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EFFECTS OF CELLULAR SHORT-TERM STARVATION ON CONVENTIONAL CHEMOTHERAPY RESPONSE IN HUMAN CANCER: UNDERSTANDING OF MOLECULAR MECHANISMS AND MICRORNAS INVOLVEMENT.

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Abstract

Abstract

Background: Short Term Starvation (STS) is a type of dietary restriction able to reduce tumorigenesis and cancer progression but molecular bases of this effect are still unclear.

Aim: In vitro analysis of STS effects in presence of chemotherapy and evaluation of microRNAs (miRNAs) involvement.

Results: STS affects the expression profiles of miRNAs involved in chemotherapy response leading to cancer cells sensitization and to healthy cells protection.

Summary

Summary

Chemotherapy is the main therapeutic strategy for cancer treatment but potentially presents serious side effects that may limit its use and/or impair its effectiveness, especially in high grade tumors. It has been shown that non-genetic approaches that limit food intake (dietary restrictions) can provide a benefit in terms of tumorigenesis and tumor progression. Considering that cancer patients are incline to weight loss, because of their debilitating disease and neoplastic cachexia, short term starvation (STS) seems to be the most suitable dietary condition because it consists of short courses of fasting and cycles with no food restriction. Previous studies indicate that STS is able to reduce tumor progression in vitro and vivo but molecular changes involved in is beneficial effect needs further consideration. Until now, it seems that a key role is exerted by nutrient signaling pathways and in particular by insulin like growth factor-1 (IGF-1) pathway.

Other experiences showed that microRNAs (miRNAs) are non-coding RNA that negatively regulate various biological processes. Recently miRNAs have been correlate to diseases onset and progression including cancer.

This manuscript aims to confirm STS effect on a triple negative breast cancer (TNBC) cell line treated with Doxorubicin and to study in deep the knowledge of the molecular basis focusing on miRNAs involvement.

TNBC cell line (MDA-MB-231) and healthy mammary cell line (MCF10A) have been cultivated in STS medium or standard medium. Once STS was established, cells were treated with Doxorubicin and cell conditions were evaluated. Their cell viability has been evaluated through CellTiter 96® AQueous One Solution and trypan blue assay and their cell proliferation has been set through growth curves. STS influence on angiogenesis has been

analyzed through real time PCR using TaqMan gene expression assay. Finally, miRNAs expression profiles has been obtained using Taqman® Array Human A microRNA Microfluidic Cards and their targets have been evaluated through online databases (Pubmed, DIANA tools, miRBase).

From preliminary data, comparing MDA-MB-231 subjected to 48h STS and Doxorubicin 10γM treatment with MCF10A in the same experimental condition, it emerges that approximately the 23,36% of miRNAs was significantly deregulated and, among these the 61,8% was downregulated. In addition, it is possible to discover a little amount of miRNAs (4.46%) that are specific only for the MDA-MB-231 cell undergone STS and treated with Doxorubicin. MiRNAs expression profiles analysis seems to explain the beneficial STS effect. Moreover, in MDA-MB-231, among upregulated miRNA there are miRNAs that increase drug sensitivity (MiR-26a, miR-149, miR-181a, miR-193b, miR-195 and miR-324-3p) while among the downregulated ones there are miRNAs that induce chemoresistance (miR-15b, miR-23a, miR-29a, miR-106b, miR-128, miR-192 and miR-494).

In MCF10A, miRNAs involved in antiblastic drug response have a heterogeneous expression levels that could explain the increased healthy cell line resistance to treatment, compared to cancer cells.

So, knowing that a miRNA acts on multiple target genes, that each target can have binding sites for miRNAs interaction, data obtained from the study need to be confirmed and insighted. However, results are encouraging and clinically managed for example by developing molecules that will target the oncomiRNAs and consequently will increase the levels of the tumor suppressor in order to make chemotherapy more tolerable.

CHAPTER 1

BACKGROUND

1.1 Dietary Restriction

Currently, chemotherapy remains the most widely adopted strategy for the treatment of a wide range of tumors [1], although it is potentially able to cause serious side effects up to lose the therapeutic efficacy, especially in advanced neoplasm stage.

Research groups are developing therapeutic approaches to make chemotherapy more tolerable by reducing the adverse effects involving both cancer cells and healthy ones. Recent in vitro and in vivo (animal models) studies have shown encouraging data about the association between chemotherapy and different types of dietary approaches that seems to have a protective effect. Literature reports various types of food restrictions that differs each other on the basis of ingested nutrients (such as the aminoacid restriction and the restriction calories) or fasting period length (long- or short-term starvation).[2]

Dietary restriction such as short-term starvations (STS), represent a non-genetic intervention with a protective role against age-related diseases (cancer included) able to increase mammalian la life-span. [3]

STS is described as an alternation of periods in which food consuming is not restricted and designated limited feeding periods[3]. Other authors described it as the period of time in which animals lose weight after initiation of food restriction but prior to rebound or weight maintenance.[4] STS is able to induce antibacterial, antimutagenic, and anticancer effects and to reduce reactive oxygen species (ROS) levels.[5] Davis et al. have observed on murine models that STS may be advantageous in presence of moderate intensity cortical impact injury.[6]

Despite the evidences of the dietary restriction-inducted beneficial effects, the underlying mechanisms are unclear. Currently, researchers have not identified a single signaling pathway or a particular molecular mechanism but appear to be partly involved nutrient-signaling pathways and in particular the growth promoting insulin-like growth factor 1 (IGF-1) receptor and its downstream effectors, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and

phosphoinositide 3-kinase (PI3K), which are known to regulate several detoxification enzymes.[7]

As demonstrated by experiments on rats, fasting also has the ability to inhibit inflammatory response, reducing the expression levels of inflammatory cytokines and chemokines (such as IL1beta, TNF-alpha and MCP-1) in various tissues.[8]

STS also influences the energy-sensing AMP-activated protein kinase (AMPK) and its downstream targets (ACC, ERK1/2, mTOR). Alterations in mTOR pathway could be associated directly to growth factors deprivation.[9]

Dietary Restriction affects cell proliferation regulating genes like the insulin signaling adaptor (Irs2) and the mitogenic hormone prolactin receptor (Prlr) in both cancer and healthy cells.[10] Lee et al observed that constitutively active mutations related to PI3K pathway are determinant in cancer response to nutrient deprivation.[11]

It's clear that STS alerts cell inducing autophagy. Autophagy is a conserved self-eating process useful to transfer nutrients from unnecessary to essential process.[11] STS stimulates autophagy through the activation of poly(ADP-ribose) polymerase(PARPA)-1, an enzyme usually activated in presence of DNA damage. In case of STS, ROS are responsible for the activation of PARP-1 required for starvation-induced autophagy.[12]

1.2 Dietary Restriction and cancer

Cancer occurrence is triggered by a series of genetic and epigenetic cell mutations that give them the ability to be self-sufficient to growth and to ignore anti-growth and pro-apoptotic stimulation while healthy cells can't grow without stimuli. It could be due to growth factors (like platelet-derived growth factor, vascular endothelial growth factor and insulin-like growth factor) produced in an autocrine way by cancer cells or because of mutations that make constitutively active proteins involved in signaling pathways or membrane receptors.[13] Alterations giving the most relevant contribution in the neoplastic transformation regards genes coding for members of the Ras/Raf/MAPK and PTEN/PI3K/AKT pathways. Mutated Ras alleles, detected in a quarter of all cancers[14] and in half of colon cancers,[15] are responsible of malignant cells independent proliferation.

It's necessary to underline that, in presence of a tumor, stroma cells generate a microenvironment that encourages disease progression (through remodeling, invasion, angiogenesis and metastasis). Cancer microenvironment is heterogeneous and it is composed of malignant cells, macrophages, adipocytes, fibroblasts, and endothelial cells that are different from the corresponding in healthy environment.[16]

Studies showed that serum starvation and short term starvation are able to reduce, respectively in vitro and in vivo, growth factor levels in healthy cells,[17, 18] decreasing the

basal cellular metabolism and, consequently, setting a quiescent status.[9] Tumor cells don't show the same ability and react to stress regulating autonomous growth stimulation in order to reprogram their metabolism and maintain a continuous proliferation.[14] Then STS causes a differential stress in healthy and malignant cells. This feature could clarify the evidence that starvation protects normal cells without interfering with the chemotherapy-dependent cancer cell death.[19, 20] It's well known in fact that several chemotherapy drugs interfering with DNA (in part through oxidative damage) causing both normal and tumor cells death.[21] In vitro and vivo studies showed that, among dietary restrictions approaches, STS is the most effective.[20]

It is noteworthy that STS may sensitize various types of cancer to chemotherapy, but it seems also to be able to attenuate side effects when followed by the treatment. An example is the cardioprotective effect after Doxorubicin utilization.[22] About that, Raffaghello et al. evaluated the effect of high-dose chemotherapy and STS in murine models. They have tested the manifestation of side effects after the administration of etoposide, a drug with a non-specific toxicity profile, in a group of mice undergone to fasting for 48 h and in a control group fed ad libitum. Unlike the fasting mice, control group showed reduced mobility, posture alterations and other signs of pain and stress. STS affected also mice vitality: in fact the mortality rate of fed group was around the 43% while the acute toxicity of etoposide caused only one dead in fasting group. The same researchers repeated the experiment using a dose of etoposide four or five times higher than the recommended one for human and increasing the fasting period from 48 h to 60 h. In this case the control group mice died or manifested toxicity while starved mice lost the 40% of their weight (recover after a week of re-feeding). STS effects have been experienced in also in ten human volunteers affected by different type of tumors starved for 48-140 h before chemotherapy and 56 h after treatment.[21] Fasting after chemotherapy seems to be important because of the association of re-feeding and DNA damage provoked by antiblastic drugs: prolonged starvation induces cell death while re-feeding trigs cell proliferation that, in presence of toxins, increase DNA damage.[23, 24]. All patients had a benefit from STS with a decrease of chemotherapy-induced side effects.[21]

Dietary Restrictions can affect tumor microenvironment and thus can become tools able to promote favorable changes.[25] Cancer-associated adipocytes are compounds of tumor microenvironment and differ from adipocyte because of a reduction of cellular markers (like HSL, APN, and resistin) and an increased inflammatory cytokines expression (such as IL-6 and IL-1β).[26] Since adipocytes represent a source of pro-inflammatory cytochines (IL-6, TNF-α), ROS, and matrix metalloproteinases, they are involved in carcinogenesis and tumor invasiveness.[27-30]

Moreover, adipocytes can secrete adipokines that recruit macrophages and endothelial cells in a way NF-kB-mediated leading to an increase of angiogenesis, fibrosis, and inflammation.[31]

Dietary approaches alter adipocytes (reducing their secretions) and thus induce changes in tumor microenvironment.[28]

Standard chemotherapy creates an environment rich of glucose and glutamine that induces cancer cells metabolism, thus it's spreading the idea to use metabolic therapy as an alternative to usual treatment. A study shows that among the metabolic therapies there is the calorie-restricted ketogenic diet (KD-R) able to improves prognosis of patients with glioblastoma multiform and brain cancer through its anti-angiogenic, anti-inflammatory, and anti-apoptotic effects.[32]

1.3 MicroRNA

Recent studies have demonstrated that microRNAs (miRNA) are able to regulate chemotherapy or target therapy sensibility/resistance.[33]

MiRNAs are small non-coding strands of RNA (long 18-25 nt-nucleotides) acting as posttranscriptional regulators (post-transcriptional gene silencing) of messenger RNA (mRNA) and binding its mRNA target promote its degradation and consequently inhibit or suppress translation.[34] MiRNAs can provoke translation inhibition or mRNA cleavage through the base pairing with the 3' untranslated region (3'-UTR) of their target mRNAs, depending on the complementarity degree between the miRNA and its target sequence.[35] In vitro analysis showed that the miRNA-mRNA recognition could occur not only at 3'UTR level but also in the 5'UTR or in coding regions even though the pairing in these sites play a marginal role in silencing.[36]

Numerous miRNA can recognize different targets and a target can be regulated by various miRNAs. This evidence explains why they represent pathways regulator involved in numerous biological functions (including cell cycle regulation, cell proliferation, differentiation, apoptosis, immune response, differentiation of stem cells and embryonic development). It has been shown that if their expression appears to be altered, miRNAs may be involved in various complex diseases, including tumors.[37] miRNAs are found aberrantly expressed in many cancer types and they can work either as tumor suppressor (TS-miRs) or as oncogenes (oncomiRs). The first category is usually deleted or silenced in tumor and this condition facilitates cancer cell growth and thus malignancy progression. The latter is found in amplified regions or is overexpressed in the tumor and this determines an increase of cell proliferation, angiogenesis, invasiveness and a reduction of apoptosis. A relevant number of MiRNA genes are located in genomic regions frequently rearranged in

tumors such as fragile sites, deleted regions (minimal region of loss of heterozygosity, LOH) or amplified (minimal amplicons) and common areas of break-point, providing further evidence of their role in the pathogenesis of cancer.[38, 39]

There are few evidences that correlate miRNAs to Dietary Restrictions. For example, researchers[40] evaluated the miRNA transcriptome in peripheral blood cells of ten obese women undergone to a 8weeks weight-loss program (the 50% of them was categorized as non-responders) observing a differential abundance of selected c-miRNAs. Other previous experiences have shown no differences before and after 14 weeks of energy-restricted diet despite the evidence of a 17% loss in body mass.[41]

In conclusion, miRNAs are emerged as possible therapeutic targets for a large number of diseases and can use as a novel clinical method to monitor the progression, prognosis, diagnosis, and evaluation of treatment responses.[42]

1.4 Objectives

Among Dietary Restriction, STS is the more suitable because of the characteristic alternating phases of fasting and feeding.[10] Since short-term fasting is able to reduce the chemotherapy toxicities, the initial project aim is to evaluate its effects in vitro. I have decided to analyze cell viability and proliferation in triple negative breast cancer cell line (MDA-MB-231) undergone to STS and treated with Doxorubicin, the aforementioned antineoplastic agent.

Triple negative breast cancer (TNBC) represents about 10% - 20% of breast cancers. It is a biologically aggressive tumor setting with high response rates to Neoadjuvant Chemotherapy (NAC) but poor outcome.[43] TNBCs are characterized by lack of expression of hormone receptors (HR, Estrogen ER and Progesterone PR) and human epidermal growth factor receptor 2 (HER2). Patients with TNBC tend to recur within the first 3 years and are considered at high risk of death in 5 years. Moreover, TNBC exhibits a higher frequency of metastasis then the other types of breast cancer, particularly in visceral and cerebral sites.[44]

Doxorubicin is a highly efficacious and well-established anthracycline chemotherapeutic agent[45] and it works as an intercalating agent inserting between the bases of DNA, blocking synthesis and transcription. It also determines the inhibition of the enzyme topoisomerase (type II). Both mechanisms lead to the rupture of DNA strands. Consequently, in order to evaluate the effect of STS on the response to conventional chemotherapy, I treated the MDA cells with two different concentrations of Doxorubicin (1 and 10μM).

Since several studies have shown that miRNAs are involved both in the development and in tumor therapy sensitivity/resistance, after evaluating cellular conditions we decided to

analyze miRNAs expression profiles in MDA-MB-231 after 48h STS and 3h Doxorubicin treatment.

The same experiment has been proposed for the healthy breast tissue cells MCF10A.

The project includes a phase of in vivo mouse models that have not been able to achieve but you may be referred to as a future perspective.

CHAPTER 2

Materials and Methods

2.1 Cell cultures

Cell lines were purchased from ATCC (American Type Culture Collection): The Global Bioresource Center (https://www.atcc.org/).

MDA-MB-231 has been starved in DMEM-GlutaMax–32430-027-ThermoFisher (4.5gr D-Glucose/Liter) with Fetal Bovine Serum (FBS-10%), Non Essential Amino Acids (NEAA-1%) and Streptomycin-Penicillin (Strepto/Pen). The medium used for the experiment of Short Term Starvation was DMEM-11966025-ThermoFisher (no glucose) addicted with glucose (0.5gr D-glucose/Liter), FBS (1%), NEAA (1%), Strepto/Pen (1%) and Hepes (2%).

MCF10A has been starved in Medium DMEM F:12 enriched with the MEGM™ Mammary Epithelial Cell Growth Medium – Lonza kit, 1gr/L Glucose and the 10% of FBS. For STS experiment DMEM medium (without glucose) consisted of 0.5gr/L Glucose, 1%FBS, 1%NEAA, 1% Strepto/Pen and 25mM Hepes.

2.2 Cell proliferation

To assess cell proliferation, it has been used the Burker Chamber, a device of manual counting of cells in the electron microscope. It consists of a rectangular slide of 7,5x3,5 cm^2 planar size and thickness of 4 mm; It has two depth cells of 0.1 mm and known surface, separated by a recess that allows the execution of the two counts on the same instrument. On them is placed a glass slide, blinded thanks to special metallic fins. The thin space that is created between the two surfaces is then filled by capillarity with a drop of cell suspension. The grating of Burker Chamber is structured into nine squares larger (camps) delimited by three parallel lines, inside squares and rectangles delimited by two parallel lines; Thanks to this structure is the count of the cells present in the sample can make.

150000 fasting cells were harvested in various Petri Plate. A group of them has been subjected to fasting while another group of cells (identified as the control group) was in a standard medium. Among both group cells, some plates have been subjected to

chemotherapeutic treatment for 3 h with Doxorubicin 10 γM and 24 h with Doxorubicin 1 γM. Cells were detached from the Petri dishes through trypsin (catalyzes the proteolytic cleavage with specificity for arginine and lysine favoring the detachment cell/plate and cell/cell) and counted Burker Chamber after 24 h, 48 h and 5 days of fasting (Fig.1).

The grid of hemocytometer Burker is structured into nine squares, delimited by three parallel lines, with inside sixteen small squares delimited by two parallel lines.

Alive and dead cells visible in the nine squares were counted and the mean of cells present in each square (M) was determined. To calculate the number of cells that were present in each well was performed the following calculation:

Cells = M x 10^4 x V (ml) x Dilution factor

2.3 Cell viability

Cell viability was assessed by two methods: Trypan blue and MTT colorimetric assay.

Assay using Trypan blue is a test used to determine the number of live and dead cells present in a suspension. It is based on the principle that alive cells, having an intact cell membrane, are able to exclude the entrance to certain dyes, just as the Trypan blue, contrary to what occurs for dead cells, which instead turn blue color. Both dead and living cells are counted by Burker Chamber.

Another methodology used to analyze cell viability is the MTT assay performed through MTT Cell Growth Kit-MILLIPORE consisting of 3-(4,5-dimethylthiazol-2)-2,5-difeniltetrazolium bromide. It is a laboratory standard colorimetric assay to measure the mitochondrial enzymes activity that reduce MTT to formazan, by making the tack with isopropanol (extraction solvent) at room temperature in the dark because it is a photosensitive compound. The intensity of the coloration of the solution obtained is directly proportional to the concentration of formazan and is therefore the expression of cell viability. The optical density (OD) is measured spectrophotometrically at a wavelength equal to 490 nm. Both the degree of cell viability and the degree of toxicity of the compound tested, can be expressed by the following formula:

% Cell viability = [OD (490 nm) compound tested / OD (490 nm) negative control] x 100

In particular, 8000 cells have been placed in 96-well multiwell and subjected to fasting and chemotherapy treatment.

2.4 Total RNA extraction and analysis

Treated cells were taken and subjected to RNA extraction through QUIAZOL and following the MiRNeasy Mini Kit – QUIAGEN protocol. This kit is useful to purify total RNA, including small RNAs (such as miRNAs), from animal cells and tissue. Then mRNA and miRNAs have been obtained from the extraction. MiRNAs were utilized for comparative analysis through the microfluidic cards while mRNAs were used for the evaluation of factor gene expressions involved in angiogenesis and of miRNAs targets. Acid nucleic integrity has been assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). MRNA and MiRNA extraction procedures provided for the reverse transcription of RNA to cDNA. In the case of miRNAs it was required the TaqMan® MicroRNA Reverse Transcription Kit-ThermoFisher Scientific. MRNA needed the High Capacity Reverse Transcription kit – ThermoFisher Scientific. Finally, cDNA analysis was realized through by RT-PCR with the 7900HT Sequence Detection System.

Since Angiogenesis (a process of new blood vessel formation from pre-existing vascular networks)[46] represents a critic step in cancer progression and in metastasis sprouting, it has been interesting evaluate it in MDA-MB-231 undergone to STS and Doxorubicin treatment. In particular, pro-angiogenic (such as VEGFA) and anti-angiogenic (THBS) factor expression levels were analyzed through TaqMan gene expression assay.

A comparative analysis between miRNA expression profiles of the different experimental conditions were performed through Taqman® Array Human microRNA Microfluidic Cards – ThermoFischer Scientific that focuses on more highly characterized miRNA. Complete coverage of Sanger miRBase v10 is enabled across a two-card set of TaqMan® MicroRNA Arrays for a total of 762 (381+381) unique assays specific to human miRNAs. Each array contains four control assays— three endogenous control assays and one negative control assay. Up- and down-regulated miRNAs that have a Fold Change increased at least to 1.5 and less than 0.5 respectively were considered significant deregulated.

To confirm MiRNAs expression levels has been used the endogenous control snRNA RNU6B. Changes in the expression of miRNAs, based on RNU6B snRNA, has been determined by the comparative Ct method, which calculates changes of coach, in order to have the changes in the fold and in percentage.

Results obtained using cards were confirmed by miRNA targets expression levels with RT-PCR assay. MiRNA targets were searched through database online (MiRbase and Targetscan) or through literature data presented on PubMed.

CHAPTER 3

Results

3.1 Short Term Starvation reduces cancer cells viability

Viability assays, MTT or Trypan Blue assay, have shown that MDA-MB-231 undergone to short term starvation (STS) were less healthy than tumor cells in standard medium (NoSTS). MTT assay was set for three days, observing a noticeable reduction in cell viability after STS in a time dependent way (Fig.2.)

As regards the chemotherapeutic treatment, for this assay two different treatments have been used: Doxorubicin 1γM for 24 hours and Doxorubicin 10γM for 3 hours (Fig.3 and Fig.4).

The association of STS and chemotherapy reduces vitality. Because of the absence of significant difference on cell viability between the treatment with both concentration of Doxorubicin, the Doxorubicin 1γM has been excluded from the experimental protocol.

The same reduction was observed with Trypan Blue assay (Fig.5).

Decreased viability was also evident in cancer cells undergone to STS and treatment for 3h with Doxorubicin 10γM (Fig.6).

In conclusion, it has been observed that Short Term Starvation provokes a reduction in MDA-MB-231 cell viability more evident after chemotherapeutic treatment.

3.2 Short Term Starvation reduces cancer cells proliferation

Proliferation curves during five days showed how fasting (STS) respect to the standard condition (NoSTS) reduces cell viability in MDA-MB-231. Reduction is directly proportional to the time spent in fasting (Fig.7).

The presence of Doxorubicin induces a reduction in cell proliferation more evident in the cancer cells previously subjected to fasting compared to the ones in standard medium (NoSTS) (Fig.8).

Thus it seems that the greatest effect occurs when Short Term Starvation is associated with chemotherapy (Fig.9).

3.3 Short Term Starvation protect mammary tissue against Doxorubicin treatment

Dietary restriction has caused a reduction in healthy cells (MCF10A) viability that was lower than the one observed in cancer cells (Fig.10 and Fig.11).

The interesting data was observed after the establishment of Short Term Starvation and chemotherapeutic treatment with Doxorubicin 10γM. (Fig.12 and Fig.13)

Thus it has been observed that Short Term Starvation induces a reduction in MCF10A cell viability that is lower than the MDA-MB-231 ones and that, in presence of chemotherapy, STS seems to have a protective effect.

STS effect on MCF10A has been confirmed with the realization of growth curves (Fig.14, Fig.15 and Fig.16).

After 3 hours of treatment with doxorubicin 10γM, cell proliferation and viability were reduced but healthy cells undergone to STS were more viable than MDA-MB-231.

3.4 Angiogenetic Evaluation

Through real time PCR and TaqMan gene expression assay, expression levels of angiogenetic factors have been evaluated: Vascular endothelial growth factor A (VEGFA), as pro-angiogenetic factor, and thrombospondin 1 (THBS1), as anti-angiogenic factor. Their expression has been analyzed in cancer and healthy cells after 24h and 48h of STS.

In MDA-MB-231, Short Term Starvation induces the upregulation of both the factor involved in angiogenesis during the first 24h but after 48h VEGFA return to basal levels (Fig.17 and Fig.18).

It is interesting that three hours of treatment with doxorubicin 10γM in presence of STS induced a downregulation of VEGFA (more evident after 48h of STS) and an upregulation of THBS1, indicating that a fasting period concomitant with chemotherapeutic treatment could inhibit angiogenesis (Fig.17 and Fig.18).

Expression levels of VEGFA and THBS1 have been observed in MCF10A after 24h and 48h of short term starvation: in detail, VEGFA and THBS1 were respectively upregulated and downregulated during 24h of STS but their expression returned to basal levels after 48h of STS (Fig.19 and Fig.20).

Similarly for the cancer cells, in healthy cells VEGFA in presence of STS and Doxorubicin 10γM at 24 and 48 hours was downregulated while THBS1 was upregulated. This indicates that even in MCF10A, the association of STS and chemotherapy inhibits angiogenesis (Fig.19 and Fig.20).

3.5 MDA-MB-231 MiRNAs analysis

Expression profiles of miRNA were analyzed through the Taqman® Array Human microRNA Microfluidic Cards – ThermoFischer Scientific and some of them were confirmed by real time PCR of their targets.

MiRNAs expression in tumor cells during 48h Short Term Starvation, in standard medium and the last two conditions but in presence of chemotherapy, has been compared. Given the purpose of this manuscript, the attention has been focused on MDA-MB-231 treated with Doxorubicin (Fig.21).

TaqMan cards assay showed that approximately the 23,36% of miRNAs was significantly deregulated and, among these the 61,8% was downregulated and 38,2% was upregulated (Fig.22). In addition to the deregulated miRNAs, it was present around the 4,46% of miRNAs expressed only in MDA-MB-231 cells undergone in STS and treated with Doxorubicin (Fig.23).

Targets of the miRNAs that observed the selection criteria were searched through online databases (such as miRBase, DianaTOOLS, miRanda and miRò) and evaluated through PubMed.

The miRNAs analysis has shown that data already published in literature are conflicting and that it depends on the cell line in which they are located, tumor grade and experimental conditions probably because each microRNA is able to influence several signaling cascades.

3.5.1 Upregulated miRNAs

Among upregulated miRNAs, some of the most relevant are miR-26a, miR-28-5p, miR-29c, miR-132, miR-149, miR-181a, miR-193a-5p, miR-193b, miR-195, miR-324-3p and miR-455-3p.

Literature data indicate them as tumor suppressors involved in cell proliferation, migration, invasion and in drugs sensitivity/resistance. To confirm card results, the expression of their target genes or the expression level of their mature form have been evaluated.

TaqMan gene expression microRNA assays have been used for a group of microRNAs: miR-MiR-132 (Hs04231496_s1), miR-149 (Hs04231523_s1), miR-181a (Hs04231460_s1), miR-193b (Hs04231604_s1) and miR-324-3p (Hs04273262_s1) (Fig.24).

The other group of miRNA has been defined through their target expression levels:

MiR-26a has been tested through E2F2 TaqMan gene expression assay (Hs00918090_m1), miR-28-5p levels has been confirmed evaluating IGF-1 expression (IGF-1 TaqMan gene expression assay Hs01547656_m1) (Fig.25), MiR-29c expression levels has been proved analyzing VEGFA through TaqMan gene expression assay (Hs00900055_m1) (Fig.17), for miR-193a-5p has been used its target mTOR (TaqMan gene expression assay

Hs00234508_m1), and miR-195 expression levels has been evaluated analyzing its target cyclin D1(CCD1TaqMan gene expression assay Hs00765553_m1). IL-1β expression levels have been assessed by real time PCR (using IL-1β TaqMan Gene Expression Assays Hs01555410) for miR455-3p confirmation (Fig.25).

3.5.2 Downregulated miRNAs

Most downregulated miRNAs act as oncomiRs influencing cell proliferation, invasion, migration and antiblastic response: miR-15b, miR-17, miR-19a, miR-19b, miR-23a, miR-29a, miR-32, miR-103, miR-106a, miR-106b, miR-128, miR-192, miR-494 and miR-503.
MiR-15b and miR-503 block inhibit the expression of Smurf2 and in fact its expression (analyzed through TaqMan gene expression assay Hs00224203_m1) is increased (Fig.25).
PTEN is a confirmed target of miR-17, miR-19b, miR-32, miR-103, miR-106a and miR-106b and its expression (evaluated using TaqMan gene expression assay Hs02621230_s1) seems correlate with their downregulation. MiR-19a downregulation was confirmed with SOCS1 TaqMan gene expression assay (Hs00705164_s1) (Fig.25).
TaqMan gene expression microRNA assays have been used for the other miRNAs: miR-23a Hs03659093_s1, miR-29a Hs03849009_s1, miR-128 Hs04231535_s1, miR-192 Hs04231449_s1, and miR-494 Hs04231605_s1 (Fig.24).

3.5.3 Specific miRNAs

Specific RNAs are significant because they identify those changes that occur simply under experimental conditions.
In this group there are: miR-148a, miR-154, miR-194, miR-361-5p and miR-449b. Two among these MiRNAs have been defined through TaqMan gene expression microRNA assay: miR-148a (Hs04273238_s1) and miR-154 (Hs04231525_s1) (Fig.24).
MiR-449b and MiR-194 expression levels have been confirmed with the evaluation of CCND1 expression levels (TaqMan gene expression assay Hs00765553_m1) (Fig.25) and miR-361-5p with VEGFA TaqMan gene expression assay (Hs00900055_m1) (Fig.17).

3.6 MCF-10A MiRNAs expression analysis

The analysis of miRNAs in MCF10A occurred evaluating expression levels of miRNAs involved in cell proliferation, migration, and invasion in response to chemotherapy using Real time PCR.
Tumor suppressors miR-132, miR-148a and miR-154 were upregulated in MCF10A. (Fig.26) MiR-361-5p and miR-449 were upregulated and this was confirmed respectively

through VEGFA TaqMan gene expression assay (Hs00900055_m1) (Fig.19) and CCND1 TaqMan gene expression assay (Hs00765553_m1) (Fig.27).

We decide to evaluate expression levels of miRNAs involved in chemotherapy response in MCF10A. Unlike in MDA-MB-231, miR-26a, miR-106b, miR-128 and miR-192 were no deregulated while the others were heterogeneously expressed. MiR-15b, miR-29a, miR-181a, miR-195, and miR-494 were downregulated and miR-23a, miR-149, miR-193b, and miR-324-3p were upregulated microRNAs. TaqMan gene expression assays that quantify miRNA levels were used to confirm MiRNAs: miR-15b (Hs04231486_s1), miR-23a (Hs03659093_s1), miR-29a (Hs03849009_s1), miR-149 (Hs04231523_s1), miR-181a (Hs04231460_s1), miR-193b (Hs04231607_s1), miR-195 (Hs03656088_s1), and miR-324-3p (Hs04273262_s1) (Fig.26).

MiR-494 downregulation was verified through PTEN TaqMan expression level assay (Hs02621230_s1) (Fig.27).

CHAPTER 4

Discussion

Short Term Starvation (STS) represents a feasible tool in preventing and protecting against the onset and the progression of age-related diseases and an ally for drugs treatment. To understand the protective effect derived from the establishment of STS, it is essential to identify genes and/or signaling pathways that are subjected to changes during nutrient deprivation conditions.

The lack of nutrient represents a trigger of autophagy, a way that allows to keep active fundamental biological processes at the expense of the superfluous ones.[12]. Poly(ADP-ribose) polymerase (PARP-1), a nuclear enzyme activated by DNA damage, is a mediator of STS-related autophagy induction. Moreover, during STS, the production of reactive oxygen species (ROS) could actives PARP-1.[47]. The latter factor is involved in ADP hydrolysis and thus a PARP-1 lack not decreases ATP levels as much as when it works. [48]. The permanence of the active state of AMPK and, consequently, the absence of mTOR signaling inhibition, cause an impaired autophagy. Some authors suggest that, in vivo, PARP-1 regulates autophagy. In conclusion STS, ROS production and DNA damage, induce PARP-1 activation that represent an important step for starvation-induced autophagy.[47]

The present study shows that STS, alone or in association with Doxorubicin treatment, inhibits cell proliferation and viability in MDA-MB-231, a triple negative breast cancer (TNBC) cell line, and, even though it is less evident, in MCF10A, a healthy mammary gland cell line. This is consistent with literature data.[2, 49]

It seems that 48 hours of STS, especially when associated with chemotherapy, affects angiogenesis reducing expression levels of the pro-angiogenetic factor, VEGFA, and upregulating the anti-angiogenetic ones, THBS1. This suggesting that a short term dietary restriction protocol could be useful reducing angiogenesis promotion inducted by nutrient lack and thus inhibiting new vessels sprouting and consequently metastasis formation.[46]

This concept can be considered innovative because, currently, there are no investigations that correlate STS approach to miRNAs expression profile analysis in MDA-MB-231 cell line.

Moreover, it contemplates comparative evaluation between MDA-MB-231 and MCF10A miRNAs expression profile.

MiRNAs are non-coding RNAs involved in different physiological and pathological processes including tumorigenesis and drugs resistance/sensitization. The understanding of miRNAs involvement can be useful to outline new strategies of target therapy directed to enhance antiblastic treatment effects modulating miRNAs activity. Actually, this assumption is unworkable because investigations related to dietary restriction approaches and miRNA functions are required. In fact, it is necessary to define STS protocols (consisting of food amount and fasting period length) suitable for humans, especially for cancer patients already prone to lose weight.

In addiction microRNAs analysis present difficulties related to their multifunctional nature: a single factor can be target of numerous miRNAs. An example is the cyclin D1that is regulated by miR-194, miR-195 and miR-449b.

Information in database online (including Pubmed) are often in conflict and show that miRNAs activity depends on cell line features. Examples are the downregulated miR-16, miR-100, miR-199a-3p, the upregulated miR-522 and the specific miR-23b. In detail, some authors [50-52] showed that miR-16 arrested cell proliferation and induced apoptosis targeting cyclin E1, Bcl-2 and FEAT in breast cancer (BC), Hepatocellular carcinoma (HCC) and lung cancer (LC), while, other researchers [53, 54] observed that miR-16 promoted cell proliferation and inhibited apoptosis in esophageal squamous cell carcinoma (ESCC) and renal cell carcinoma (RCC) targeting RECK and SOX6 mRNA. MiR-100 induced epithelial mesenchymal transition (EMT) and inhibited tumorigenesis, migration, invasion in BC targeting SMARCA5 (downregulating E-cadherin) and HOXA1 [55] but it inhibited apoptosis regulating MTMP3-p27 pathway in SK-BR-3 cells compared with other human BC cell lines (MCF7, MDA-MB-453, T47D, HCC1954 and SUM149).[56] MiR-199a-3p improved gastric cancer (GC) progression targeting ZHX1 [57] and inhibited apoptosis in CRC silencing nemo like kinase (NLK)[58] while it acted as a tumor suppressor in ovarian cancer (pmid:25839163) and blocked glioma cell progression regulating Akt/mTOR signaling pathway.[59] MiR-522 correlated with an increased tumor cell proliferation in HCC by targeting dickkopf-1 (DKK1) and secreted frizzled-related protein 2 (SFRP2)[60] but provoked cell cycle arrest in G1 step, cell detach without anoikis and the acquisition of properties mesenchymal in different BC phenotypes[61] DIANA tools point out that Cyclin-dependent kinase 6 (CDK6) is a target of miRNA and this can explain cell cycle arrest. Finally, MiR-23b acted as tumor suppressor in RCC[62] while it improved gastric cancer progression.[63]

Preliminary analysis of the results obtained in this work indicate that STS has an effect on miRNAs expression profiles that maybe influence cell proliferation, migration, invasion and

drugs response. In particular, it seems that a short fasting period induces the expression or the upregulation of microRNAs with tumor suppressive activity and the downregulation of those that stimulate tumor progression.

Among upregulated miRNA there are miR-28-5p, miR-29c, miR-30b, miR-130a, miR-132, miR-193a-5p, miR-195, miR-331-3p, miR-374 and miR-455-3p.

MiR-28-5p inhibited metastasis and tumor growth inactivating PI3K/Akt through its target IGF-1.[64] MiR-29c is a tumor suppressor able to inhibit cell proliferation, migration, invasion in lung cancer cell line 95C, silencing integrin β1 and MMP2 [65] and angiogenesis in glioma targeting VEGFA.[66] MiR-30b inhibited NSCLC cells through a negative regulation of Rab18[67] and plasminogen activator inhibitor 1 (PAI) in non-small cell lung cancer (NSCLC) and gastric cancer (GC).[68] MiR-130a inhibited cell proliferation, migration and invasion targeting RAB5A in BC.[69] MiR-132 is a tumor suppressor able to inhibit cell proliferation, invasion, migration and metastasis in BC by targeting Hematological and Neurological Expressed 1 (HN1)[70, 71] Similar results have been found in osteosarcoma, through the silencing of SRY-Box 4 (Sox4)[72] and HCC by the inhibition of Phosphoinositide-3-Kinase Regulatory Subunit 3(PIK3R3). [73] Literature data describe miR-193a-5p levels lower in colorectal cancer (CRC) than in healthy tissue and more reduced in presence of lymph node metastases. Authors have observed that it suppresses metastasis in osteosarcoma repressing Rab27B and SRR through inhibition of TGFβ, Myc/Max and ATF2/ATF3/ATF4 signaling pathways.[74] Moreover miRNA-193a-5p upregulation in non-small cell lung cancer (NSCLC) inhibited migration, invasion and epithelial mesenchymal transition (EMT). MiR-193a-5p, together with miR-193a-3p, acts as tumor suppressor probably inactivating Akt/mTOR signaling. Unlike the miR-193a-5p, miR-193a-3p seems not to be influenced by STS.[75] MiR-195 is a miRNA identified as tumor suppressor in different studies and it inhibited cell proliferation, invasion and migration in NSCLC [76] and in prostatic cancer (PC) targeting breast cancer-overexpressed gene 1 (BCOX1),[77] Ribosomal protein S6 kinase beta-1 (RPS6KB1)[78] and Checkpoint kinase 1 (Chk1). Moreover, miR-195 was downregulated in BC and its reset suppressed cell proliferation and invasion and increased apoptosis through the silencing of the proto-oncogene Raf1, cyclin 1 (CCDN1), Bcl-2 and P-glycoprotein.[79] MiR-331-3p has been indicated as a tumor suppressor that inhibited cell proliferation in glioblastoma targeting neuropilin-2 (NRP-2) and in GC, silencing E2F1.[80, 81] MiR-374b suppressed cell proliferation and promoted apoptosis in t-cell lymphoblastic lymphoma repressing Akt1 and Wnt16.[82] Direct interaction of miR-455-3p with IL-1β is predicted by DIANA-microT and miRBase servers. IL-1β gene located on chromosome 2 and encoding a protein of 269 aminoacids is a chief regulator of the body's inflammatory response and is produced as a consequence of an injury and antigenic challenge. A previous experience showed that IL-1β

secreted by macrophages, an important stromal component in many breast cancers, stimulated a ROS/Src/MAPK/AP-1 pathway inducing the increased COX-2 levels.[83] Moreover, levels of IL-1β are higher in invasive breast cancers than in ductal carcinoma in situ or benign lesions, and IL-1β content in BC correlated with the degree of macrophage infiltration.[84].

As regards downregulated miRNAs after 48h STS and 3h Doxorubicin treatment there are miR-503, miR-15b, miR-17, miR-18a, miR-19a, miR-19b, miR-21, miR-103, miR-106a, miR-135, miR-200b and miR-744.

Literature data showed that miR-503 improves tumor progression in CRC [85] and in esophageal cancer.[86] Li Y. et al indicated that SMAD specific E3 ubiquitin protein ligase 2 (smurf2) and SMAD family member 7 (SMAD7) are miR-503 targets. Smurf2 and SMAD7 are two regulators of TGF-β that has an oncogenic activity promoting metastasis in breast cancer. Another miRNA that targets Smurf2 is miR-15b that promotes EMT in pancreatic cancer.[87] Thus miR503 and miR-15b suppressed Smad7 and Smurf2 leading to enhanced TGFβ signaling and metastatic capability of breast cancer cells.[88] These results seem to confirm the downregulation of miR-503 and miR-15b.

MiR-17 suppresses tumor growth and metastasis in osteosarcoma.[89] MiR-18a increased cell proliferation, migration and invasion in GC (targeting IRF2 leading to p53 suppressing)[90] and it weakened miRs biogenesis in nasopharyngeal cancer regulating Dicer1.[91] Moreover the inhibition of miR-18a in U87 and U251 glioblastoma cell lines upregulated neogenin leading to a reduced cell proliferation, invasion and migration and to an improved apoptosis.[92] MiR-19a resulted upregulated in different types of malignant cancer (such as CRC,[93] RCC,[94] GC[95], NSCLC[96] and bladder cancer[97] where provokes tumor necrosis factor α (TNFα) induced EMT, metastasis formation through activating PI3K/Akt pathway and inactivating STAT3 through its target SOCS1. MiR-19b increased the expression of PI3K acting like an oncogene.[98, 99] MiR-21 was upregulated in BC (including TNBC), CRC, and other types of malignancy where acted like oncomiR targeting STAT3 and promoting PI3K/Akt pathway[100-103]

MiR-32 that improves tumorigenesis (targeting Kruppel-like factor 4) and cell proliferation[104, 105] and of miR-103 that promotes tumor progression and metastasis in CRC targeting both also death-associated protein kinase (DAPK) and Kruppel-like factor 4 (KLF4).[106, 107] MiR-106a acts inducing cell proliferation and inhibiting apoptosis by the regulation of JNK/MAPK pathway in glioma[108] and improves migration and invasion in NSCLC though phosphatase and tensin homolog (PTEN) silencing.[109] MiR-135 improved cell proliferation in endometrial cancer, targeting FOXO1[110], in BC, suppressing midline1 (MID1) and mitochondrial carrier 200homolog 2 (MTCH2),[111] and in CRC targeting TGFBR2.[112] MiR-200b is another oncomiR that in CRC promoted cell

proliferation reducing reversion-inducing cysteine-rich protein with kazal motifs (RECK) and leading to the upregulation of S-phase kinase-associated protein 2 (SKP2) and the degradation of p27.[113] MiR-744 increased tumorigenesis in pancreatic cancer cells targeting negative regulators of cancer activating Wnt/β-catenin pathway, such as secreted frizzled-related protein1 (SFRP1), glycogen synthase kinase 3β (GSK3β) and transducing-like enhancer of split (TLE3)[114] and in nasopharyngeal carcinoma through the silencing of Rho GTPase activating protein 5 (ARHGP5).[115]

Of note, the majority of specific miRNAs acts as tumor suppressor providing encouraging results like miR-148a, miR-154, miR-194, miR-296-5p, miR-361-5p, miR-362-5p, miR-429, miR-449b and miR-652.

MiR-148a suppressed metastatic process in both human and mouse triple negative breast cancer cells by targeting two oncogenes: (Wnt family member 1) WNT1 and (Neuropilin-1) NRP1.[116] MiR-148a seems able to inhibit invasion and migration also in ovarian cancer through sphingosine-1-phosphate receptor 1. [117] Moreover, this miRNA was observed correlated with the carcinogenesis in HCC and was downregulated compared with healthy control.[118] Historically, MiR-154 is considered a tumor suppressor. MiR-154 inhibited cell proliferation, migration and invasion in HCC[119], NSCLC[120], CRC[121] and prostatic cancer.[122] This activity is due to its target zinc finger E-box binding homeobox 2 (ZEB2), toll like receptor 2 (TLR2) and High Mobility Group AT-Hook 2 (HMGA2). Another tumor suppressor is miRNA-194 and it affected PI3k/Akt/FPXP3a pathway reducing CCD1 and increasing p21.[123, 124] It also inhibited cell proliferation in HCC and in CRC by targeting MAP4K4.[125, 126] MiR-296-5p reduced cell proliferation and viability in NSCLC, BC and PC. Some of its targets are polo-like kinase 1(PLK1) and Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1(PIN1).[127-129] MiR-361-5p is a tumor suppressor that arrested cell cycle in NSCLC and in PC targeting signal transducer and activator of transcription 6 (STAT6)[130, 131] and inhibited angiogenesis decreasing VEGFA. MiR-362-5p inhibited cell proliferation and migration in neuroblastoma targeting PI3K-C2β.[132]. MiR-429 prevented cell migration and invasion in CRC[133], in glioma[134], in MDA-MB-231[135] and in cervical cancer[136] silencing big mitogen-activated protein kinase 1 (BMK1), and consequently GSK3β, ZEB1 and CRKL, a kinase harboring SH2 and SH3 domain. MiR-449b that inhibited cell proliferation in CRC (SW1116) through the downregulation of Cyclin-D1 (CCND1) and E2F Transcription Factor 3 (E2F3).[137, 138] Moreover, high levels of miR-449 was associated with recurrence after prostatectomy [139] and act as tumor suppressor also in retinoblastoma.[137] Finally, miR-652 inhibited ZEB1 leading to a suppression acidity-induced EMT in Prostatic cancer cells.[140]

In oncology it is essential to develop a strategy that makes more effective and tolerable chemotherapeutic treatment. Literature data showed that MiRNAs are able to affect chemotherapy response and it seems that short term starvation influence their expression. In MDA-MB-231 cells undergone to 48hours of STS and treated with doxorubicin, the expression profiles of miRNAs are modulated so as to make the cancer cells more sensitive to antiblastic therapy.

Among upregulated miRNAs in STS+Doxo condition there are: MiR-26a, miR-149, miR-181a, miR-193b, miR-195 and miR-324-3p.

MiR-26a sensitized nasopharyngeal carcinoma to irradiation blocking IL-8/STAT3 pathway[141] and increased gastric cancer cells response to cisplatin targeting NRas and E2F2.[142]

MiR-149 is a tumor suppressor able to increase chemosensitivity to Temozolomide treatment through a remodeling of cellular cytoskeleton RAP1B-mediated in glioblastoma[143] and suppressed metastasis in BC and in HCC targeting actin-regulatory protein (PPM1F)[144, 145]

MiR-181a sensibilized GC cells to Cisplatin treatment,[146] and enhanced Adryamicin-inducted apoptosis targeting Bcl-2.[147]

MiR-193b levels were lower in BC Doxorubicin-resistant. The restoring of its expression reduced myeloid cell leukemia-1 (MCL-1) and sensitized cancer cells to Doxorubicin.[148] Moreover miR-193b has been indicated downregulated in MDA-MB-231 and MCF-7[149] so it is evident that the combination of Doxorubicin and STS acts by increasing it.

MiR-195 increased Adriamycin sensibility downregulating Raf.[150]

Finally, miR-324-3p was downregulated in chemoresistant patients compared to those sensitive and its target is SMAD7, an antagonist of TGFβ receptor, associated with lung-, pancreas, skin cancer.[151]

Among MicroRNAs downregulated involved in chemotherapy response there are: miR-15b, miR-23a, miR-29a, miR-106b, miR-128, miR-192 and miR-494.

MiR-15b was upregulated in lung cancer cell line A549 Cisplatin-resistant and one of its target is phosphatidylethanolamine-binding protein 4 (PEBP4).[152]

MiR-23a improved cisplatin chemoresistance and prevented cisplatin-inducted apoptosis in tongue squamous cell carcinoma through twist. MiR-23a and Twist individually increased cisplatin chemoresistance.[153]

MiR-29a conferred Adriamycin and docetaxel resistance in BC[154] and it induced EMT downregulating tristetraprolin (TTP).[155]

MiR-106b induces radioresistance in cancer cell through the silencing of PTEN and p21 leading to an increased tumorigenesis in CRC.[156] Moreover it was associated with high risk of recurrence, lymph node metastasis and tumor progression in BC.[157]

MiR-128 induces chemoresistance in BC cells targeting BAX (pro-apoptotic).[158]
MiR-192 conferred Cisplatin-resistance in lung cancer cells A549/DDP and inhibit apoptosis binding BIM 3'-UTR.[159]
Finally, miR-494 acts as an oncomiR, promoting cell proliferation, migration and invasion, and increases Sorafenib-resistance in HCC targeting PTEN. As shown in Fig.39, PTEN expression levels support miR-494 downregulation.[160, 161]
Whereas many chemotherapy treatments exert their activities interacting with DNA bases, Medina et al. that evaluated the activity of miRNA-221 and miRNA-222 showing that miRNAs promote cell proliferation and induce quiescence preventing the premature entry into S phase of this action, in the absence of nutrients, may lead to cell death.[162]
Ending, from the experience of my project, I could verify that STS beneficial effects in terms of reduction of cell proliferation, migration, invasion and angiogenesis could be explained by the remodeling of microRNA expression profiles that promotes the expression of tumor suppressor and inhibits that of oncomiR. Also, the results show those miRNAs that let the MDA-MB-231 cells chemosensitive are upregulated. These evidences could lead to the development in the next future of a new class of drugs able to interfere with the oncomiR and/or to improve the expression of selected miRNAs with tumor suppressor activity.
Further analysis is needed involving the expression profiles of miRNAs of MCF10A healthy cell lines to confirm the purposed results and to develop new strategies and tools to protect healthy tissue from chemotherapy cytotoxic effect.

CHAPTER 5

Figures

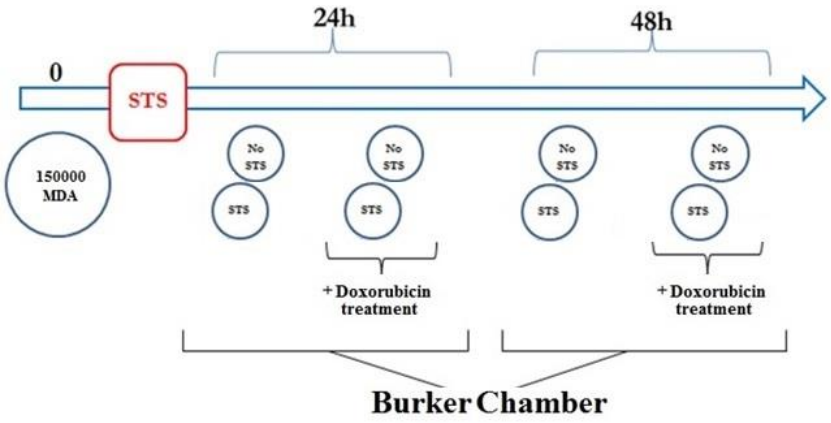

Fig.1: Procedure used to create growth curves

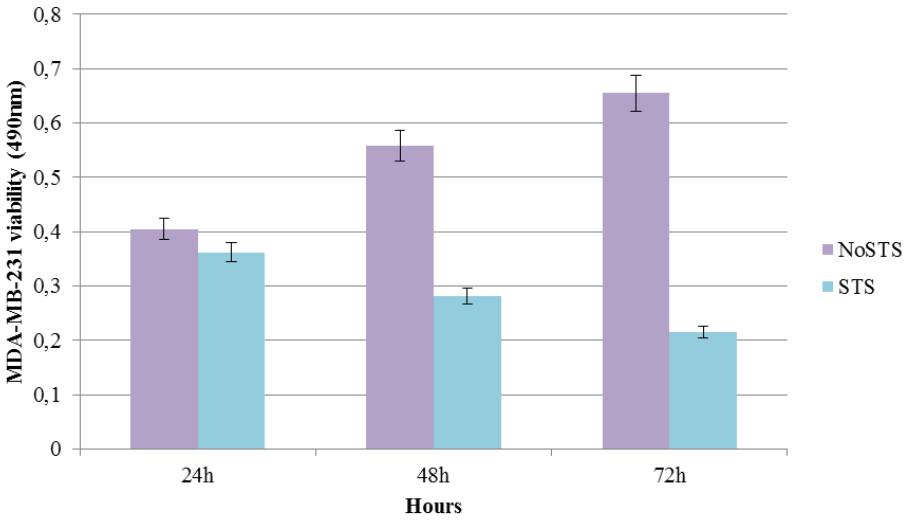

Fig.2: MDA-MB-231 cell viability after 24h, 48h and 72h STS through MTT assay

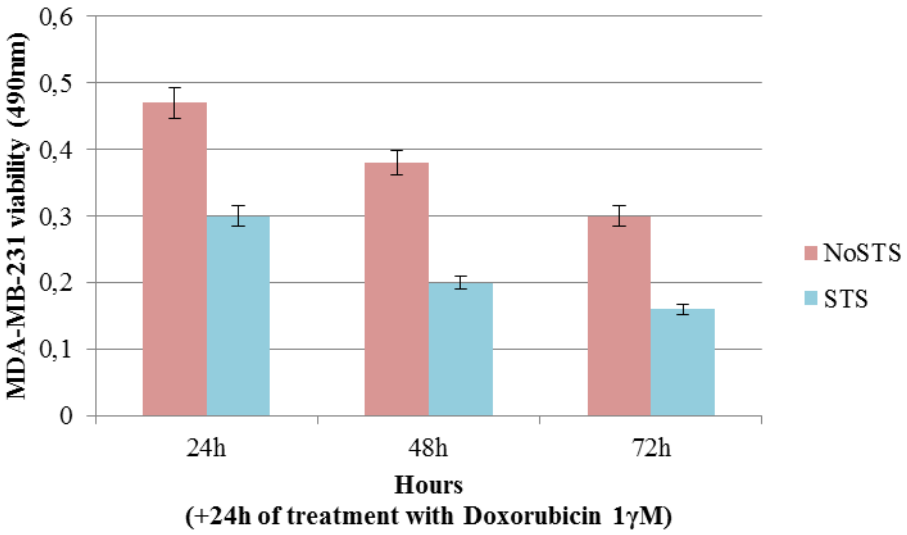

Fig.3: MDA-MB-231 cells viability after 24h, 48h and 72h STS and 24h Doxorubicin 1γM treatment through MTT assay

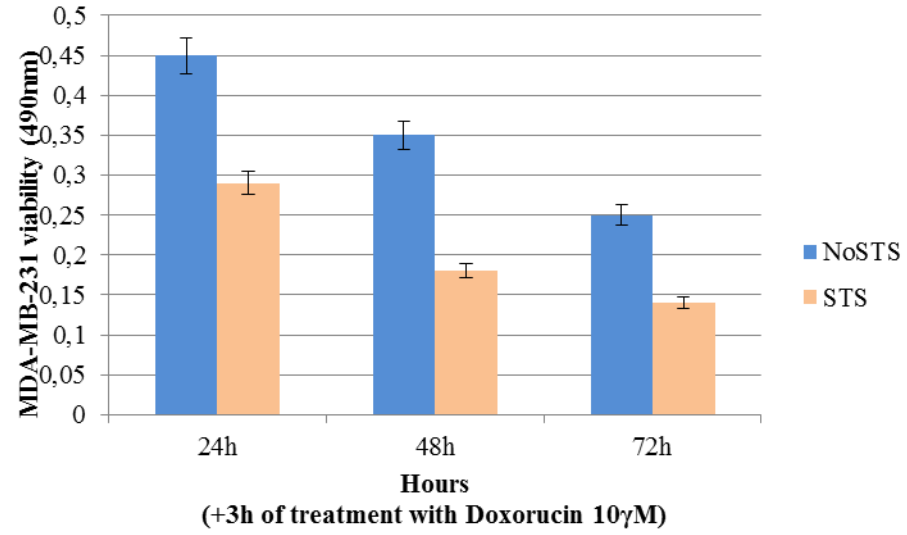

Fig.4: MDA-MB-231 after 24h, 48h and 72h STS and 3h Doxorubicin 10γM treatment through MTT assay

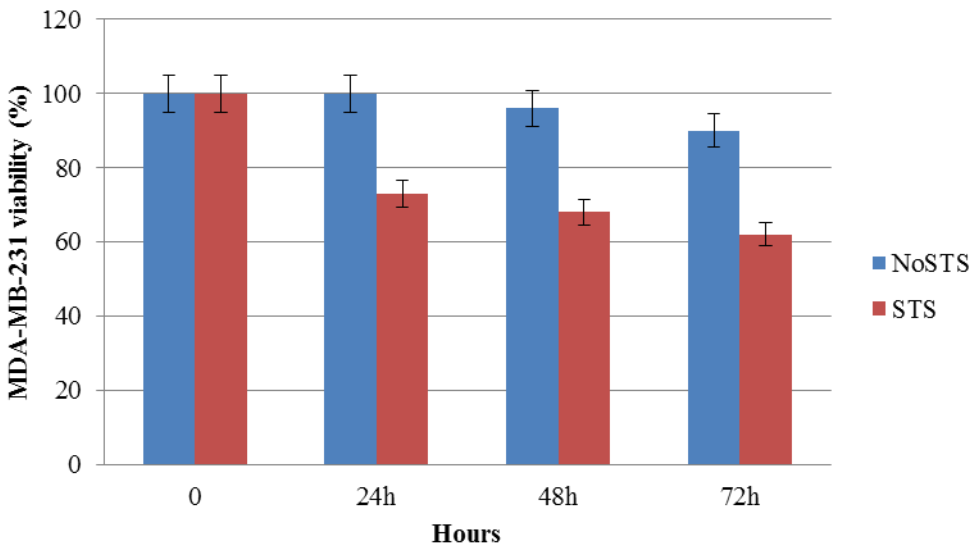

Fig.5: MDA-MB-231 viability after 24h, 48h and 72h STS through trypan blue assay

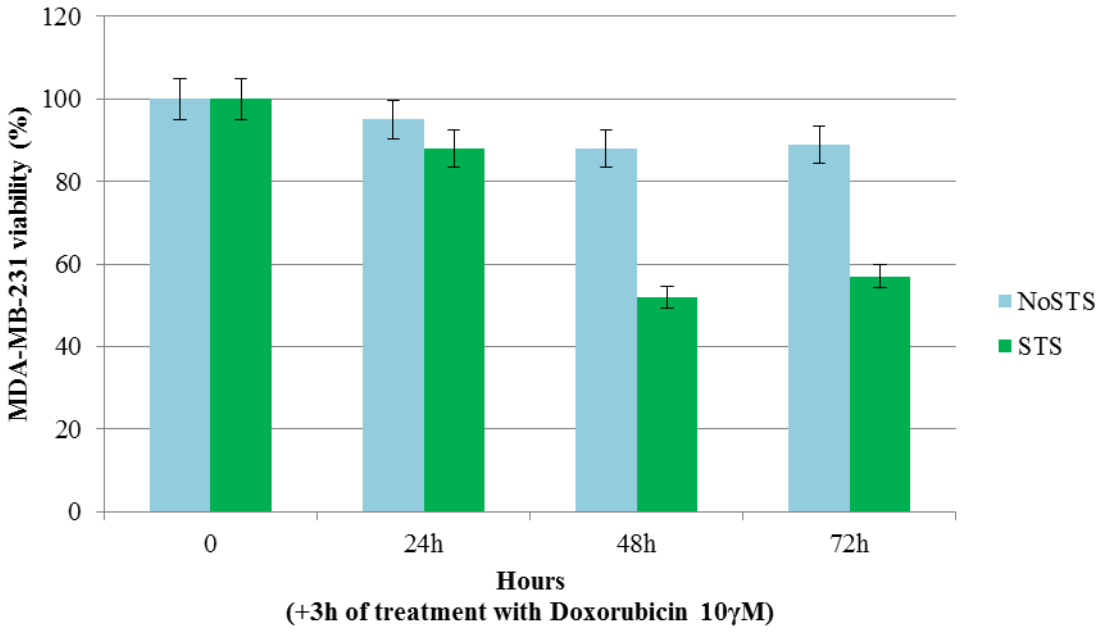

Fig.6: MDA-MB-231 cell viability after 24h, 48h and 72h STS and 3h Doxorubicin 10γM treatment through trypan blue assay

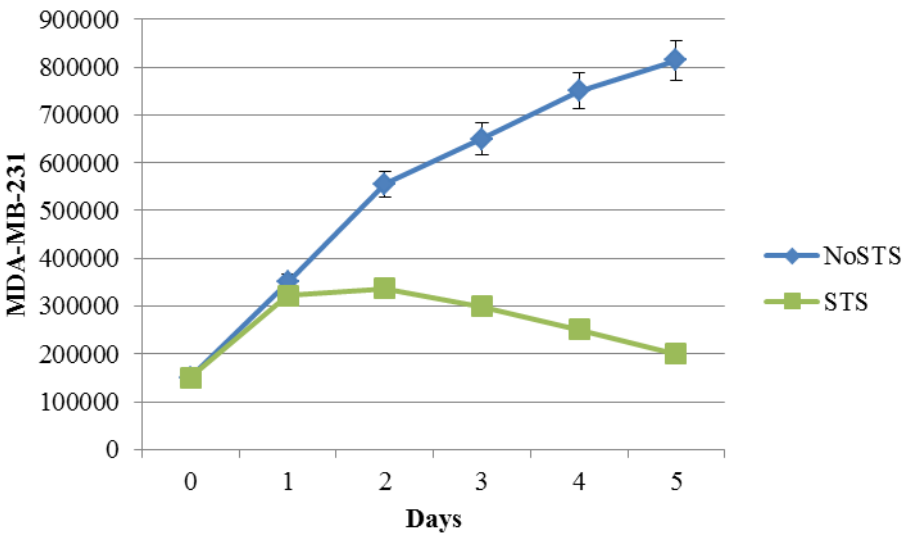

Fig.7: Growth curves of MDA-MB-231 after 1,2,3,4 or 5 days STS

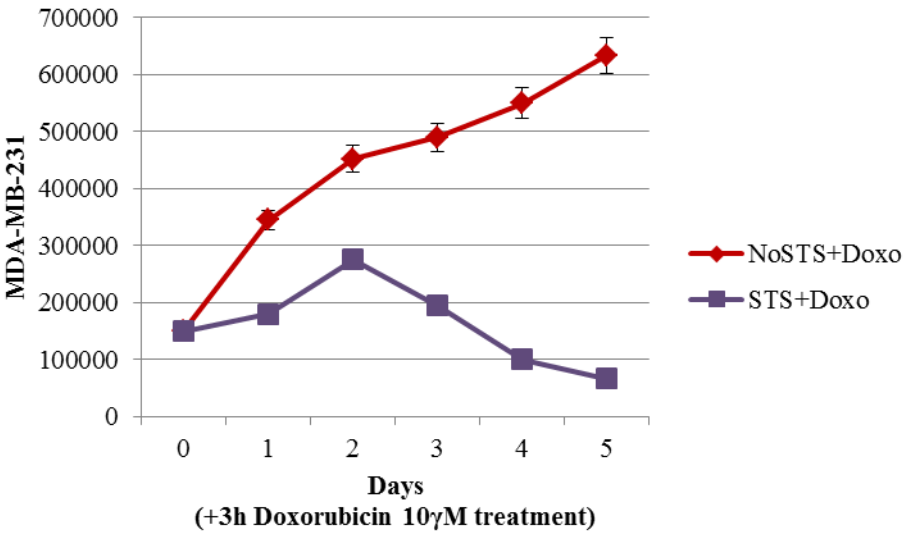

Fig.8: Growth curves of MDA-MB-231 after 1,2,3,4 or 5 days STS and 3h Doxorubicin 10γM treatment

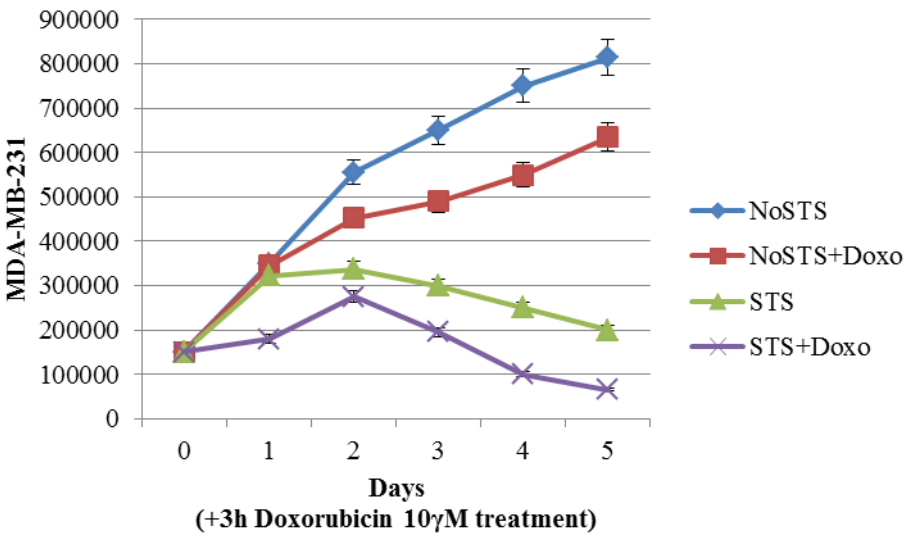

Fig.9: Growth curves of MDA-MB-231 after 1,2,3,4 and 5 days STS with and without 3h Doxorubicin 10γM treatment

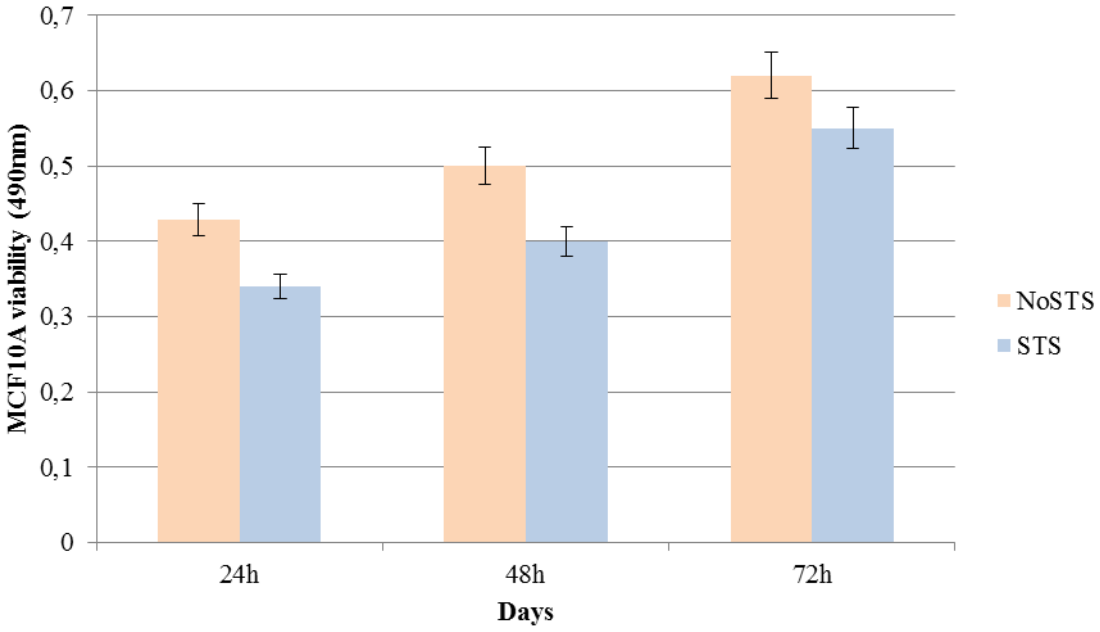

Fig.10: MCF10A viability after 24h, 48h and 72h STS

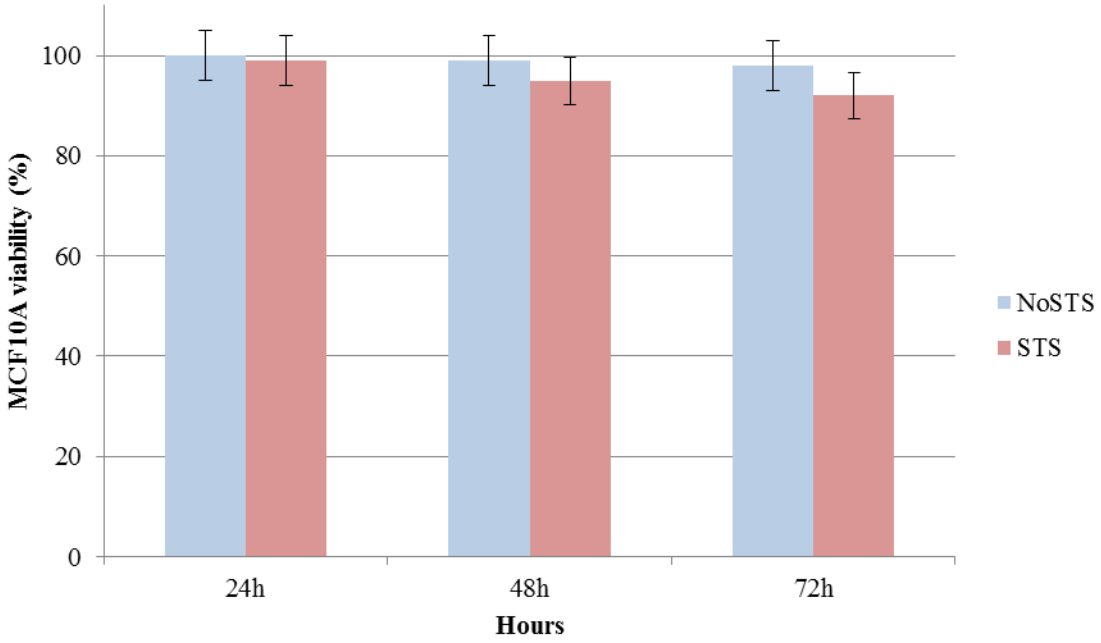

Fig.11: MCF10A viability after 24h, 48h and 72h STS through MTT assay

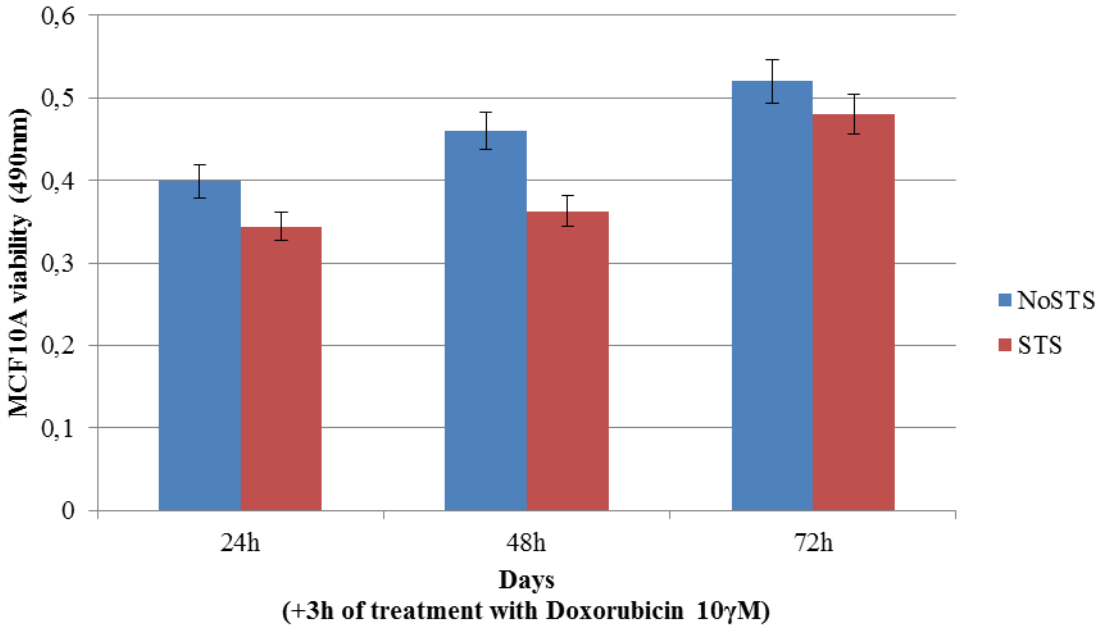

Fig.12: MCF10A cell viability after 24h, 48h and 72h STS and 3h Dororubicin treatment through MTT assay

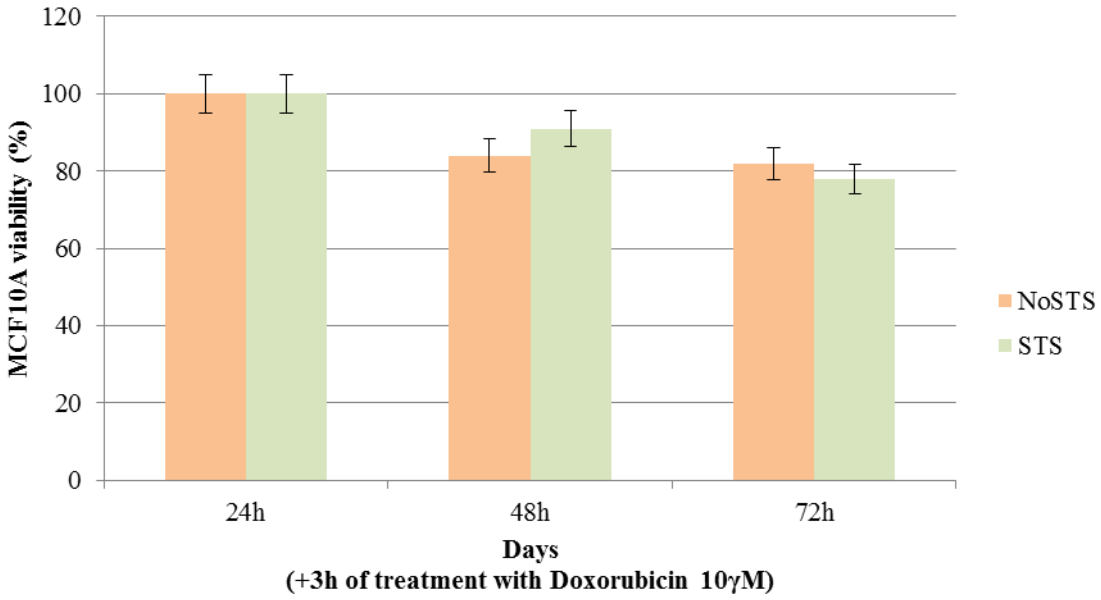

Fig.13: MCF10A cell viability after 24h, 48h and 72h STS and 3h Doxorubicin 10γM treatment through trypan blue assay

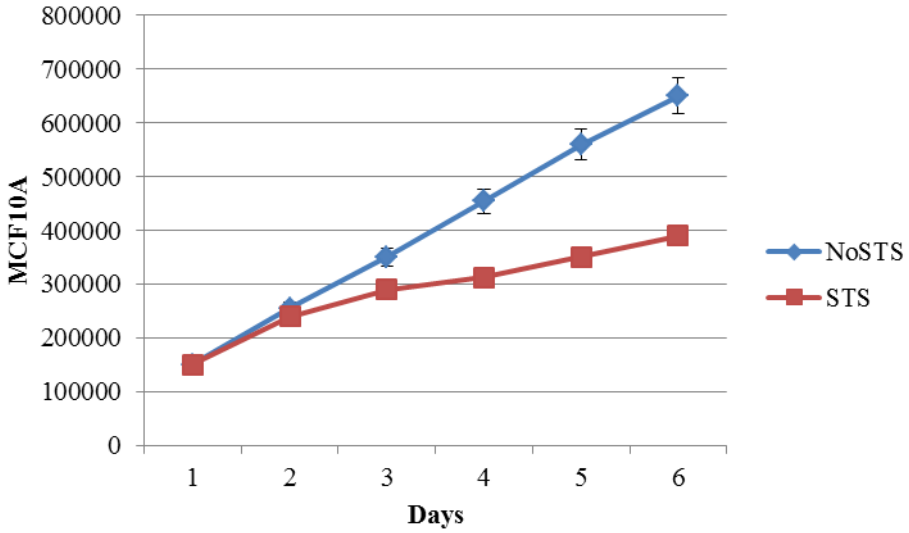

Fig.14: Growth curves of MCF10A after 1,2,3,4 and 5 days STS

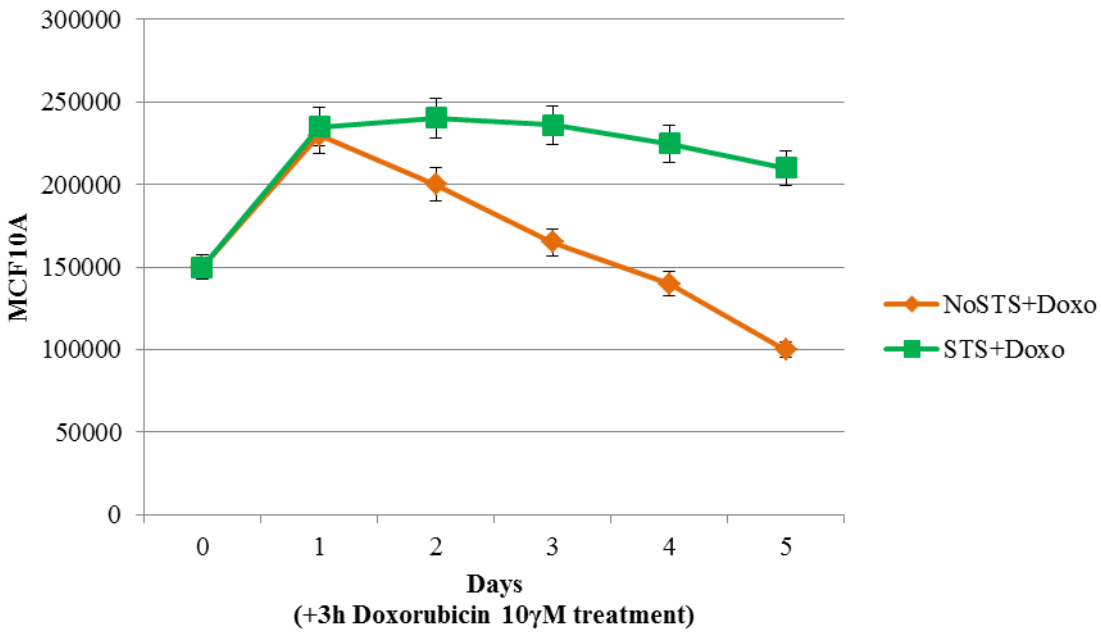

Fig.15: Growth curves of MCF10A after 1,2,3,4 and 5days STS and 3h Doxorubicin 10γM treatment

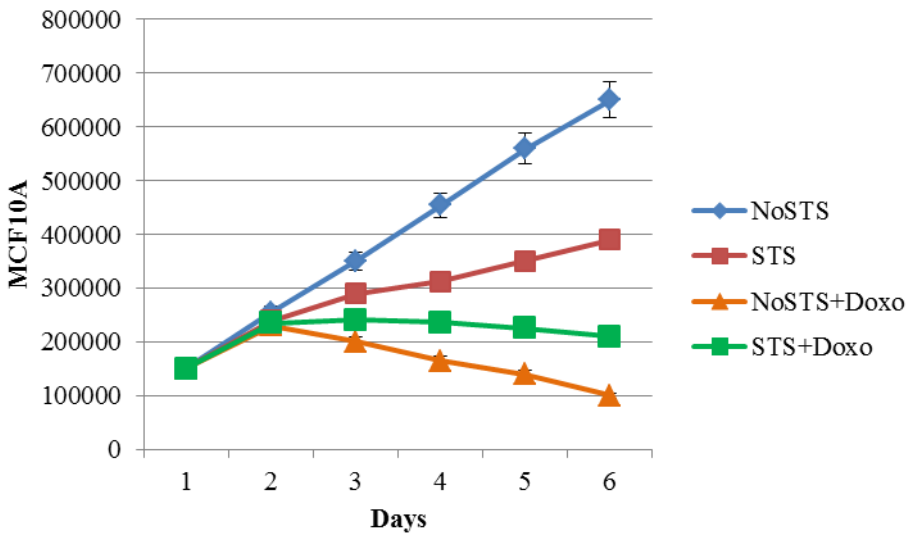

Fig.16: Growth curves of MCF10A after 1,2,3,4 and 5 days STS with and without 3h Doxorubicin 10γM treatment

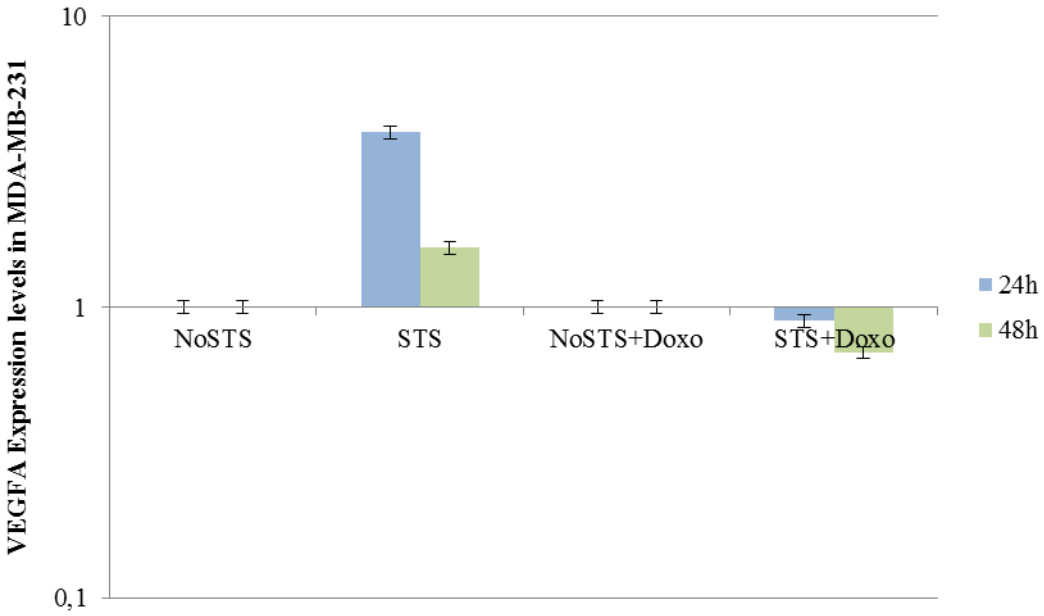

Fig.17: VEGFA expression levels in MDA-MB-231 after 24h and 48h STS and 3h Doxorubicin Treatment

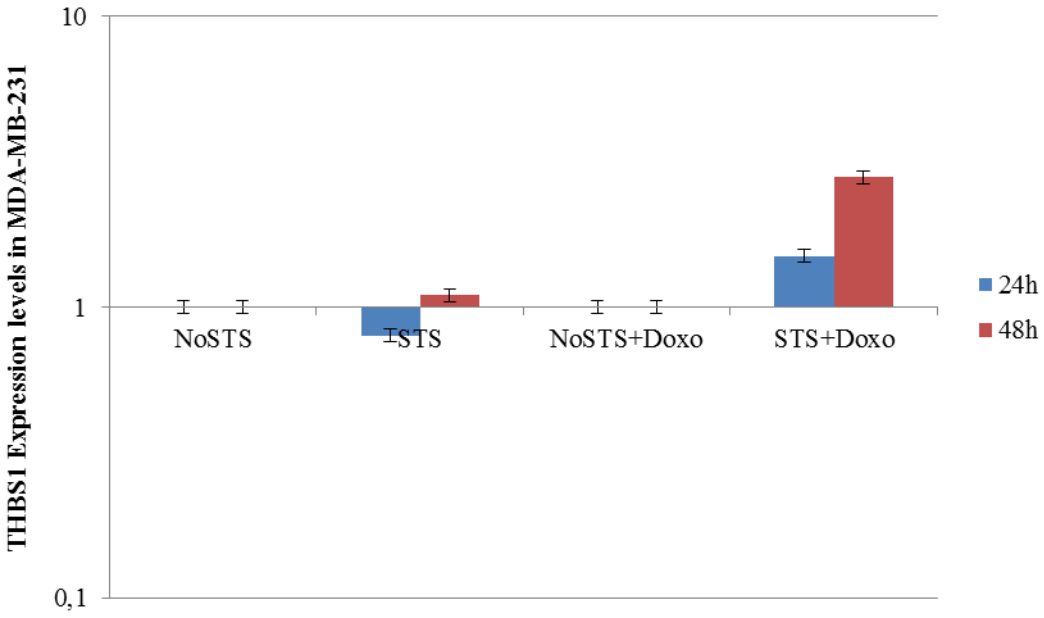

Fig.18: THBS1 expression levels in MDA-MB-231 after 24h and 48h STS and 3h Doxorubicin10γM treatment

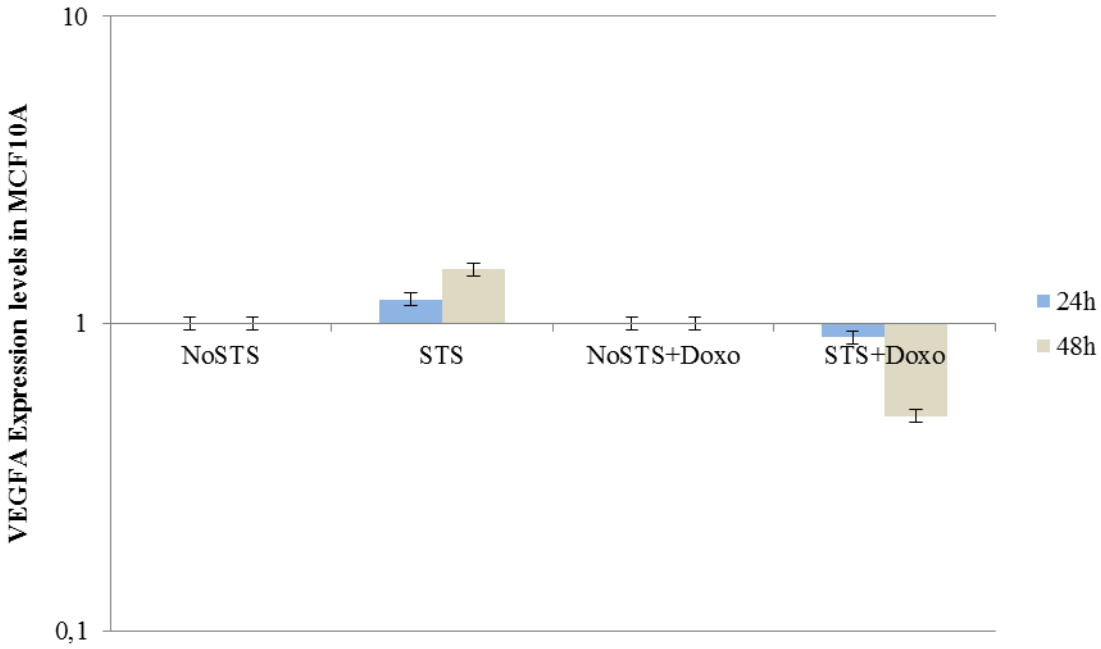

Fig.19: VEFGA expression levels in MCF10A after 24h and 48h STS and 3h Doxorubicin treatment

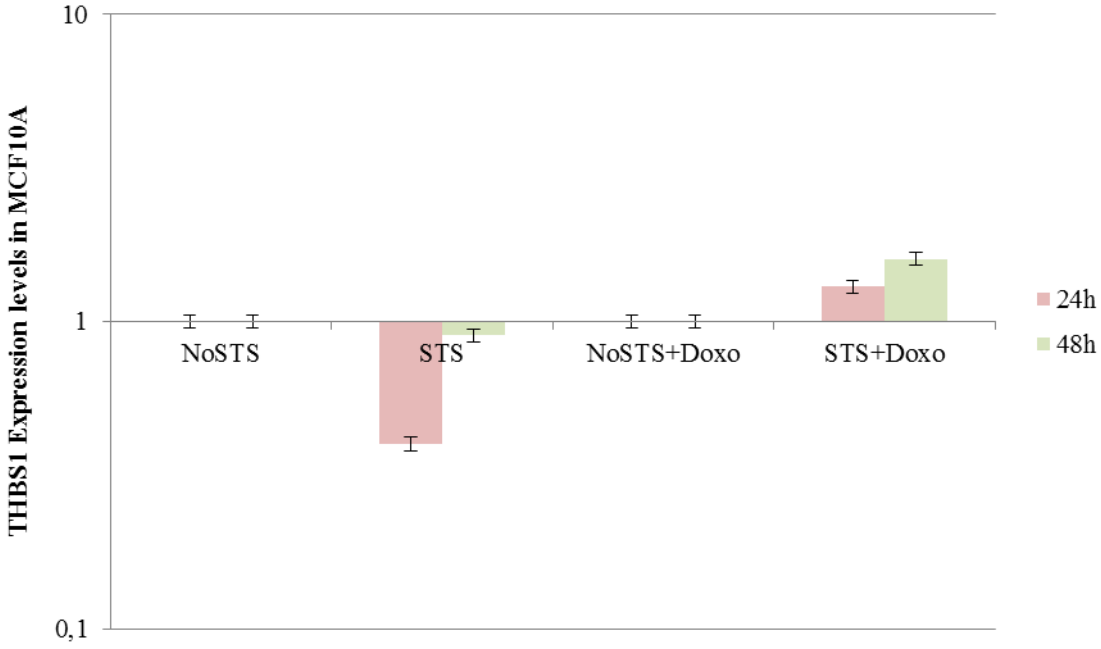

Fig.20: THBS1 expression levels in MCF10A after 24h and 48h STS and 3h Doxorubicin treatment

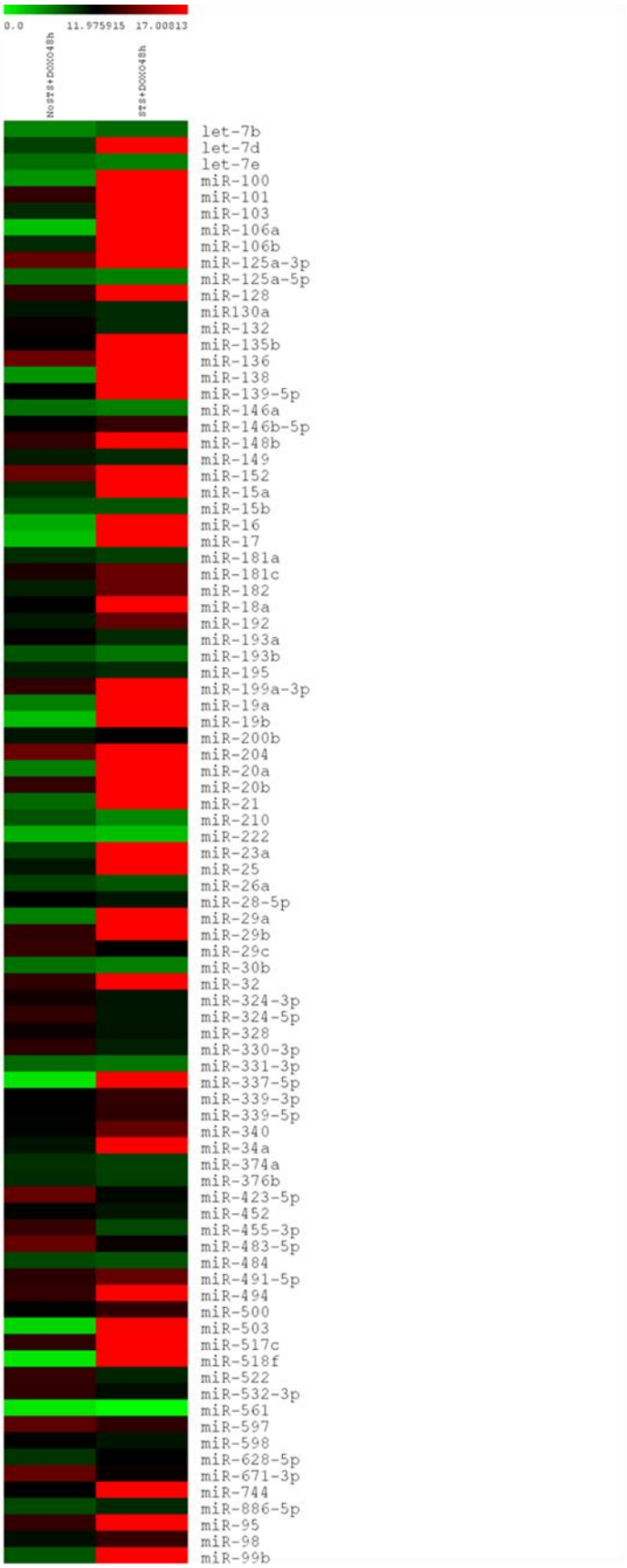

Fig.21: Heatmap of MDA-MB-231 after 48h STS and 3h Doxorubicin 10γM treatment

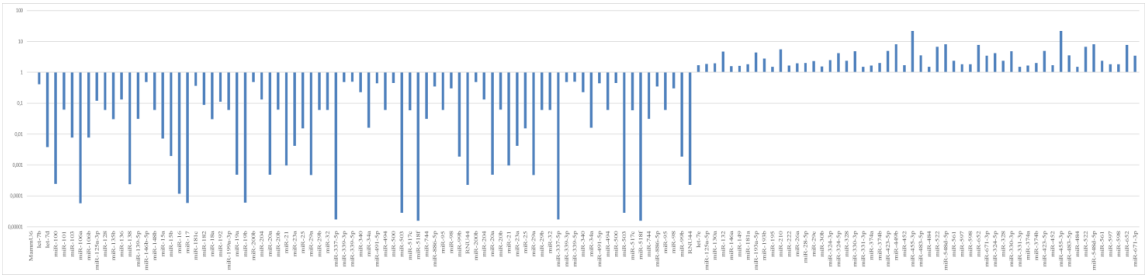

Fig.22: Deregulated miRNA after 48hSTS and 3h Doxorubicin 10γM treatment

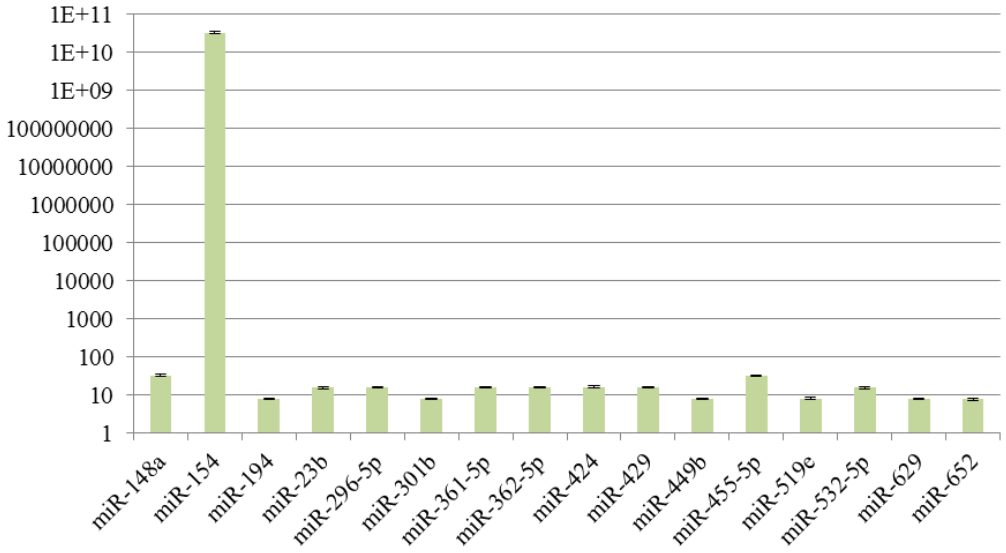

Fig.23: MiRNA specifically expressed in MDA-MB-231 undergone to 48h STS and 3h Doxorubicin treatment

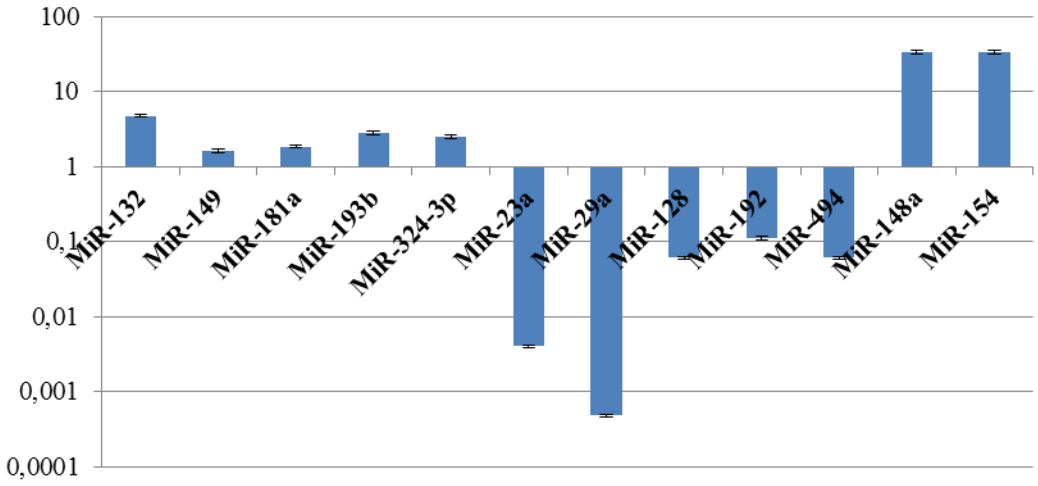

Fig.24: MiRNAs confirmed in MDA-MB-231 after 48h STS and 3h Doxorubicin treatment

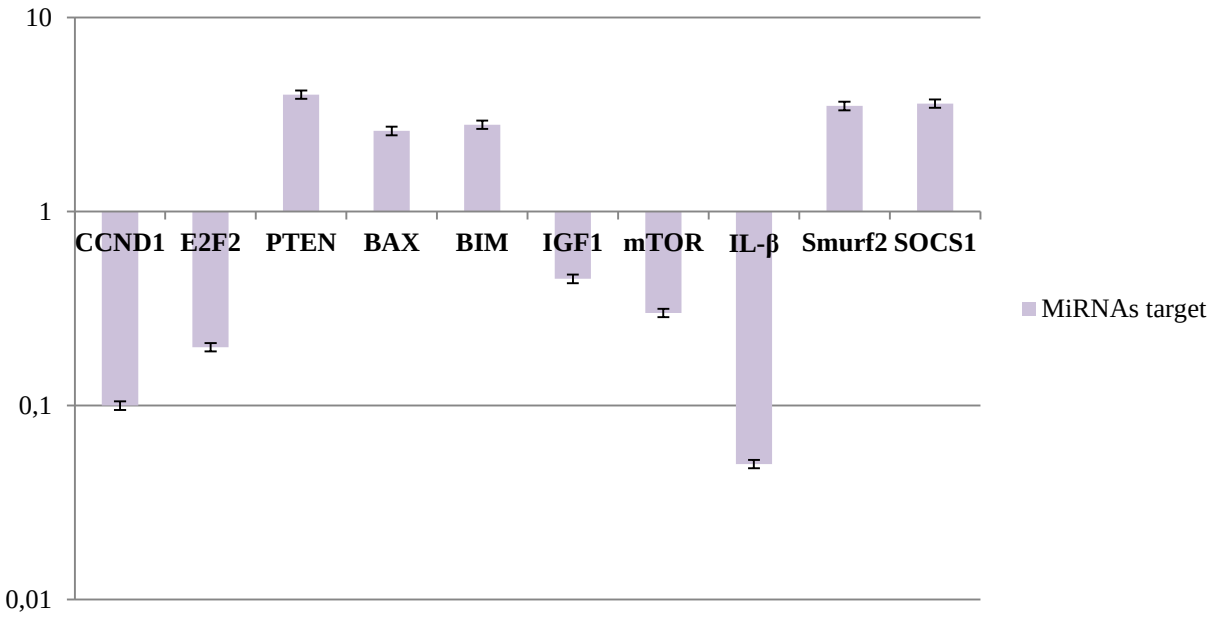

Fig.25: MiRNAs target expression levels in MDA-MB-231 after 48h STS and 3h Doxorubicin treatment

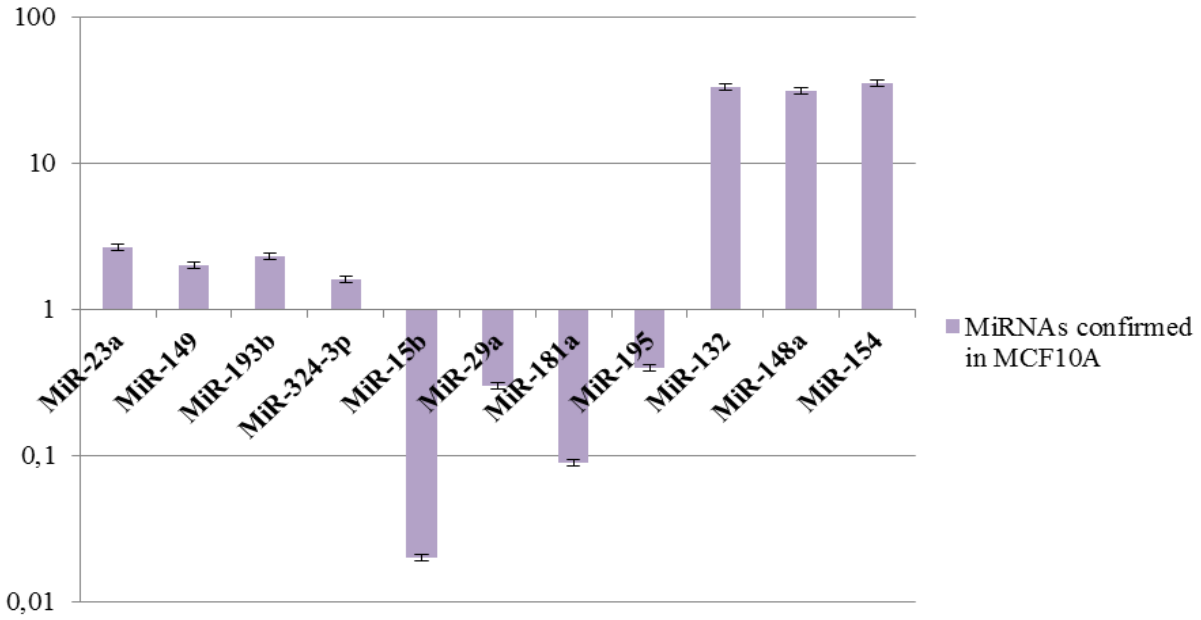

Fig.26: MiRNAs confirmed in MCF10A after 48h STS and 3h Doxorubicin treatment

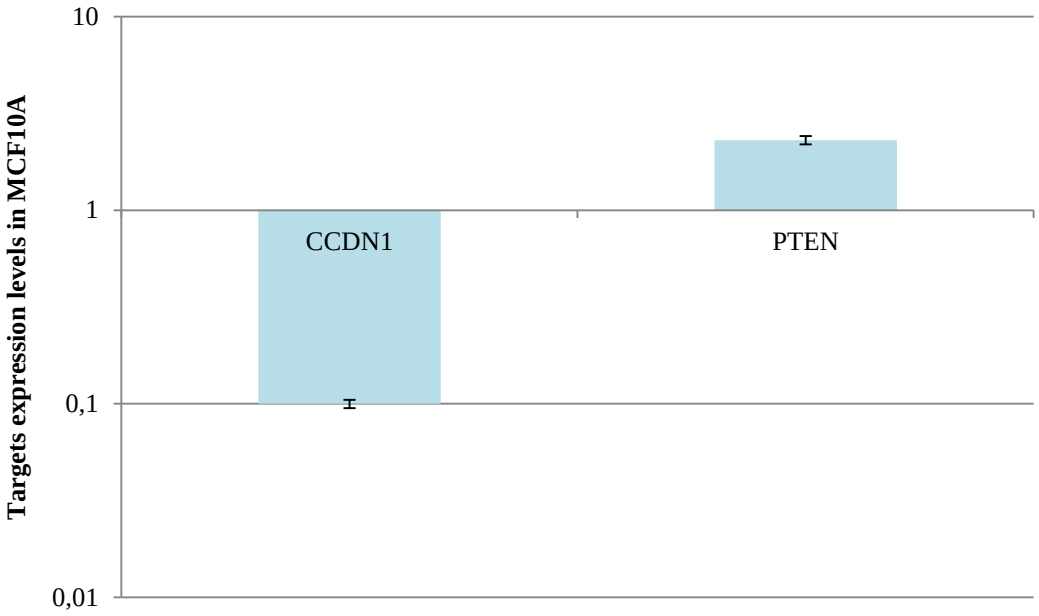

Fig.27: MicroRNAs expression levels in MCF10A after 48h STS and 3h Doxorubicin treatment

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