 ºC. After cooling to room temperature for about 15 min, the DTT solution was replaced with a solution 110mM of iodoacetamide (IAA) in 25mM NH_4HCO_3 (20µL). Incubation with the alkylation agent was performed for 35 min at room temperature in the dark. After reduction and alkylation steps, the gel pieces were submitted once more to the washing

and dehydration procedure with water and acetonitrile, in the same way as described above. Subsequently, the gel pieces were completely dried in a vacuum centrifuge. The dried protein spots were incubated with a solution of trypsin 25 ng/µL in 12.5 mM NH_4HCO_3 (15µL) in an ice bath for 30 min to rehydrate the gel and to allow enzyme penetration into it. In-gel protein digestion was performed at 37ºC overnight. After collecting the supernatant to clean vials, further peptide extraction was performed by addiction of a solution of trifluoroacetic acid 0.1% (v/v) in acetonitrile 50% (v/v) (25 µL) to the gel pieces and incubation at room temperature with shaking for 10 min. This step was repeated twice. All extracts were pooled and evaporated to dryness. The samples were re-suspended with 10 µL of trifluoroacetic acid 0.1% (v/v).

MALDI-TOF-MS ANALYSIS

Prior to MALDI analysis, the sample was mixed with an equal volume of the MALDI matrix solution, 10 mg/ml α–CHCA in trifluoroacetic acid 0.1% (v/v) and acetonitrile 50% (v/v). An aliquot of the sample/matrix solution (0.5 µL) was hand-spotted onto the MALDI sample plate and the sample was allowed to dry. The mass spectrometric analyses were performed using the Applied Biosystems MALDI-TOF-MS system model Voyager-DE PRO Biospectrometry Workstation equipped with a nitrogen laser radiating at 337 nm (Applied Biosystems, Foster City, USA) and the laser intensity was set just above the threshold for ion production. MALDI mass spectra were acquired in positive ion reflectron mode, with an accelerating voltage of 20 kV, a grid voltage of 15 kV, 0.4 V of guide wire and an ion extraction delay of 100 ns. The MS spectra for each sample were based on the average of 700 laser shots per spot with an acquisition rate of 2 ns. MS acquisition data was calibrated externally using the ProteoMass Peptide MALDI-MS Calibration Kit.

DATA ANALYSIS AND DATABASE SEARCHING

All data were processed using DataExplorer 4.5 software from Applied Biosystems. Peptide Mass Fingerprint (PMF) data were used to search for candidate proteins using the MASCOT database search (http://www.matrixscience.com) engine. SwissProt database was selected by default for all Mascot searches. NCBInr database was used each time no significant identification was obtained with SwissProt. Database searches were, by default, performed with no taxonomy restriction and allowing up to a maximum peptide mass tolerance of 100 ppm. The number of allowed missed cleavages for trypsin was set to one. Carbamidomethylation of cysteine and methionine oxidation were selected as fixed and variable modifications, respectively. In order to provide accurate results, protein identification was considered positive for MASCOT protein scores higher than 77 ($p<0.01$) that present a sequence coverage higher than 20% and a minimum of 4 peptides matching.

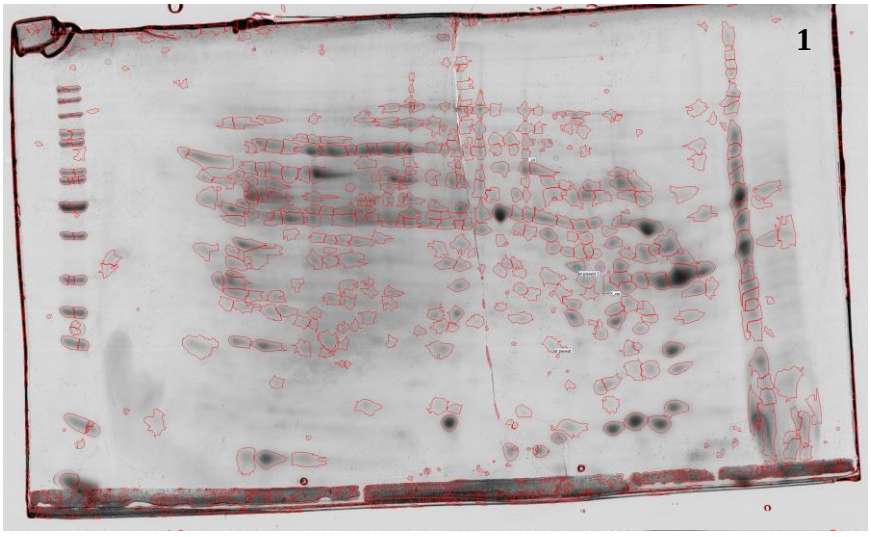
RESULTS

RESULTS

In order to understand which proteins are potentially involved in glioma tumor, we analyzed the protein expression from glioma cells, with and without conditioned medium, by means of bidimensional polyacrylamide gel electrophoresis under denatured conditions (2D-PAGE) followed by mass spectrometry analysis of the selected spots. These spots were analyzed by matrix assisted laser desorption ionization time-of-flight/mass spectrometry (MALDI-TOF-MS). The non-conditioned glioma cells (NCGl) were used as the control group for the conditioned glioma cells (CGl).

ANALYSIS OF DIFFERENTIALLY EXPRESSED PROTEINS

After 2D-PAGE, images were analyzed using Image2D Master Platinum software (Amersham). Among 1192 matched protein spots, 5 spots were significantly over-expressed in CGl (CGl/NCGl $\geq$ 2, p$\leq$ 0.01), 3 spots were under-expressed in the CGl (CGl/NCGl $\leq$ 0.5, p$\leq$ 0.01), 2 spots only present in NCGl control group and 1 spot only present in the CGl (Table 1).



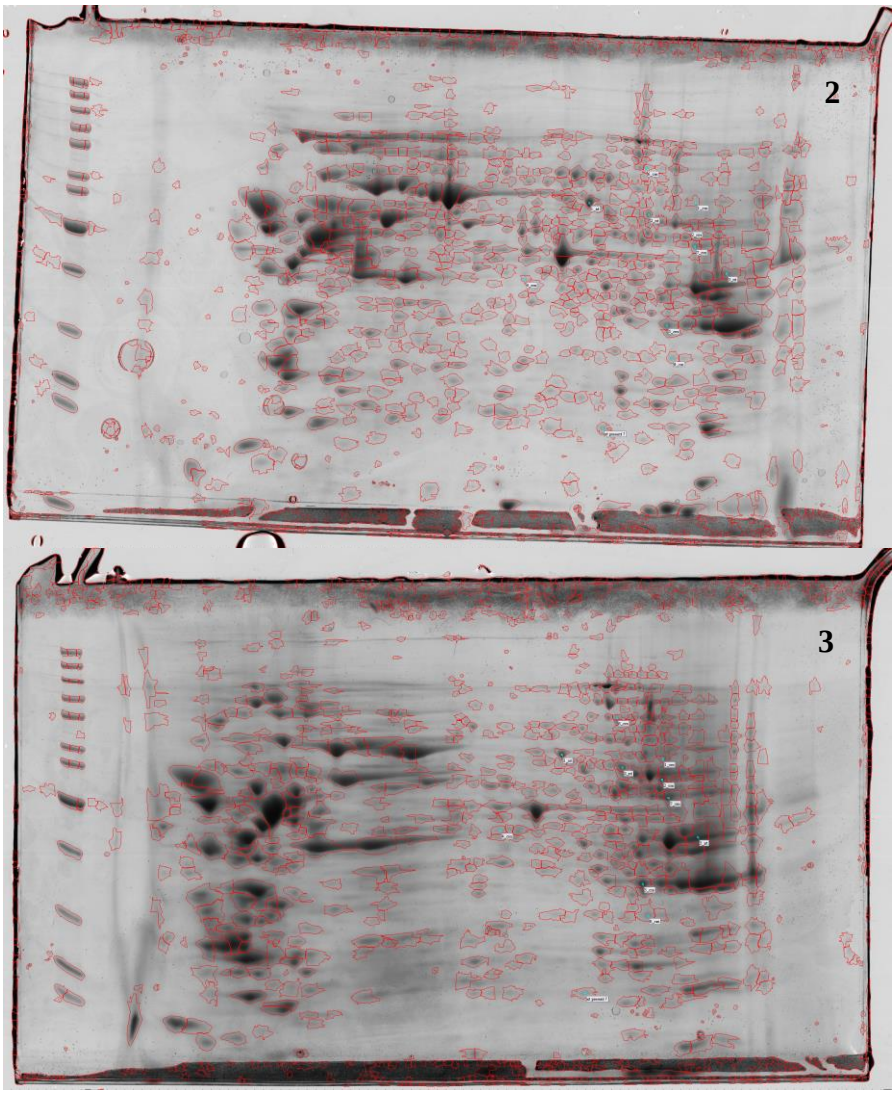
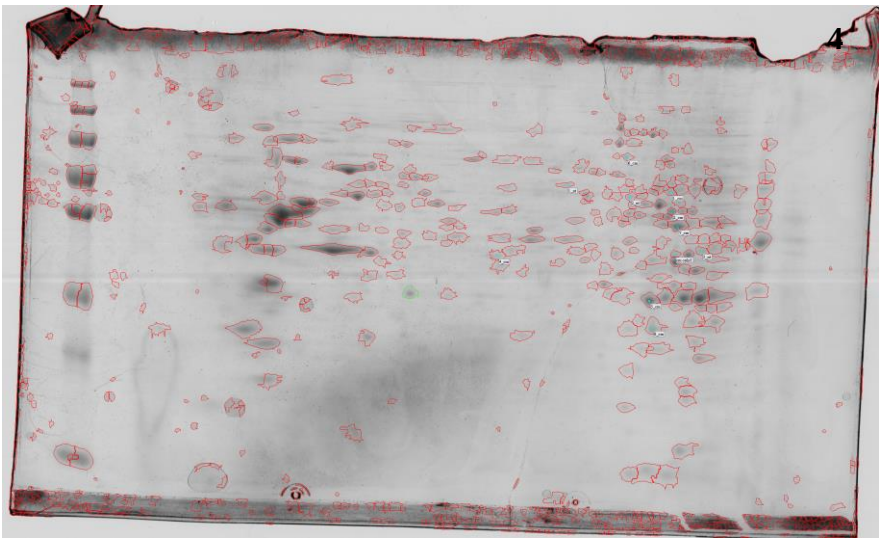


Figure 5 – Images 1, 2 and 3 are 2D gels from the non-conditioned glioma cells (NCGl).



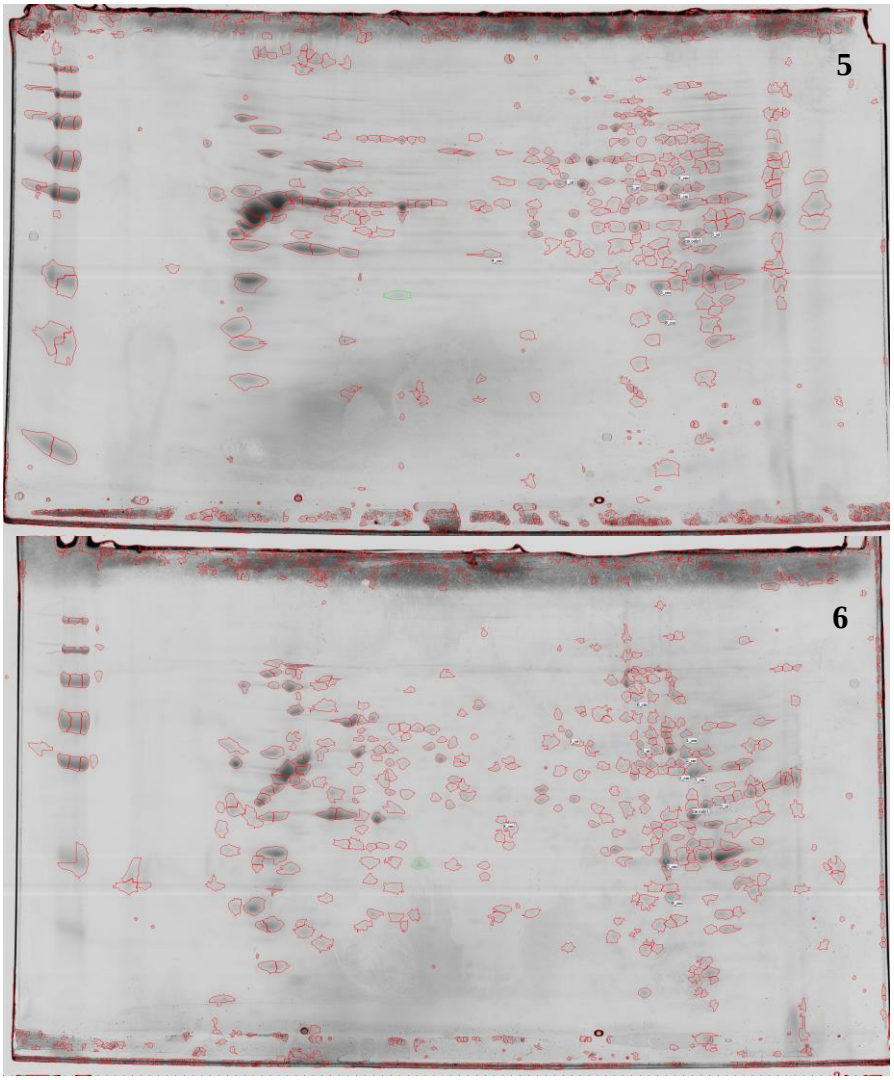


Figure 6 – Images 4, 5 and 6 are 2D gels from the conditioned glioma

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS

Eleven differentially expressed spots were identified and its detailed information is presented in Table 1.

There are 5 over-expressed proteins presented as 5 spots on the CG1. RFC1 corresponds to spot-1 with 9.38 isoelectric point, 126705 relative molecular mass and presenting a 2.32 ratio. KIF5C corresponds to spot-2, with 5.86 isoelectric point, 109777 relative molecular mass and 2.91 ratio. ANXA2 corresponds to a spot-3 protein spot, with 7.55 isoelectric point, 38937 relative molecular mass and 2.23 ratio. NRAP, corresponds to spot-4, with 9.34 isoelectric point, 196716 relative molecular mass and 2.82 ratio. RACK 1, corresponds to spot-5, with 7.60 isoelectric point, 35511 relative molecular mass and 2.07 ratio (Table 1).

The 3 spots significantly under-expressed in CGl corresponding to 3 proteins identified proteins: STI1, corresponds to spot-6 and it presents a 6.4 isoelectric point, 63170 relative molecular mass and 0.33 ratio; hnRNP L, corresponds to spot-7 spot, with 8.33 isoelectric point, 64550 relative molecular mass and 0.66 ratio; PGK1, corresponding to spot-8, with 8.02 isoelectric point, 44921 relative molecular mass and 0.4 ratio.

Only 2 spots were present in NCGl control group were *aldose reductase*, corresponds to spot-9, with 6.71 isoelectric point, 36052 relative molecular mass, and *carbonic anhydrase*, corresponds to spot-10, with 6.45 isoelectric point, 30124 relative molecular mass.

The only spot present in the CGl was *Citrate Synthase* that corresponds to spot-11, with 8.72 isoelectric point and 51988 of relative molecular mass.

Table 1 – Protein identification results retrieved for MALDI-TOF-MS/PMF queries in Mascot database search engine.

Protein spot	Mascot MOWSE score [1]	Expectation value [2]	Database	N matches (n queries)	% sequence coverage[3]	pI[4]	Mr[5]	Ratio[6]	Protein ID	Abreviation
Spot-1[7]	69	2.3e-03	Swissprot	14 (43)	14	9.38	126705	2.32 (↑)	Replication factor C subunit	RFC1
Spot-2	80	4.9e-03	Swissprot	25 (122)	25	5.86	109777	2.91 (↑)	Kinesin heavy chain isoform 5C	KIF5C
Spot-3	102	3.4e-05	Swissprot	20 (107)	52	7.55	38937	2.23 (↑)	Annexin A2	ANXA2
Spot-4	90	5.2e-04	Swissprot	37 (109)	24	9.34	196716	2.82 (↑)	Nebulin-related-anchoring protein	NRAP
Spot-5	125	1.7e-07	Swissprot	14 (67)	53	7.60	35511	2.07 (↑)	Guanine nucleotide-binding protein subunit beta-2-like 1	RACK1
Spot-6	100	5.8e-05	Swissprot	18 (68)	35	6.40	63170	0.33 (↓)	Stress-induced phosphoprotein 1	STI1
Spot-7	93	2.7e-04	Swissprot	17 (65)	35	8.33	64550	0.66 (↓)	Heterogeneous nuclear ribonucleoprotein L	hnRNP L
Spot-8	93	2.9e-04	Swissprot	21 (128)	61	8.02	44921	0.4 (↓)	Phosphoglycerate Kinase 1	PGK1
Spot-9	110	5.4e-06	Swissprot	14 (72)	48	6.71	36052	Only in NCGl	Aldose reductase	-
Spot-10[8]	79	7.1e-03	Swissprot	10 (54)	40	6.45	30124	Only in NCGl	Carbonic anhydrase	-
Spot-11[7]	64	6.0e-03	Swissprot	8 (58)	17	8.72	51988	Only in CGl	Citrate synthase, mitochondrial	-

[1] scores greater than 77 are significant for $p<0.01$

[2] number of matches with equal or better scores that are expected to occur by chance alone

[3] represents the percentage of the protein's sequence represented by the peptides identified in the MS run

[4] isoelectric point

[5] relative molecular masses

[6] Ratio = CGl/NCGl (CGl – conditioned glioma cells, NCGl – non-conditioned glioma cells)

[7] Obtained by limiting the research to entries from *Mus musculus*. In this case, scores greater than 62 are significant for $p<0.01$.

[8] Obtained allowing up to 2 missed cleavages

DISCUSSION

DISCUSSION

High grade gliomas are the most common and malignant brain tumors in adults and their morbility and mortality nature makes them the fourth major cause of cancer death. It is crucial to invest in neuro-oncology research to find new glioma biomarkers, to grade specific protein signatures to provide treatment efficacy, and to identify novel effective drug targets against malignant glioma (7).

The proteomic analyzes of the current work has allowed the identification of eleven well differentiated proteins whose molecular role on cell and cancer biology will be discussed in the following text.

UNDER-EXPRESSED PROTEINS

Stress-induced phosphoprotein 1 (STI1), also known as HOP (an abbreviation for *Hsp70-Hsp90 organizing Protein)*, is a co-chaperone which mediates the association of the molecular chaperones Hsp70 (an abbreviation for heat shock proteins 70) and Hsp90 and can be found in diverse cellular locations. STI1 belongs to the large group of co-chaperones which brings and holds together Hsp70 and Hsp90 but also modulates the activities of these major chaperones (40). In addition to its role in the Hsp70/Hsp90 protein folding system it seems to participate in several other cellular processes including transcription, protein translocation, viral replication, signal transduction, and cell division (40–42). It has been reported that STI1 is secreted by different types of cancers such as hepatocellular carcinoma (HCC), ovarian cancer, pancreatic cancer and glioblastoma, being able to promote tumor cell proliferation and invasion (34,35,41,42). In neuronal cells it was revealed that STI1 is produced by normal astrocytes (43). However, and according to Erlich *et al*. STI1 is expressed by a glioblastoma cell line (A172), promotes proliferation of distinct glioma cell lines and mediates the ERK (Extracellular signal-regulated kinases) and AKT (also known as Protein Kinase B-PKB) signaling pathways, induce proliferation and conversely does not induced proliferation of normal astrocytes (42).

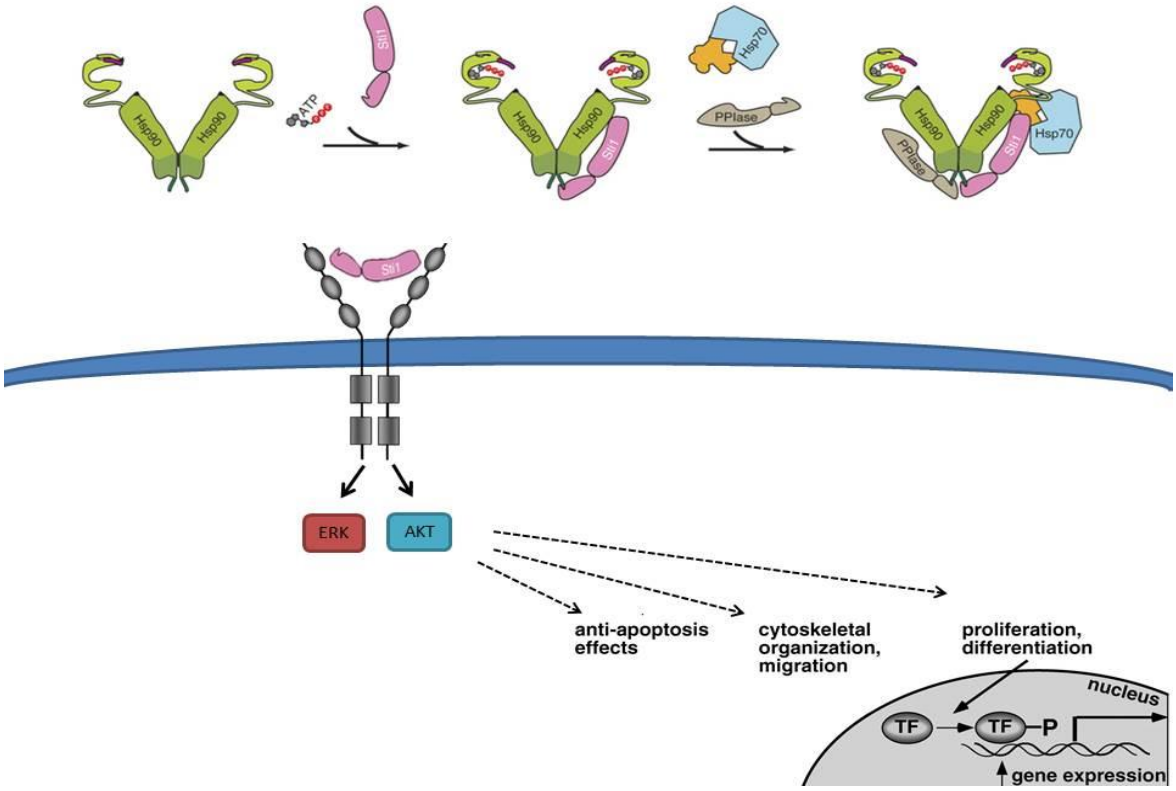


Figure 7 – Possible mechanism that suggests that STI1 promotes proliferation of distinct glioma cell by ERK and AKT signaling pathway.

Heterogeneous nuclear ribonucleoprotein complexes (hnRNPs) is a family of proteins that plays a central role in the RNA metabolism as formation, packaging, processing, splicing and function of mRNA in the cytoplasm (44–46). Proteins of this complex are termed A1 to U and many hnRNPs genes may be regulated by oncogenes (46). hnRNPL is present in the nucleoplasm as part of the hnRNPs but has also been identified outside of the nucleoplasm (44,46). The hnRNP proteins are aberrantly expressed in cancer cells supportting the theory that they are implicated in tumor development. HnRNP L has been shown to regulate the expression of VEGF under hypoxic conditions. It also binds a specific base region of VEGF mRNA and increase VEGF protein production, only in cells under hypoxic stress. This interesting association protein-VEGF mRNA suggest that hnRNP L plays an important role in angiogenesis and tumorigenesis (44,46).

Phosphoglycerate Kinase 1 (PGK1) is an enzyme that catalyzes a reversible chemical reaction that converts 1,3-disphosphoglycerate to 3-phosphoglycerate, producing one molecule of adenosine triphosphate (ATP) during the breakdown of glucose. PGK1 overexpression has been associated with gastric cancer, lung adenocarcinoma, prostate cancer, pancreatic tumor, breast cancer, multidrug resistant cancer and astrocytoma radioresistance (47–51). Researchers suspect that PGK1 is involved in many pathophysiological mechanisms like radioresistance, tumorigenesis, DNA replication and repair and angiogenic processes (49,51,52).

Our findings revealed lower expression of STI1 (0.33 fold), hnRNP L (0.66 fold) and PGK1 (0.4 fold) when glioma cells were exposed to mature adipocytes conditioned medium. Regarding multiple effects of STI1, this under-expression might reflect that the adipokines secreted by mature adipocytes might be able to stop some important pathways in carcinogenesis. In what concerns to PGK1 it may be under-expressed on the glioma cells under conditioned medium due to a mechanism of remodeling the oxidative metabolism toward the utilization of fatty acids substrates instead of glucose. This interpretation may be supported by the increased expression of mitochondrial citrate synthase.

SUPPRESSED PROTEINS

Two enzymes were suppressed on the glioma cells cultured with conditioned medium.

Aldose reductase is an enzyme that belongs to aldo-keto reductase superfamily and reduces glucose to sorbitol in the presence of NADPH, the first step in polyol pathway of glucose metabolism. The second and last step in the polyol pathway is catalyzed by sorbitol dehydrogenase, which catalyzes the NAD-linked oxidation of sorbitol to fructose. Thus, the polyol pathway results in conversion of glucose to fructose with use of NADPH and production of NADH (53). Recently, the potential physiological role of aldose reductase has been reassessed from a different perspective. Recent studies suggested that aldose reductase, besides reducing glucose, also efficiently catalyzes the reduction of reactive oxygen species (ROS), lipid peroxidation generating lipid aldehydes and their glutathione conjugates. The elevated levels of ROS with high chemical reactivity cause lipid peroxidation and oxidation of proteins and nucleic acids, which can increase the risk of mutagenesis. Aldose reductase is activated and overexpressed under conditions of oxidative stress and inflammation. This protein is a regulator of ROS signals induced by activation of transcription factors such as NF-κB and AP1. It has been shown that aldose reductase can be one of the main mediators of inflammatory signals and proliferation of cancer cells. Indeed, a large number of recent studies point that aldose reductase inhibition prevents oxidative stress-induced activation of NF-κB and AP1 signals and, consequently, inflammatory complications of cancer (53,54).

Carbonic anhydrase is a family of enzymes that participate in a variety of biological processes, including respiration, calcification and acid-base balance, because they catalyze the reversible hydration of carbon dioxide. It is one of the transcription products of hypoxia inducible factor-1α (HIF-1α) and is over-expressed in hypoxic solid tumors. Carbonic anhydrase plays an important role in tumorigenesis and drug resistance because regulates many pathways including angiogenesis, glycolysis, growth factor signaling, apoptosis, genetic instability, metastasis and tissue invasion. Carbonic

anhydrase is identified as to be one of the best cellular biomarkers of hypoxia. Furthermore, some recent studies are exploring the association between carbonic anhydrase levels and the capacity to induce resistance to cytotoxic therapy (55).

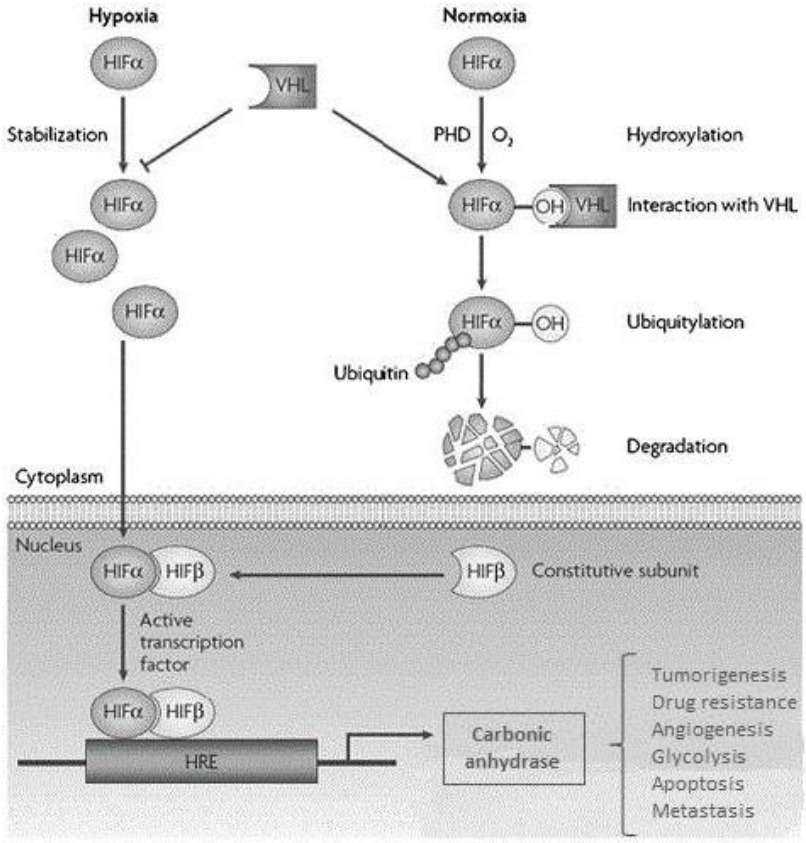


Figure 8 – At normal oxygen levels (normoxia) hypoxia inducible factor-alpha (HIFalpha). is degraded by the ubiquitin–proteasome system. Under hypoxia, carbonic anhydrase is one of the transcription products of HIF-1α.

Our research revealed that aldose reductase and carbonic anhydrase were present only in glioma cells and that their expression was completely blocked when glioma cells were exposed to mature adipocytes conditioned medium. Our findings suggested that this blockage may reflect a total inhibition of aldose reductase and carbonic anhydrase, possibly associated to oxidative stress response prevention, inhibition of inflammation and cancer proliferation, drug resistance, angiogenesis and carcinogenesis, which may suggests some kind of protective mechanism related to obesity.

OVER-EXPRESSED PROTEINS

Replication factor C subunit 1 (RFC1) participates in eukaryotic replication as a clamp loader. Its role as clamp loader involves catalysing the loading of *Proliferating Cell Nuclear Antigen* (PCNA) on to DNA, encircles DNA in a ring. It binds to the 5' end of the DNA and spends ATP to open the ring of PCNA so that it can encircle the DNA. ATP hydrolysis releases the PCNA-DNA complex, with concomitant clamp loading onto DNA (56–58). RFC1 also plays an important role in maintaining the stability and the integrity of the genome and by coping with various genomic stresses as well. This mechanism is a DNA repair pathway and prevents fatal genomic rearrangements. Genome instability is a hallmark of cancer cells and plays a critical role in generating the variability that allows cancer cells to evolve during tumor progression (59). Considering RFC1 over-expression by a 2.32 fold in glioma cells cultured with conditioned medium, RFC1 may be involved in protector reparative mechanisms under obesity environment.

Kinesin heavy chain isoform 5C (KIF5C) is a novel kinesin motor protein, firstly thought to be expressed exclusively in neurons, preferentially in motor neurons. This motor kinesin is also linked to synaptic vesicle components in neuronal cells where it plays a role in anterograde trafficking of mitochondria and vesicles. In the absence of this protein, KIF5C knockdown mice revealed a decrease in brain mass (60). Chicken embryos studies unveiled functional KIF5C in non-neuronal tissues, however, has not yet been described it’s complete function. More recently KIFC5 has been involved in apical protein transport of polarized MDCK (canine) cells. It is important to mention that KIF5C phosphorylation plays a key role in the regulation of these motor proteins because pharmacological treatment of kinases and phosphatases results in altered vesicle motility (61). Moreover, a link has been established between APC tumor suppressor protein and KIF5C with overlapping distributions along microtubules segments, as well as in cell periphery (62). Since KIF5C is over-expressed in glioma under condition conditioned medium it may suggests that obesity may play some role on proliferation and axonal transport on cancer cells.

Annexin A2 (ANXA2), a member of the annexin family, is a calcium-dependent phospholipid-binding intracellular protein found on various cell types. ANXA 2 heterotetramer consists of two subunits of ANXA2 and two subunits of p11 (member of S100 family). ANXA 2 is involved in a diverse range of physiological cellular processes. ANXA 2 has anti-inflammatory and anticoagulation role and is also involved in endocytosis and exocytosis, signal transduction, cell proliferation, differentiation and apoptosis. Increased ANXA2 expression results in increased plasmin generation and enhances cancer invasion and metastasis (63,64). On the cell surface (ANXA2) binds to t-PA and activates plasminogen conversion to plasmin. ANXA2 is over-expressed in hepatocellular carcinoma, colorectal cancer, breast cancer, ovarian cancer, pancreatic cancer, acute promyelocytic leukemia, renal cell carcinoma and glioma when activated the tPA/plasminogen proteolytic system. In glioma, ANXA2 play a central role in cell motility, migration, invasion and angiogenesis (63–65). On the other hand, it is under-expressed in prostate cancer, esophageal squamous carcinoma and nasopharyngeal carcinoma and sinonasal adenocarcinoma (63,64). Our analysis revealed that ANXA2 expression (2.23 fold) was more pronounced when glioma cells were exposed to adipocytes conditioned medium. This finding is in agreement with previous studies that also revealed that ANXA2 is elevated in glioma (63–65).

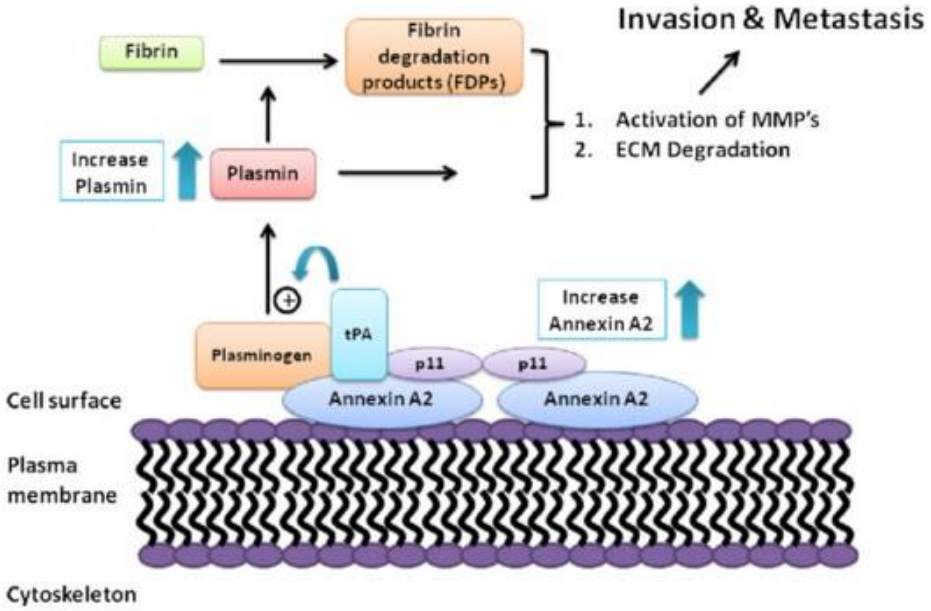


Figure 9 – Proposed mechanism of annexin A2 promoting cancer metastasis in the plasminogen activation system. Increased annexin A2 expression results in increased plasmin generation and enhances cancer invasion and metastasis. From Lokman N *et al*. 2011.

Nebulin family is known for some different cellular functions namely in cytoskeletal stability, cell migration, as well as protein scaffolding (66). *Nebulin-related-anchoring protein* (N-RAP), one of nebulin family members, is highly over-expressed in animal models of *dilated cardiomyopathy* (DCM), which exhibited an early increase in N-RAP expression before the manifestation of DCM. Authors suggested that it could be an adaptive response to strengthen the link between the myofibrils and the membrane at intercalated discs (66). Furthermore, it has been found over-expressed in human pancreatic cancer. Wu et al. have associated N-RAP with loss of heterozygosity (LOH) in glioblastoma (67). Thus, our protein expression of 2.82 fold can be related to a cellular mobilization and reorganization typical in a tumor microenvironment.

Receptor for activated C kinase 1 (RACK1) is a highly conserved intracellular adapter protein in multiple intracellular signal-transduction pathways. RACK1 integrates inputs from distinct signaling pathways, being critical for fundamental cellular activities such as cell proliferation, migration, adhesion and spreading, transcription and protein synthesis, as well as many neuronal functions (68). Many reports indicate that RACK1 plays an important role in cancer progression and that it is over-expressed during angiogenesis and in many human carcinomas (69). RACK1 is over-expressed in hepatocellular carcinoma, colon cancer and metastic melanoma (70–72). RACK1 is also implicated as a key player in ovarian cancer (73), prostate cancer (74) and in cancers caused by pathogens such as human papillomavirus 16 (HPV 16) (75) and *Helicobacter pylori* (76). The focal adhesion kinase/RACK1 complex is needed for cell spreading, polarity and chemotactic invasion affecting spreading initiation and cancer cell polarity (77). Specifically, RACK1 expression is being assessed as a biomarker and a prognostic indicator in breast cancer (78). In pulmonary adenocarcinomas, increased RACK1 expression is associated with pathological stage and tumor size and is also a potential diagnostic biomarker (79). In our assays, glioma cells exposed to adipocytes conditioned medium increases RACK1 expression by 2.07 fold. Given the potential deleterious effect of up regulated RACK1 expression in several tumors, which may be related with a less favorable prognosis, we might assume the potential use of this protein as a potential biomarker to the glioma invasiveness and/or

prognosis determination.

CITRATE SYNTHASE WAS PRESENT ONLY THE CGL

Citrate synthase is a mitochondrial enzyme that exists in nearly all living cells and catalyzes the first reaction of the tricarboxylic acid (TCA) cycle -the condensation of acetyl-CoA with oxaloacetate to yield citrate- which regulates energy generation in mitochondrial respiration, and is generally assumed to be the rate-limiting enzyme of the cycle (80). Cancer-associated alterations in metabolism are no longer viewed as an indirect response to cell proliferation and survival signals (81). The 'Warburg effect' and metabolic reprogramming has attained the status of a hallmark of cancer (81,82) and citrate seems to play a central role in the metabolism of cancer cells (83). Low levels of citrate and the loss of citrate synthase is associated with the so-called aerobic glycolysis, as well as with malignancy in cervical cancer (84). The growth of various human cancer cells stopped when cultured with citrate (83,85,86). Citrate can also sensitize cancer cells to chemotherapy (86). Conversely, an enhanced citrate synthase activity has also been reported in human pancreatic cancer (87). Our findings revealed that citrate synthase expression was only found when glioma cells were treated with adipocytes secretome. According to previous studies, metabolic patterns of most cancer cells rely on the aerobic glycolysis. This increase in citrate synthase expression may reveal, in our opinion, a normalization of aerobic metabolism with the stimulation of TCA cycle and oxidative phosphorylation to obtain energy more efficiently. Given the multiple metabolic pathways involving citrate (83), the overexpression of citrate synthase might represent a change of substrate for respiration, like lipids of other anapleurotic intermediates over carbohydrate substrates.

CONCLUSION

CONCLUDING REMARKS

In conclusion the present study presents a paradoxical relationship between obesity and cancer. In a first view, conditioned medium (adipokines-enriched medium) may present a protective effect against glioma, since STI1, hnRNPs and PGK1 that are generally overexpressed in several types of cancer are underexpressed. Similarly, both *carbonic anhydrase* and *aldose reductase* that play an important role in cell physiology, inflammation and cancer metabolism are even suppressed in glioma cells that grown under adipokines-enriched environment. However this view may not be completely unexpected. A couple years ago, a team led by Steven Lehrer hypothesize that obesity might protect against benign brain tumors based on several epidemiological studies on the USA (88). They found that there was a significant inverse correlation between percent obesity versus percent benign brain tumors in 19 USA states ($r = 0.666$, $p = 0.002$).

Contrariwise, RFC1, KIF5C, ANXA2, N-RAP and RACK1 that are generally involved in the matrix remodeling, proliferation, migration, invasion of cancer cells are overexpressed in GL261 cell in the presence of the adipokines-enriched medium.

Also the presence of citrate synthase only in glioma cells cultured under conditioned medium and the decreased expression of PGK1 supports the idea that under an obesity environment, glioma cells may reshuffle their metabolic patterns towards increased lipid consumption for aerobic ATP production over glucose and other simple sugars.

Conditioned medium may presents both anticarcinogenic proteins and procarcinogenic proteins.

RFC1, Aldose reductase and PGK1 reveal protective function in cancer initiation, although in an advanced stage they seem to be involved in its progression. PGK1 and carbonic anhydrase look like to sensitize tumoral cells to therapeutic. KIF5C, ANXA2, RACK1 and N-RAP are important in tumor progression and invasion of cancer cells.

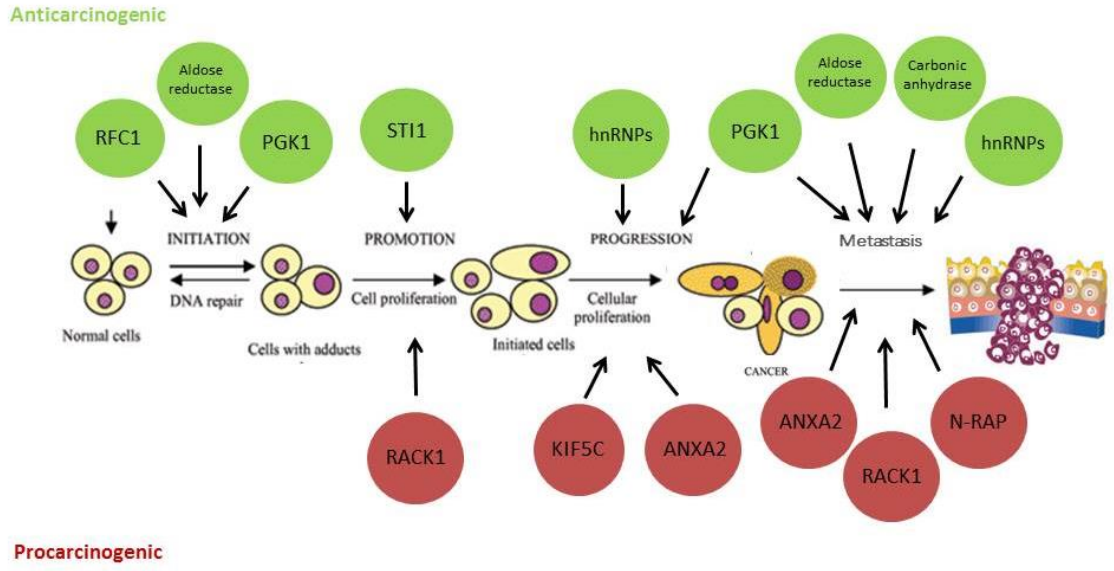


Figure 10 – Interpretive model for the mechanism proposed that conditioned medium may presents both anticarcinogenic proteins and procarcinogenic proteins.

It is clear that additional work will be required before complete understanding of the implication of obesity in malignant brain tumors biology. Meanwhile, the present work presents an interesting *in vitro* model for the study of glioma biology under a "obesity" environment, that can be explored for the understanding of cancer cells biology, for the search of biomarkers, prognostic markers and therapeutic approaches.

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