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**14. ABSTRACT**

**Corticotropin Releasing Factor (CRF) is the major mediator of hypothalamic response to stress. CRF has been detected not only in the central nervous system but also in peripheral tissues and organs and in sympathetic neurons. Even though systemic stress has been reported to affect cancer cell growth primarily due to suppression of the immune response against cancer cells, no information was available on the impact of peripheral CRF on breast cancer. Aim of our studies was to determine the impact of peripheral CRF on breast tumor growth and propose a novel potential mechanism on the crosstalk between the stress-response axis and tumors. The results indicated that CRF induced the expression of beta-catenin and Smad2 affecting Wnt and Tgf-β signaling pathways. CRF also induced actin polymerization and stress fiber formation supporting an effect on cell motility. In vivo administration of a CRF antagonist in a mouse mammary tumor model where mice were subjected to chronic stress, indicated that inhibition of CRF resulted in reduced tumor burden, angiogenesis and metastasis in the liver. Overall, the present work supports that CRF mediates stress-induced breast cancer growth and metastasis.**

**15. SUBJECT TERMS**
Stress, Corticotropin Releasing Factor, Wnt, 4T1 mammary epithelial cells

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## Table of Contents

**Page**

## Introduction

Aim of the grant proposal was to investigate the role of peripheral CRF on breast cancer cell growth and metastasis in vivo, as a potential mediator of the crosstalk between the stress axis and cancer cells. CRF is a neuropetide that mediates the hypothalamic response to stress but it has been reported to be present not only in the central nervous system but also in peripheral tissues and organs (1). CRF exerts its effect primarily via CRF1 and at a lesser extent via CRF2 receptors (1). A recent report demonstrated that Urocortin2, a peptide homologous to CRF and ligand for CRF2 receptor, suppresses tumor growth by inhibiting endothelial cell proliferation and angiogenesis (2). In the present work we tested the role of peripheral CRF on breast cancer cell growth in vitro and in vivo. We therefore utilized a mouse model of orthotopic injection of breast cancer cells and studied the effect of stress on tumor growth and metastasis. In the first part we evaluated the effect of CRF on breast cancer cell growth and motility in cell culture models. In the second part we exposed CRF deficient mice to stress and in the third part we utilized a CRF receptor antagonist to inhibit CRF signaling and determined its effect on tumor growth in the presence or absence of stress exposure.

## BODY

*Task 1: Determine the mechanism of CRF action on tumor cells*

*Results:* 4T1 and 67NR mouse breast cancer cells were cultured and the expression of CRF receptors was analyzed by RT-PCR. The results showed that 67NR cells did not express CRF1 or CRF2 receptors while 4T1 cells expressed high levels of CRF1 receptor and very low levels of CRF2 receptor type b (CRF2b) (**Figure 1**). We, therefore, focused our studies on 4T1 cells, since this would be the cell line responsive to CRF. 4T1 cells were stably transfected with a construct expressing GFP under the control of the CMV promoter. Cells expressing high levels of GFP were selected using neomycin and by selecting single-cell clones that express high levels of GFP. The expression of CRF1 and CRF2b receptors was also confirmed in 4T1 cells that had been selected for GFP expression.

4T1 cells were treated with CRF for different time points and its impact on proliferation was determined. The results indicated that there was a significant induction of 4T1 cell proliferation in the presence of CRF as compared to cells not treated with CRF, becoming evident 48 hours following stimulation (**Figure 2**). To evaluate the effect of CRF on the expression of genes involved in tumor cell growth and metastasis, RNA from CRF treated 4T1 cells was analyzed using a gene-specific oligo microarray for 113 genes known to be involved in tumor growth and metastasis (Superarray, cat# OMM-028). Samples from different time points were analyzed. Among the genes analyzed, significant difference was observed in the expression of several genes as shown in **Figure 3**.

In addition to the genes depicted in Figure 3, differences were observed in the expression of beta-catenin. The potential effect of this stress neuropeptide on beta-catenin and subsequently Wnt signaling may confer a novel mechanism for crosstalk between breast cancer cells and stress neuropeptides. We, therefore, further investigated the impact of CRF on beta-catenin expression at the protein level. The results indicated that CRF rapidly induced beta-catenin expression at the protein level (**Figure 4**). Beta-catenin mediates Wnt signals and affects the transcription of several genes regulated by the TCF/LEF transcription factor complex (3). Since CRF increased beta-catenin expression we expected that it may affect Wnt signaling. For this purpose we employed a reporter construct containing three TCF binding sites upstream of the luciferase gene. A construct with mutant TCF binding sites was used as a control. The plasmids were transfected in 4T1 cells and treated with CRF. Activation of Wnt signaling was

evaluated by measuring luciferase activity in cell lysates. The results indicated a moderate induction of Wnt signaling by CRF (**Figure 5**) providing a potential mechanism for the action of CRF on breast cancer epithelial cells.

GSK-3 is a serine/threonine kinase that phosphorylates nuclear proteins such as β-catenin. GSK-3 negatively regulates β-catenin by phosphorylation, which leads to ubiquitination and degradation by cellular proteases, preventing it from entering the nucleus and activating transcription factors (4, 5). We therefore investigated whether increased β-catenin expression was the result of GSK-3 inactivation in response to CRF. To determine if the observed increased protein levels of β-catenin seen after CRF treatment are caused by increased phosphorylation and thus inhibition of GSK-3, 4T1 cells were treated for 10, 20, 40 and 60 minutes with CRF and western blots for GSK-3 and phosphorylated GSK-3 were performed. As shown in **Figure 6**, CRF treatment did not affect GSK-3 phosphorylation, suggesting that the increased level of β-catenin seen after CRF treatment is not due to increased GSK-3 phosphorylation.

Another gene identified as a target of CRF in the microarray analysis was Smad2. Smad2 is a downstream mediator of TGF-β signaling (6). Smad2 expression was induced at the mRNA level. To confirm that the same effect was evident at the protein level 4T1 cells were treated with CRF for 2, 4, 6, 12, and 24 hours and western blots for the expression of SMAD2 were performed. As shown in **Figure 7**, Smad2 protein expression increased after 6, 12 and 24 hour of CRF treatment. Smad2 is a major component of TGF-β signalling. TGF-β controls proliferation, differentiation and invasiveness in normal cells and plays an important role in cancer (6). In normal cells TGF-β stops the cell cycle at the G1 phase to inhibit proliferation, induce differentiation or promote apoptosis. Malignant cells however, often become refractory to TGF-β-mediated growth inhibition and frequently utilize TGF-β to promote proliferation via overexpression of Smad7, an inhibitory Smad that blocks the pro-apoptotic effect of TGF-β (7). Moreover, they may also overexpress TGF-β, which causes immune suppression and angiogenesis. To determine the effect of CRF in TGF-β induced proliferation, MTT assays were performed with 4T1 cells treated with CRF in combination with TGF-β. As shown in Figure 4, CRF slightly increased TGF-β induced proliferation after 24 and 48 hours treatment. In addition, CRF significantly inhibited the reduction of cell count due to cell death, observed after 72 and 96 hours of culture in the presence of TGF-β (**Figure 8**), suggesting that it may also inhibit apoptosis.

TGF-β promotes cell motility and invasiveness in epithelial cancer cells. In addition, beta-catenin is also involved in cytoskeletal changes characterized by actin polymerization, cell adhesion and motility (8). We, therefore, examined the impact of CRF on cytoskeletal changes in 4T1 breast cancer cells. To determine the effect of CRF on tumor cell actin polymerization, 4T1 cells were treated with CRF or UCN1 and stained with rhodamine-phalloidin. The toxin phalloidin, conjugated to the fluorescent dye rhodamine, binds specifically to polymerized actin allowing us to visualize the architecture of actin in the cell. Cells treated with CRF showed more intense staining compared to the untreated controls, most extensively seen after 4h treatment (**Figure 9A**). In addition, CRF treated cells showed increased actin stress fibers (**Figure 9B**). This suggests that the altered actin structures seen after CRF and UCN treatment might be associated with an increase in cancer cell motility, a process necessary for tumor cells to invade and metastasize.

Since CRF induced actin polymerization and expression of Smad2 and β-cateninin in 4T1 cells, processes important in invasion, we determined the effect of CRF on 4T1 cell invasiveness. We used a Boyden-chamber assay in which 4T1 cells were plated on an extracellular matrix (ECM) – coated transwell plate to determine the effect of CRF on the invasion towards the lower compartment. As shown in **Figure 10**, CRF did not alter invasion of the cells through the ECM.

It is likely that even though CRF induces molecules and processes that could potentially affect metastasis it does not promote invasiveness in culture because additional factors are required that may derive from the tumor stroma or the infiltrating immune cells. Immune and stroma cells are affected by factors secreted from cancer cells. We, therefore, tested an array of cytokines and chemokines to determine which factors are secreted from cultured 4T1 cells and which of those are affected by CRF. To this end, cells were stimulated with CRF for different time points and culture supernatants were analyzed using a bead-based cytokine/chemokine array (lincoplex-luminex, Millipore). The results indicated that among 13 factors analyzed 4T1 cells expressed IL-6, MIP1a, TNFα, KC and IP-10 and from those MIP1a was significantly suppressed by CRF (**Figure 11**). MIP1a promotes the recruitment of macrophages, thus CRF may inhibit recruitment of activated macrophages that could eliminate the tumor.

*Tasks 2and 3:* E*valuate the significance of peripheral CRF and its antagonists in tumor growth and metastasis in vivo.*

*Results:* CRF-/- and CRF+/+ mice were canulated in the lateral ventricle by performing a stereotactic approach. Canulated animals were allowed to recover for 5 days. To induce an anxiety response cortagine (9, 10) was administered ICV and the levels of corticosterone were measured in blood samples collected 15 minutes and 1 hour post administration. No effect on corticosterone levels was observed indicating that cortagine was not effective in inducing stress or anxiety in our experimental setup. The experiment was repeated twice with the same results. We, therefore, focused on analyzing in greater extent the molecular mediators of CRF on tumor growth (Task 1) and the effect of peripheral inhibition of CRF actions using Antalarmin, a synthetic CRF1 receptor antagonist (11, 12).

For this purpose, 4T1-GFP cells were implanted in the mammary fat pad of 40 Balb/c mice. From those 10 were not treated and served as a control group. Another 10 were injected with Antalarmin every 3 days. A third group was exposed to restraint stress for 3 hours for 4 consecutive days following a 5 day interval. The fourth group was exposed to the same type of restraint stress and in addition received Antalarmin every three days. We used Antalarmin for inhibition of CRF receptors since CRF functions primarily via CRF1, the target of Antalarmin, and CRF1 was the CRF receptor isoform expressed in 4T1 cells **(Figure 1)**. Mice that did not receive antalarmin received an injection of saline at the same time points. Tumor growth was monitored every 4 days using a Fluorescent Molecular Tomography approach (FMT) (13) as shown in **Figure 12**. During the period of the experiment samples were collected to measure corticosterone in the plasma. The experiment was terminated 6 weeks later. At the end of the experiment mammary glands were visualized on the animal to determine the extent of neoangiogenesis and then excised and fluorescence was quantified to allow us determining the size of the tumor (**Figure 13**). Lungs were isolated and visualized for the presence of metastatic (GFP expressing) cells. In addition, liver tissue was isolated and single cell suspension was prepared to analyze by Flow cytometry for the presence of GFP-expressing tumor cells. Tumor samples were collected from the different groups and histological analysis was performed. RNA was also isolated from tumor samples to determine the expression of the molecules affected by CRF.

Analyzing blood samples collected from the different groups of mice indicated that the levels of corticosterone were significantly increased upon stress. Antalarmin had no effect on corticosterone levels in the serum, suggesting that when it is administered peripherally it does not affect corticosterone production (thus the Hypothalamus-Pituitary-Adrenal axis response), at least under conditions of severe stress **(Figures 14, 15)**. We measured corticosterone on the $4^{th}$ day of the interval that followed the last exposure to stress, to determine whether our experimental setup indeed resembled chronic stress. Indeed, costicosterone plasma levels were still increased **(Figure 14)** suggesting that these mice were exposed to

chronic stress. No difference in corticosterone was observed between mice injected with saline or Antalarmin and exposed to stress **(Figure 14)**. To determine the levels of corticosterone immediately following exposure to stress we measured cortiocsterone in the serum of mice immediately after the last exposure to stress. We found that stress resulted in significant increase of corticosterone and no difference was observed between mice that received saline or Antalarmin and exposed to stress **(Figure 15)**. We could, therefore, conclude that peripherally administered Antalarmin affects only peripheral actions of CRF and does not alter corticosterone levels in the serum.

Histological and FMT analysis of the tumors revealed that in mice not exposed to stress, administration of antalarmin resulted in reduced tumor burden. Upon stress tumor burden was increased compared to non-stressed animals **(Figure 16)**. Administration of antalarmin in stressed animals resulted in significant reduction of tumor burden, comparable to that of antalarmin-treated non-stressed mice **(Figure 16)**. Representative photographs of mammary tissues stained with Haematoxylin-Eosin are shown in **Figure 17.**

To determine presence of metastatic cells in the lung, FMT analysis was performed following sacrifice of the mice. Visualization for fluorescent cells in the lung did not reveal any GFP positive cells in the lungs of any group, suggesting that no lung metastasis occurred in any of the group studied (data not shown).

To detect potential metastatic cells in the liver we performed FACS analysis in liver homogenates, since the volume of the liver and the presence of red blood cells does not allow the visualization with FMT. Liver homogenates were prepared and red blood cells were removed by lyzing them with a hypo-somolar solution. FACS analysis revealed the presence of GFP positive 4T1 cells in the liver, indicating that metastasis had occurred. Metastatic cells were increased in the mice that were exposed to stress while treatment with antalarmin abolished this increase, suggesting that CRF inhibition may suppress the metastatic process (**Figure 18**). Antalarmin did not affect metastasis in the absence of stress, suggesting that the levels of CRF in the absence of stress are not adequate to contribute to metastasis.

The preceding cell culture data showed that treatment of 4T1 breast cancer cells with CRF resulted in increased expression of beta-catenin, thus augmenting WNT signaling. To evaluate whether beta-catenin was also increased in vivo in mice that were exposed to stress, we dissected mammary glands from all the groups and isolated RNA. Real time-RT PCR analysis for the expression of beta-catenin revealed that tumor free mice had less beta catenin than tumor-bearing mice. In addition, Antalarmin-treated mice exposed to stress also had lower levels of beta-catenin than mice exposed to stress and not treated with antalarmin **(Figure 19)**.

Angiogenesis is a hallmark of tumor growth and metastasis. We, therefore, evaluated the extent of neoangiogenesis in the 4T1 tumors and the impact of stress and CRF inhibition. To quantitatively measure angiogenesis a new method was established. This method utilized the contrast of light excitation between the mammary tissue and the blood vessels. Blood vessels absorbed light, while mammary gland and mammary tumors exited light **(Figure 20)**. Software exclusively developed for our study by Dr. J Ripoll, member of our research team, quantified the area of blood vessels in the tissue. The method was evaluated using the tumors generated in the present study. The results showed that tumor-bearing mammary glands had increased angiogenesis compared to normal mammary glands and angiogenesis was significantly increased when mice were exposed to stress **(Figure 21)**. Treatment of mice exposed to stress with Antalarmin resulted in reduced angiogenesis. Our results suggest that stress augments neoangiogenesis in breast tumors and a potential mediator is peripheral CRF, since treatment with Antalarmin suppressed stress-induced neoangiogenesis.

**Key Research Accomplishments**

- *The Wnt signaling pathway is augmented by the stress neuropeptide CRF via induction of beta-catenin expression in breast cancer cells*
- *CRF induces Smad2 expression and affects TGF-β signaling by inhibiting the anti-proliferative actions of TGF-β in breast cancer cells*
- *Stress augments tumor growth and metastastasis in the liver. Inhibition of peripheral CRF by the CRF1 receptor antagonist Antalarmin partly reverses this effect*
- *A novel methodology using an imaging approach has been developed to quantitatively evaluate the extent of neoangiogenesis in tumors*
- *Stress increases neoangiogenesis in breast tumors in vivo. Inhibition of peripheral CRF suppresses stress-induced neoangiogenesis*

**Reportable Outcomes**

1. *Presentation of the results at the "2008 Era of Hope" Conference, Baltimore, MD (presentation # P 63-6, page 423)*
2. *MSc thesis of Ms Berber Mol, MSc student of the Oncology program in Vrije University, Amsterdam, Netherlands. (Work was conducted at the laboratory of Dr. C. Tsatsanis at the University of Crete, Greece, as part of collaboration between the two institutions).*
3. *Submission of an abstract reporting the methodology for evaluating neoangiogenesis in breast tumors using a new imaging approach, to be presented in the "Congress of the European Society for Molecular Imaging – ESMI" in May 2009.*

**Personnel that received pay under this project**

Alicia Arranz de Miguel, PhD, Postdoctoral Researcher
Ariadne Androulidaki, PhD, Postdoctoral Researcher

**Conclusion**

In the present study we analyzed the impact of a stress-neuropeptide CRF on breast cancer cells. The results showed that CRF induced the expression of beta-catenin, augmenting Wnt signaling in breast cancer cells, providing a potential mechanism for pro-tumorigenic and pro-metastatic properties of this stress neuropeptide. In addition, we showed that CRF induces Smad2 expression and suppressed TGF-β – mediated suppression of proliferation. Orthotopic transplantation of 4T1 cells in the mammary fat pad of mice and subsequent exposure to chronic stress showed that stress augmented tumor growth and metastasis in the liver. In vivo inhibition of peripheral CRF using the selective CRF1 receptor antagonist Antalrmin, indicated that inhibition of CRF1 receptor resulted in reduction of tumor growth and suppression of liver metastasis. In addition, we developed a novel methodology using an imaging approach to evaluate neoangiogenesis in tumors. The results showed that stress augmented

neoangiogenesis, which was abolished by the CRF antagonist Antalarmin. We could, therefore, conclude that stress-induced peripheral CRF mediates tumor-growth and neoangiogenesis via the CRF1 receptor.

Overall, the present study showed that chronic stress augmented tumor growth and that CRF is a central mediator of the impact of stress in breast cancer. Inhibition of peripheral CRF may, therefore, be beneficial for suppressing stress-induced breast tumor growth.

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# Appendices

## Appendix I:

### *Abstract # P 63-6, at the "2008 Era of Hope" Conference, Baltimore, MD, June 2008 (page 423 in the meeting proceedings)*

***Title: The impact of stress on tumor growth; the significance of peripheral Corticotropin Releasing Factor***

Alicia Arranz, Maria Venihaki, Olga Rassouli, Andrew N. Margioris and Christos Tsatsanis
*Department of Clinical Chemistry-Biochemistry, School of Medicine, University of Crete, Heraklion, Crete, Greece*

**Background and Objectives:** Corticotropin Releasing Factor (CRF) is the primary neuropetide that mediates the hypothalamic response to stress. CRF has been detected not only in the central nervous system but also in peripheral tissues and organs. CRF is secreted from autonomic neurons in peripheral tissues upon stress affecting the surrounding cells but it has also been detected in epithelial cells and immune cells. Even though systemic stress has been reported to affect cancer cell growth primarily due to suppression of the immune response against cancer no information was available on the impact of peripheral CRF on breast cancer cell proliferation and metastasis. Aim of our studies is to determine the impact of peripheral CRF on breast cancer growth and invasiveness and propose a novel potential mechanism on the crosstalk between stress and tumor growth.

**Methodology:** We employed the mouse breast cancer cell line 4T1 to test the impact of CRF on breast cancer cell motility and invasiveness in culture and in vivo. In culture cells were treated with CRF and the expression of tumor-related cytokines and chemokines was determined by ELISA-based array and of genes related to metastasis was determined by gene expression arrays. For the in vivo experiments 4T1 breast cancer cells were transferred orthotopically in mice and tumor growth was assessed in the presence or absence of CRF agonists and antagonists.

**Results to date:** We first characterized the CRF receptors expressed on 4T1 cells and found that they express only CRF1 receptor, the primary receptor for CRF. Treatment of 4T1 cells with CRF revealed that it had no effect on cell proliferation and apoptosis. We further tested the effect of CRF on several tumor-derived cytokines and chemokines using array technology and found that CRF significantly suppressed the expression of Macrophage Inflammatory Protein -1 alpha (MIP-1alpha), a chemokine known to induce macrophage recruitment. Low levels of MIP-1alpha have been associated with impaired immune response against the tumor. We then used a boyden chamber invasiveness assay and found that CRF promoted cell invasiveness through extracellular matrix.
To validate the effect of peripheral CRF on breast cancer cell growth we transfected 4T1 cells with a plasmid expressing the Green Fluorescent Protein (GFP) and implanted them in the mammary fat pad of mice in order to monitor tumor growth in vivo using laser fluorescence tomography. Mice were treated with CRF receptor agonists and antagonists to determine tumor growth, angiogenesis and invasiveness.

**Conclusions:** Our preliminary findings suggest that CRF has a direct effect on breast cancer cell invasiveness. The results of the study when completed will allow us to determine the significance of peripheral CRF on breast cancer and suggest novel therapeutic approaches using CRF receptor antagonists. Moreover, the results will provide a novel link between stress and tumor growth.

## Appendix II:

## Abstract accepted to be presented at the "4th European Molecular Imaging Meeting", Barcelona, Spain, May 2009

**TOPIC:** New Developments in Optical Probes and Instrumentation

***Title:*** *Spectral Imaging as a tool for accurate measurement of Vascularization in mice.*

**Author and affiliation** (underline presenting author)**:** Alicia Arranz (+), Ariadne Androulidaki (+), Berber Mol (+), Ana Sarasa-Renedo (*), Christos Tsatsanis (+) and Jorge Ripoll (*); (+)Laboratory of Clinical Chemistry-Biochemistry, School of Medicine, University of Crete, Heraklio 71003, Greece; (*) Institute for Electronic Structure and Laser, Foundation for Research and Technology-Hellas, 71110 Heraklion, Greece.

**Introduction:** The development of new blood vessels is essential in several biological processes, such as the healing of damaged tissues, but also in the progression of numerous diseases, including cancer. In this sense, methods to estimate tissue vascularization are crucial for the observation of blood vessels changes in the course of in vivo models, as well as, the development of potential treatments.
Current 3D optical methods exist that can provide information on oxygen saturation and blood volume in-vivo in the intact animal [1], however suffer of low spatial resolution (>1mm). Other methodologies employ more advanced techniques such as MRI and are therefore not suitable for studies where large numbers need to be analyzed. In order to obtain measurements as accurate as possible in -vivo, in this work we study the potential of intravital imaging for vascularization measurements.

**Methods:** A Fluorescence Molecular Tomography (FMT) setup developed at FORTH [2] has been adapted to perform intravital measurements on small animals. The setup consists of several laser sources with wavelengths (458nm, 488nm, 514nm, 635nm and 780nm) that are guided by mirrors and scanned in reflection mode on the whole surface of the animal with its mamary fat pads exposed. The laser is scanned while the CCD camera is exposing, varying the exposure time and laser speed to obtain optimal signal to noise ratios. For each excitation wavelength, several emission filters are measured, building a library of emission-excitation images. These images are then combined to provide the optimal source of contrast to distinguish blood vessels from surrounding tissue. An in-house software was designed to vary the contribution of each excitationemission image and apply a threshold, which was later used to measure, with pixel-size accuracy, the vascularization area. During the measurements, Balb/c mice where anesthetized with Isofluorane, and the mammary fat pads were selected as the area of interest. Measurements took approximately 10 mins, after which all animals were sacrificed.

**Results:** We have found that the combination of several spectral (emission-excitation) measurements in the visible rage enhances the contrast of blood and surrounding tissue, enabling the accurate measurement of vascularization. By combining autofluorescence in the 600nm range when excited with 488nm and absorption at 800nm, we have been able to measure the vascularization area in a total of 5 mice.

**Conclusions:** Even though results are preliminary, we have seen that the combination of several excitation/emission pairs greatly enhances the contrast of haemoglobin against surrounding tissue, enabling the measurement of vascularization area with pixel-size resolution (in the order of 0.001 cm in our case). Since this technique is simple and fast, involving commercial laser sources, we believe it will serve as an additional tool in biological studies where changes in vascularization are crucial for the outcome.

**Acknowledgements:** This work as supported by the Integrated Project Molecular Imaging LSHGCT-2003-503259, the EU collaborative project FMT-XCT and the Bill and Melinda Gates Foundation. A.A. acknowledges support from US-DOD-BC062715 CA.
**References:** [1] Zhou Chao, Choe Regine et al, Journal of biomedical optics 2007;12(5):051903.
[2] A. Martin et al, Mol Imaging. 2008 Jul-Aug;7(4):157-67.

**SUPPORTING DATA**

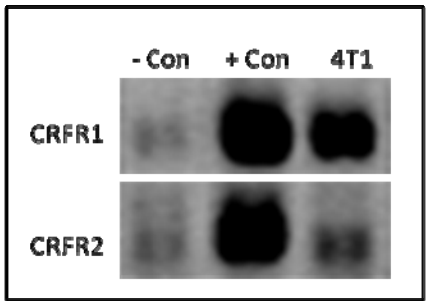


**Figure 1. CRF receptor expression in 4T1 cells**. Reverse transcription-PCR for CRFR1 and CRFR2 was performed. 4T1 cells express CRFR1 and low levels of CRFR2. – Con indicates negative control, + Con indicates positive control (mouse brain).

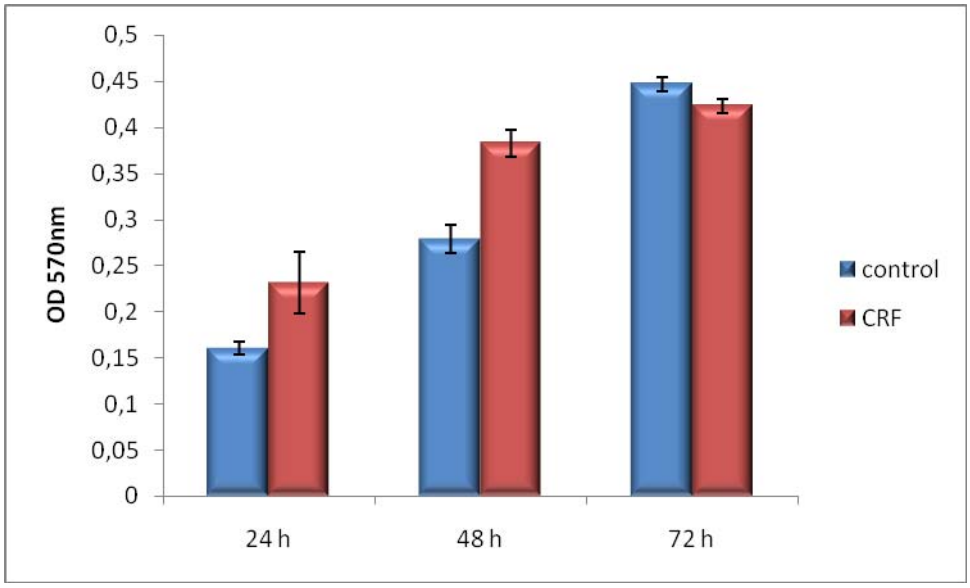


**Figure 2.Effects CRF on the proliferation of 4T1 mouse mammary tumour cells**. 4T1 cells were treated with $10^{-8}$ M CRF for 24, 48 and 72 h and MTT assays were performed. Error bars represent the standard error of the mean, asterisks indicate significant difference ($p < 0.05$) compared to untreated control, OD, optical density

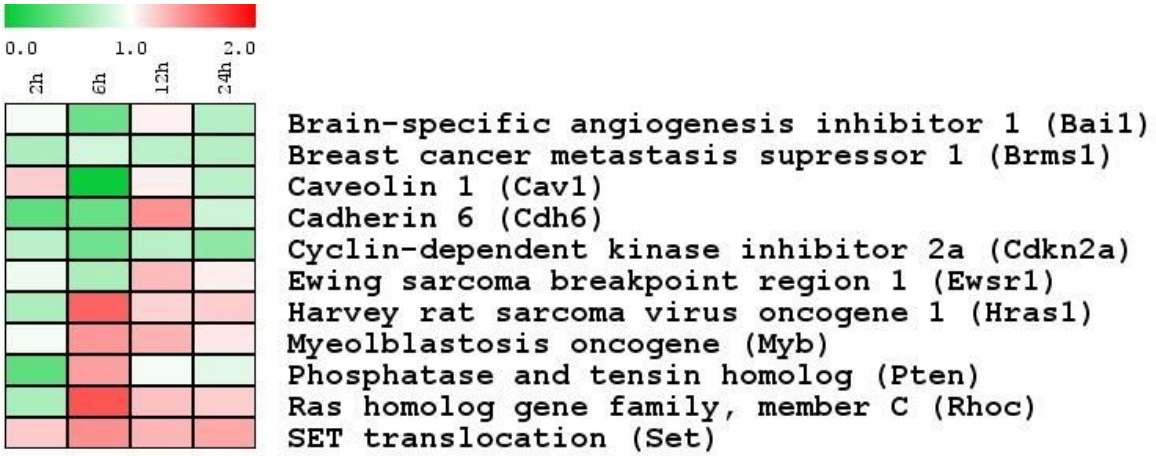


**Figure 3. CRF modifies the expression of several genes involved in cell cycle, growth, proliferation and metastasis**. 4T1 cells were treated with CRF for different time points and Oligo GEArray® Mouse Tumor Metastasis Microarrays were performed. The ratio between treatment and control groups was transformed into color intensity using the TIGR MultiExperiment Viewer V4.0.

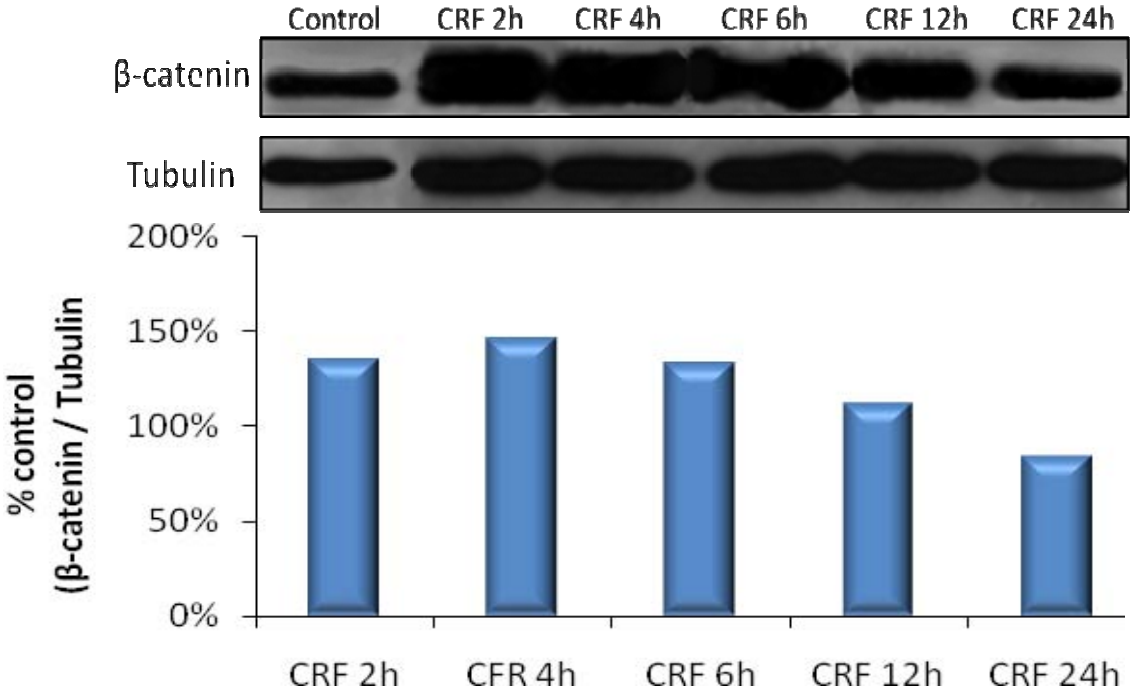


**Figure 4. Effect of CRF on β-catenin production.** 4T1 cells were treated with or without (control) $10^{-8}$ M CRF for 2, 4, 6, 12, and 24h. Cells were lysed and supernatants were electrophoresed and probed by Western analysis with anti-β-catenin antibodies followed by anti-tubulin antibodies. CRF increased β-catenin at 2, 4, and 6h treatment. Data are expressed as a percentage of control and represent three independent experiments

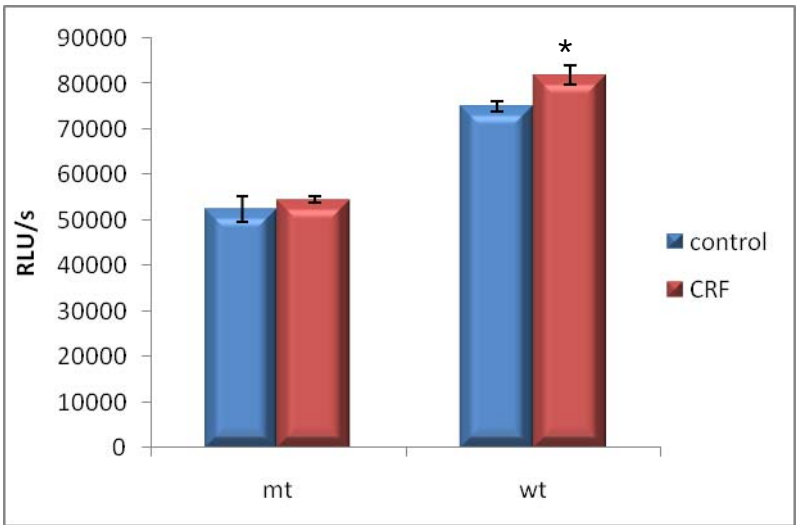


**Figure 5. Effect of CRF on the Wnt signaling pathway.** 4T1 cells were transfected with a construct containing 3 TCF binding sites (wild type) or a point mutation the TCF binding site (mutant) linked to the luciferase gene and transcriptional activity was measured 18 h following stimulation with CRF. CRF did not induce transcriptional activity of the Wnt signaling pathway. Results are expressed as relative light units per second (RLU/sec). Each bar corresponds to the average of three individual transfections done in parallel, error bars represent the standard error of the mean, Control, untreated cells; wt, wild type; mt, mutant; *$p<0.05$

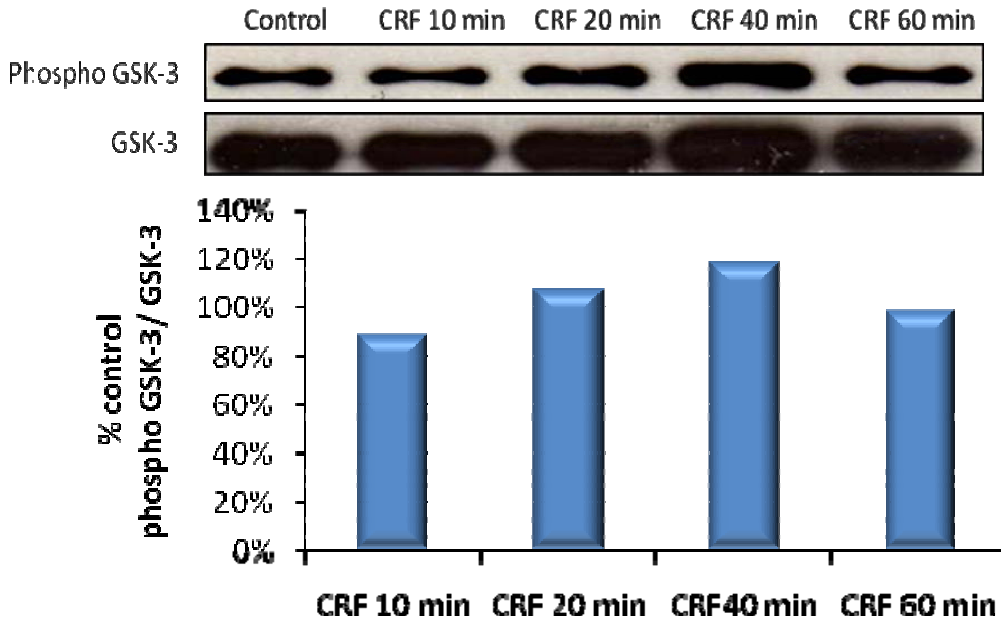


**Figure 6. Effect of CRF on GSK-3 phosphorylation**. 4T1 cells were treated with $10^{-8}$ m CRF for 10, 20, 40 and 50 minutes. Cells were lysed, and supernatants were electrophoresed and probed by western analysis with anti-GSK-3 antibody, and, after stripping, with anti-phospho-GSK-3 antibody. CRF treatment did not have an effect on GSK-3 phosphorylation.Data are expressed as a percentage of control.

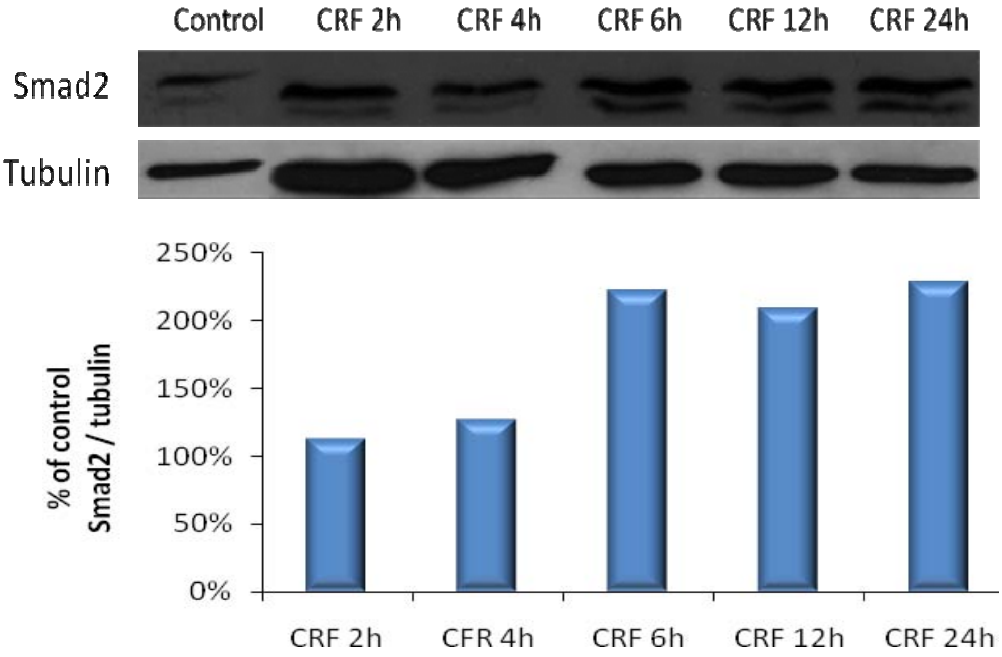


**Figure 7. Effect of CRF on Smad2 expression.** 4T1 cells were treated with or without (control) 10-8 M CRF for 2, 4, 6, 12, and 24h. Cells were lysed and supernatants were electrophoresed and probed by Western analysis with anti-Smad2 antibodies followed by anti-tubulin antibodies. CRF increased Smad2 at 6, 12, and 24h treatment. Data are expressed as a percentage of parallel controls and represent three independent experiments.

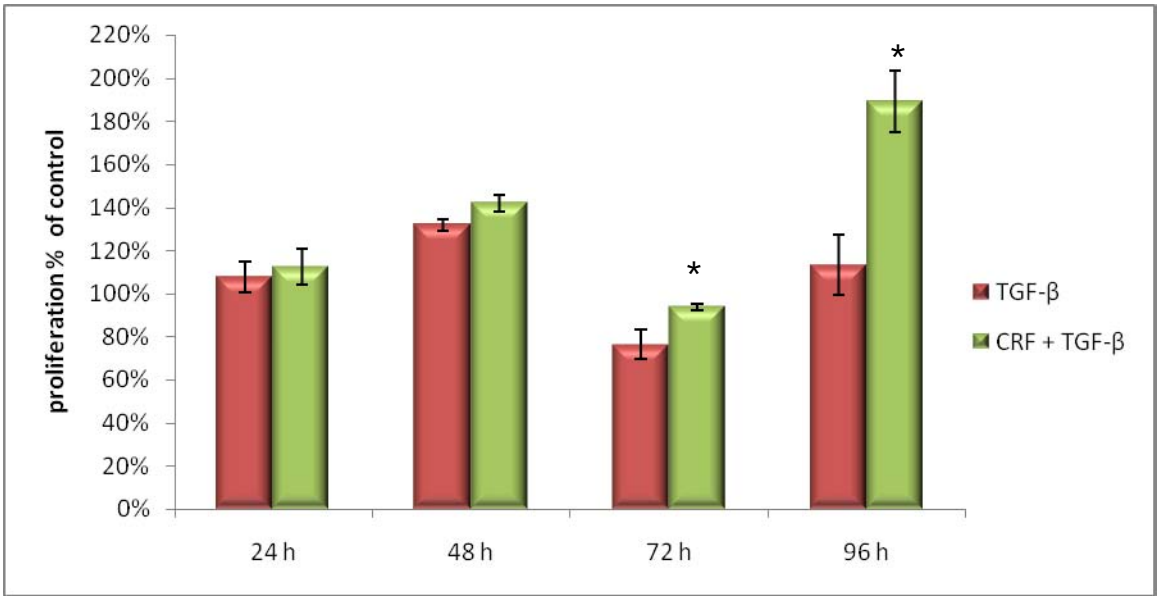


**Figure 8.Effects CRF on TGF-β induced tumour cell proliferation**. 4T1 cells were treated with 5 ng/ml TGF-β alone or in combination with 10-8 M CRF for 24, 48, 72, and 96 h. The percentage of cell survival was determined using the MTT assay, results are expressed as percentage of control and error bars represent the standard error of the mean, asterisks indicate significant difference ($p < 0.05$) compared to untreated control

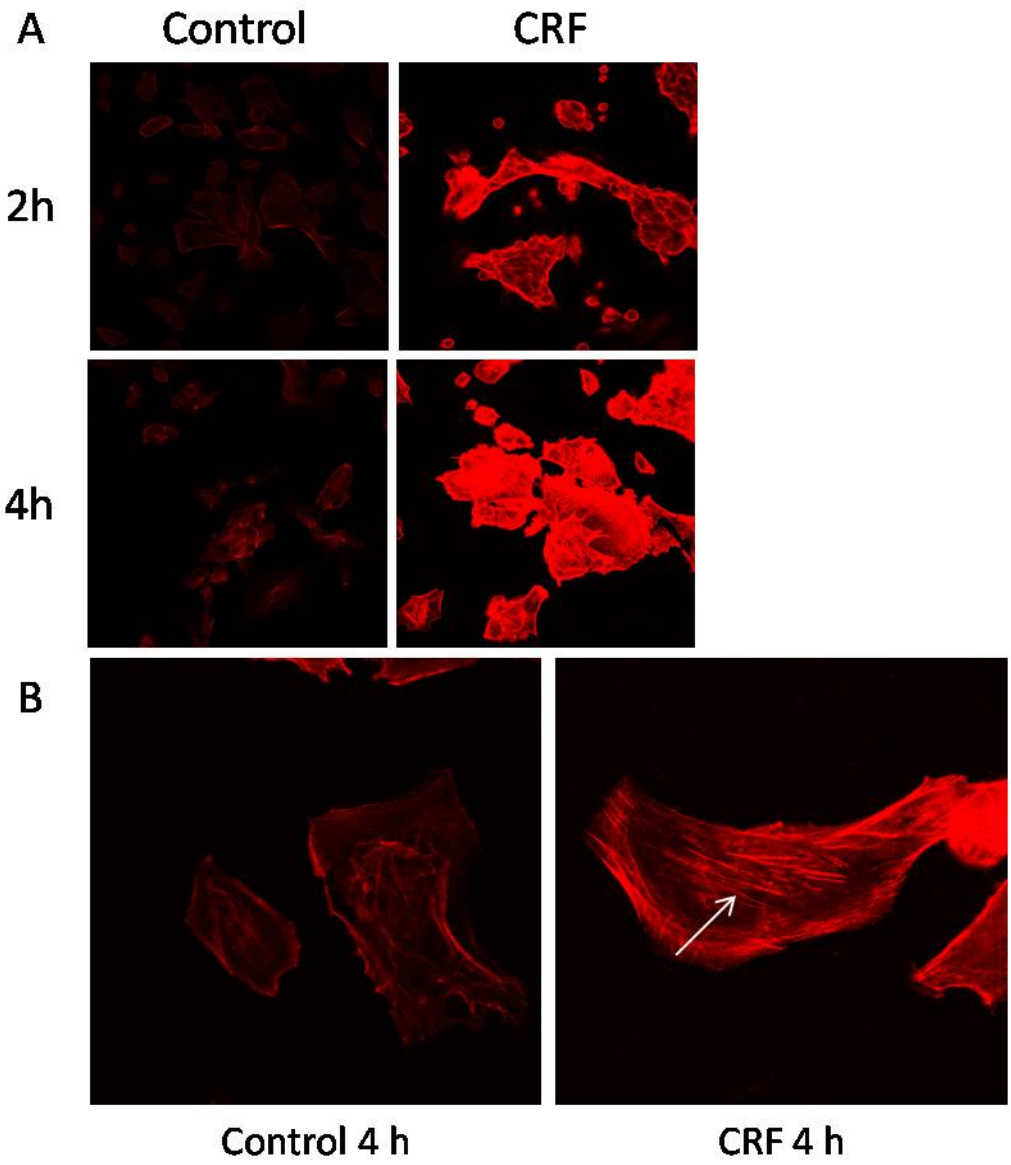


**Figure 9. The effect of CRF on actin structure of mouse mammary tumour cells.** 3 x 104 4T1 cells were cultured overnight on coverglass. Subsequently cells were stimulated with 10-8M CRF or UCN1 for 2 or 4 hours. After fixation in 3.7% formaldehyde, cells were permeabilized with acetone and stained for F-actin with rhodamine-conjugated phalloidin. **A.** CRF and UCN1 treated 4T1 cells show more intense actin staining compared to untreated control. **B** CRF treated cells showed more pronounced actin stress fibers compared to untreated control (indicated with white arrow). Results are representatives of two independent experiments.

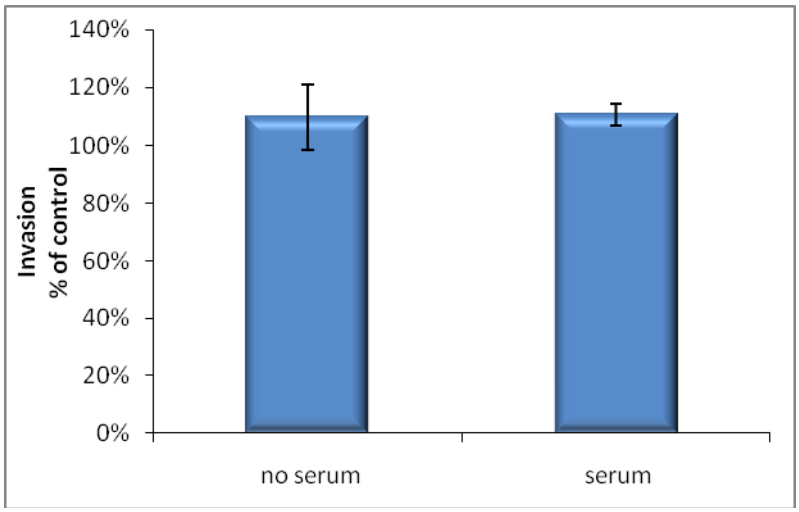


**Figure 10. CRF does not effect the invasion of 4T1 cells through ECM.** 4T1 cells were stimulated with 10-8 M CRF and the invasion assay was performed according to the manufactotrer's instruction, with (serum) or without fetal bovine serum (no serum) in the invasion chamber. Results are the average of two independent experiments where each treatment was performed in triplicate. Results are expressed as percentage of the vehicle treated control, error bars represent the standard error of the mean.

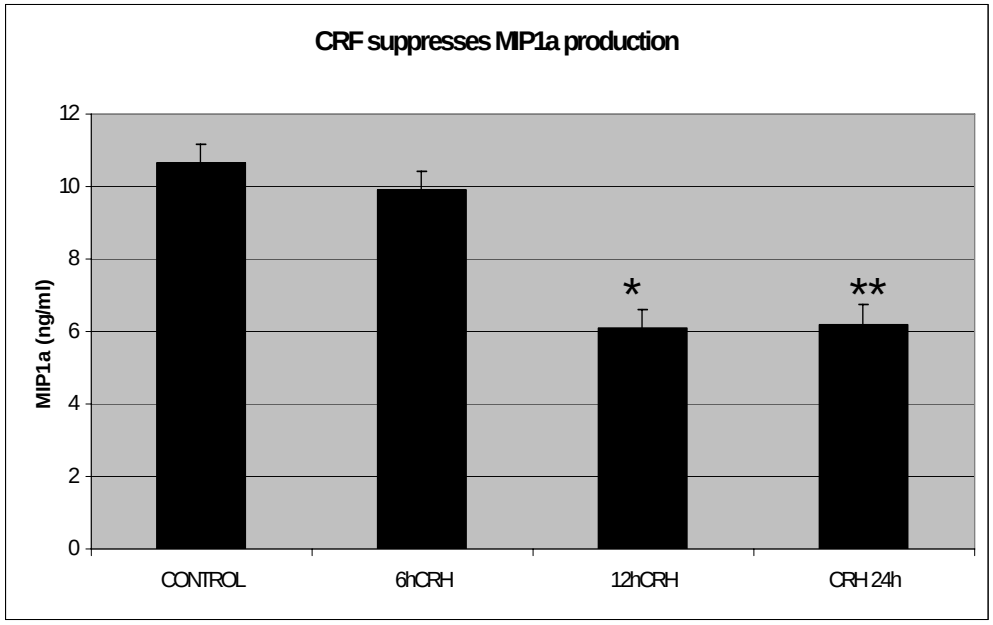


**Figure 11.Effects CRF on MIP1a secretion**. 4T1 cells were treated with $10^{-8}$ M CRF for 6, 12 and 24 h and supernatants were collected and analyzed using a cytokine array (lincoplex, Millipore). Error bars represent the standard error of the mean, asterisks indicate significant difference (**p < 0.01) compared to untreated control.

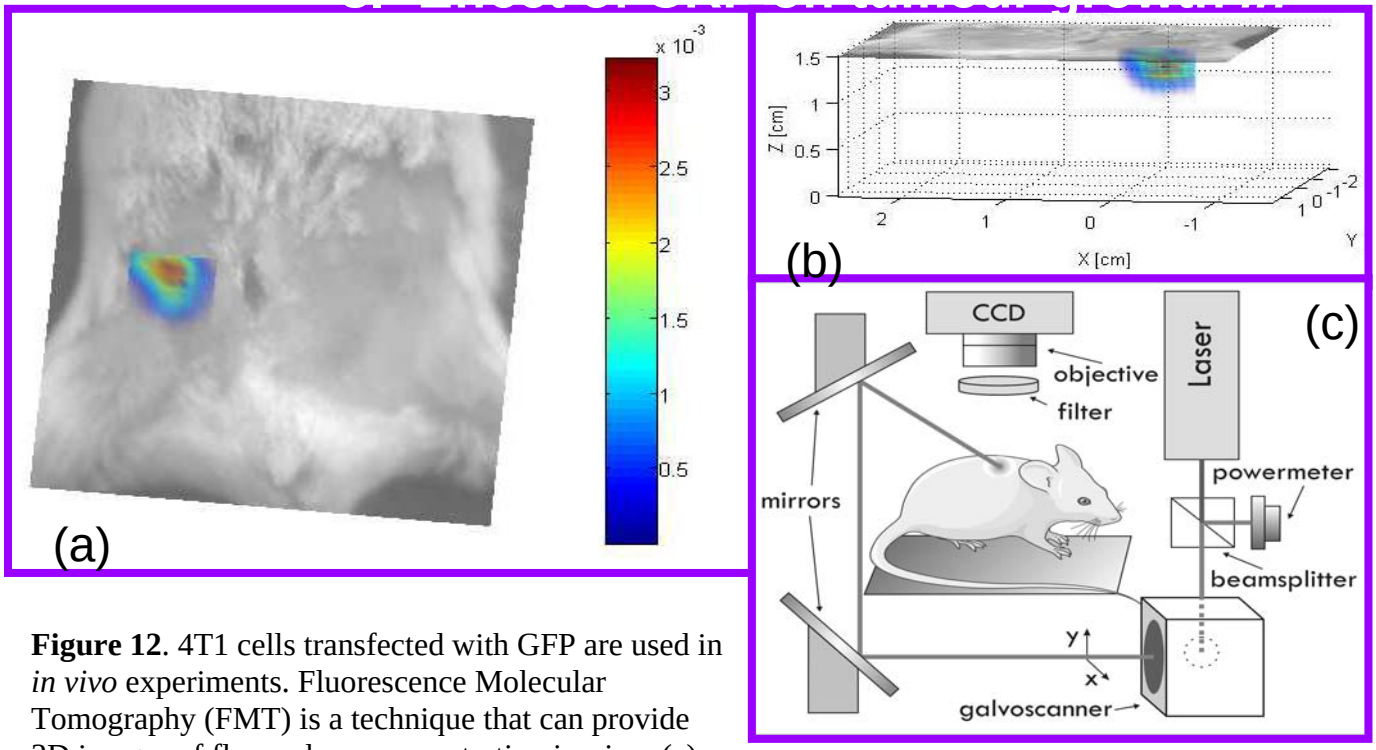


**Figure 12**. 4T1 cells transfected with GFP are used in *in vivo* experiments. Fluorescence Molecular Tomography (FMT) is a technique that can provide 3D images of fluorophore concentration in-vivo. (a) shows a top view and (b) a side view presenting the depth information. Color scale is proportional to GFP concentration. A schematic of the setup is shown in (c).

**Ex 488nm; Em: 540nm** **Ex 488nm; Em: 615nm**

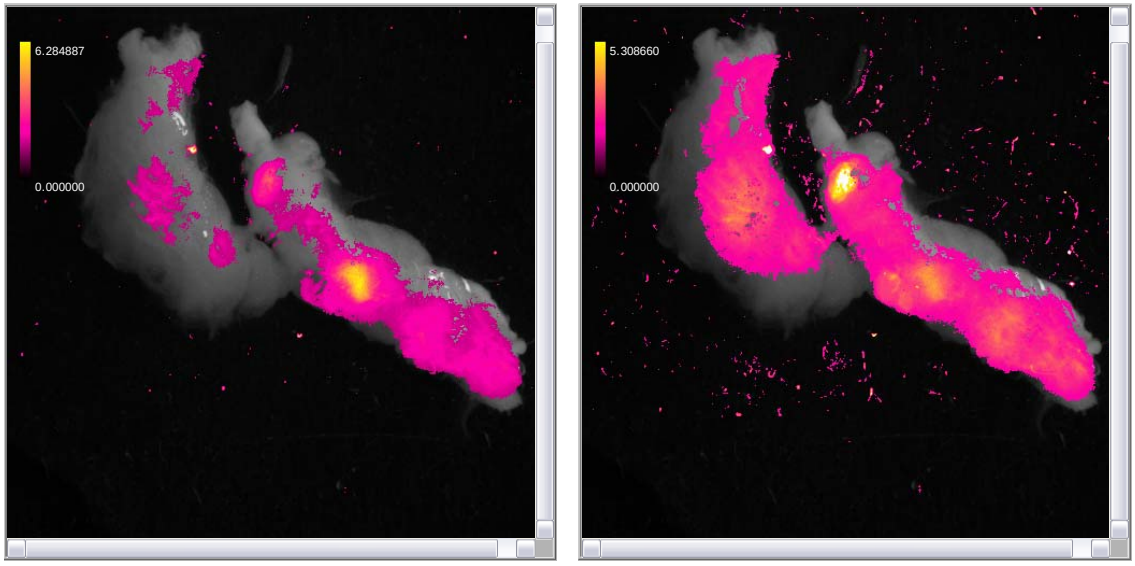


**Figure 13**. 4T1 cells transfected with GFP were orthotopically tranferred to Balb/c mice. Fluorescence Molecular Tomography (FMT) analysis shows fluorescence of the tumor mass ex vivo.

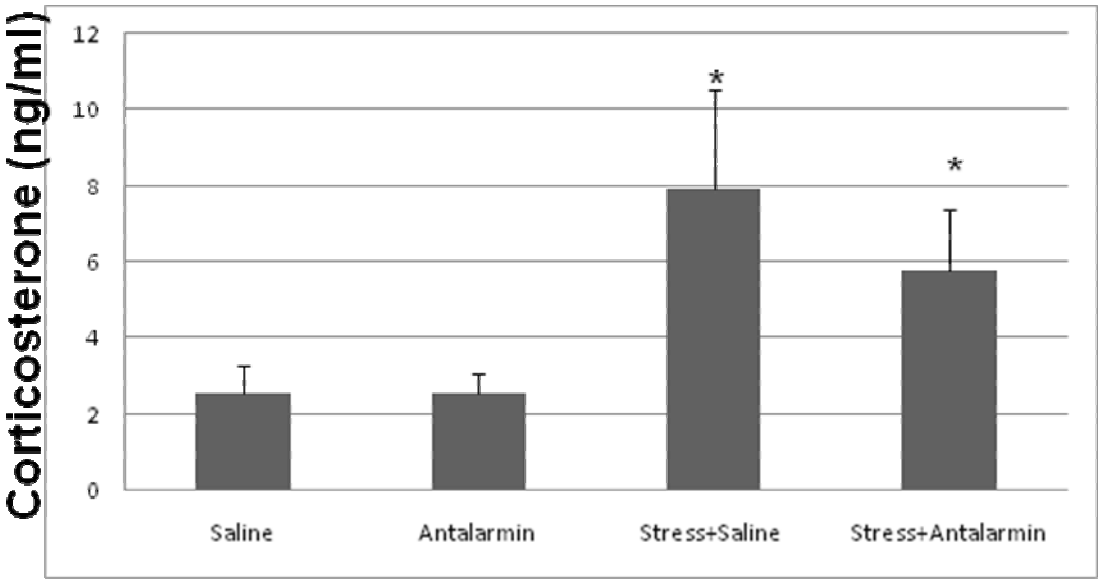


**Figure 14.** Corticosterone levels in the serum of mice 3 days following exposure to stress. The increase observed indicates that these animals were under chronic stress. *p<0,05 compared to animals not exposed to stress.

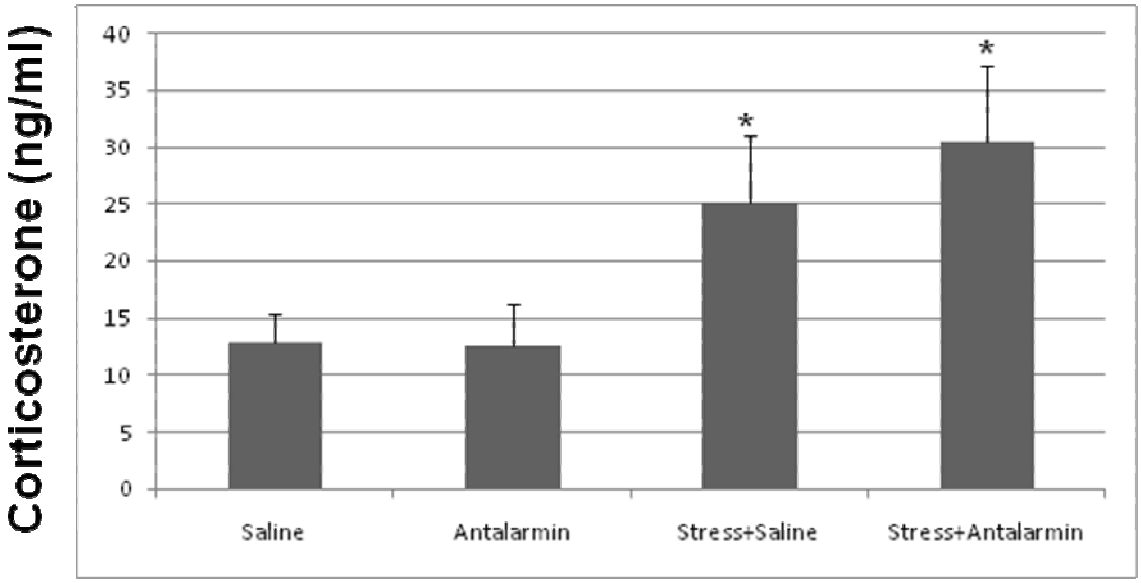


**Figure 15.** Corticosterone levels in the serum of mice 12 hours following exposure to stress. The increase observed confirms that these animals were under stress. *p<0,05 compared to animals not exposed to stress.

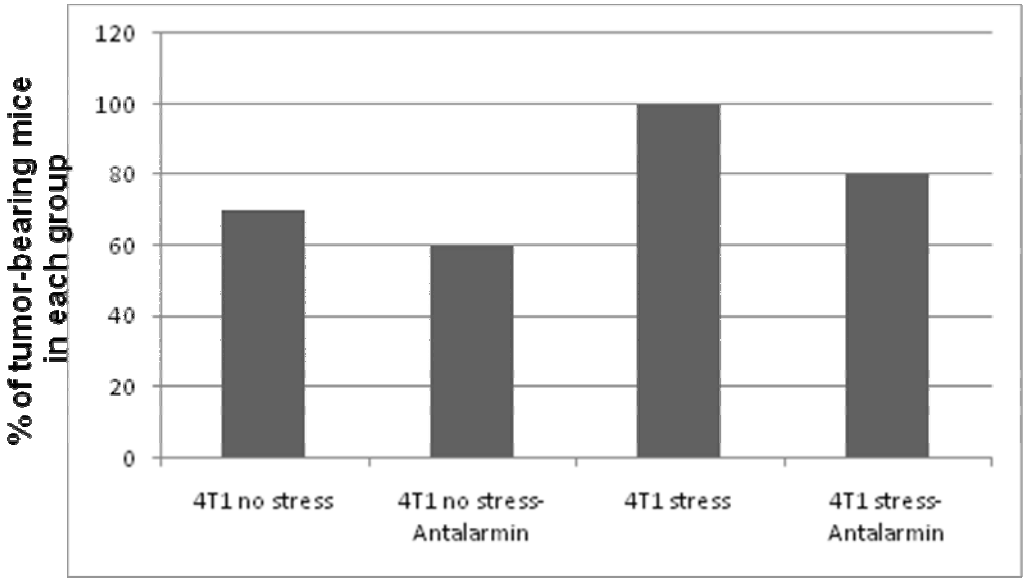


**Figure 16.** Histological analysis of the mammary glands revealed that not all mice were bearing tumors. Depicted is the precentage of tumor-bearing mice in each group.

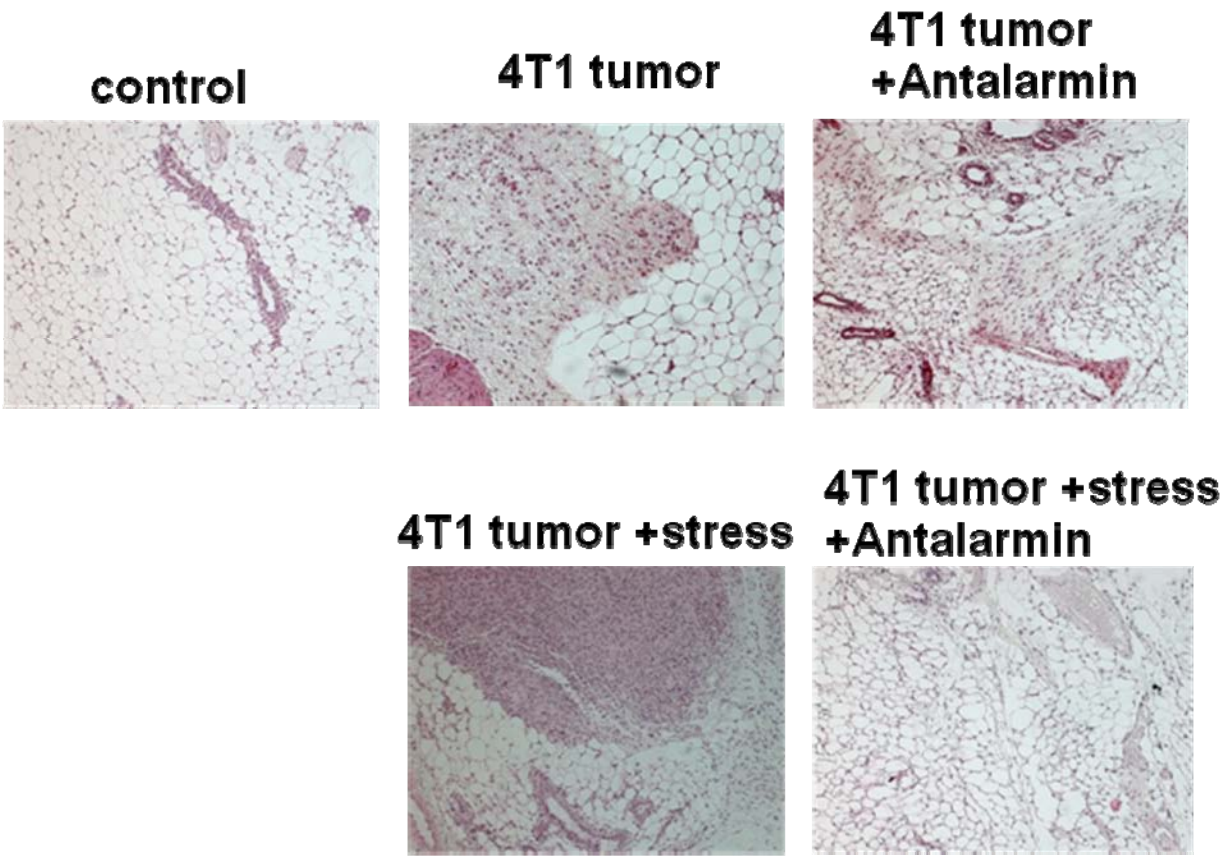


**Figure 1**". Histological analysis of the tumor samples from all mouse groups.

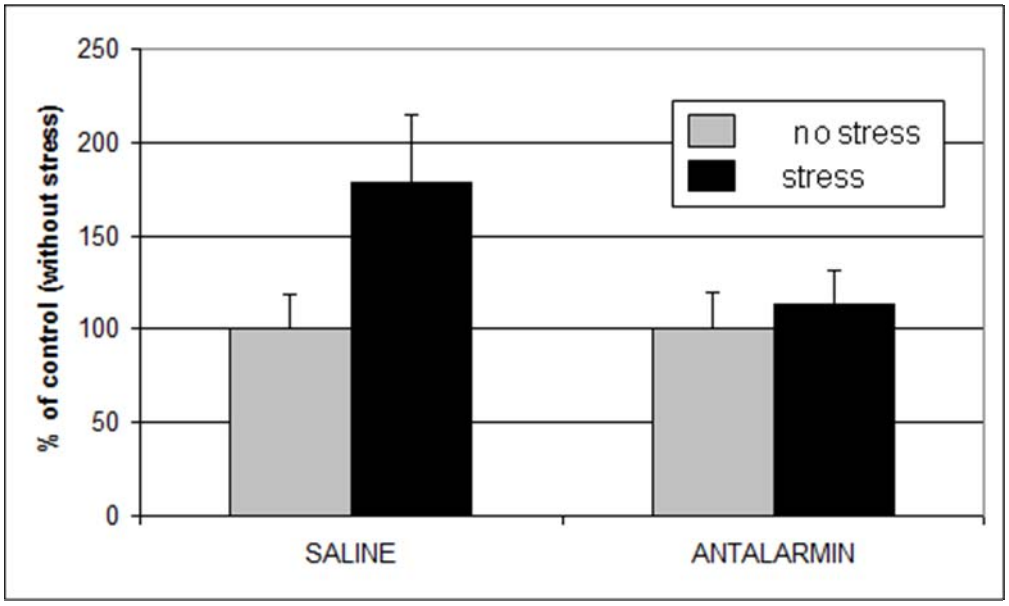


**Figure 18**. Liver samples were analyzed by flow cytometry for the presence of GFP-expressing 4T1 cells. Mice that underwent stressed had increased numbers of GFP-postive cells while treatment with Antalarmin abolished this increase.

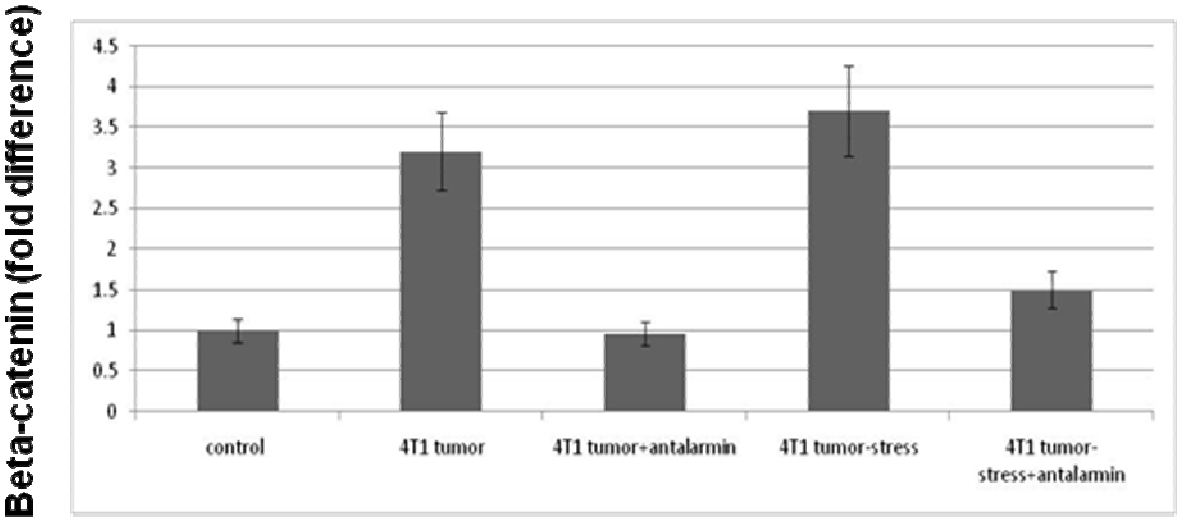


**Figure 19**. Expression of beta-catenin in mammary tissues isolated from mice orthotopically injected with 4T1 cells or non-injected tissues (control). Results represent the average of tissues from 7 individual mice in each group.

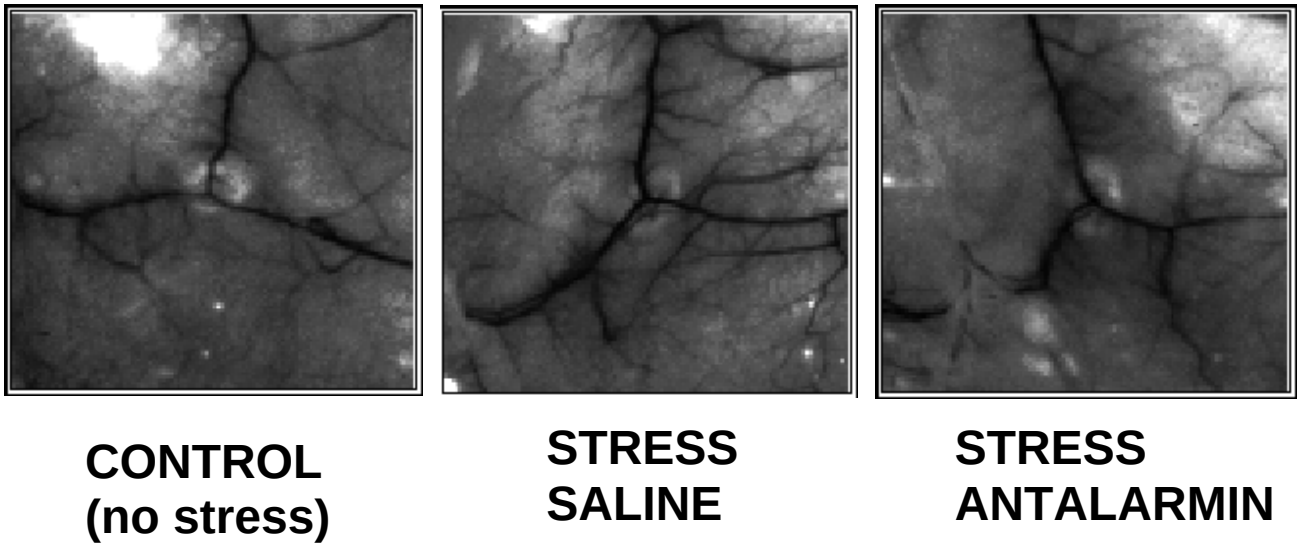


**Figure 20.** Representative images of blood vessels on the tumor-bearing mammary glands. The software calculated the area of blood vessels.

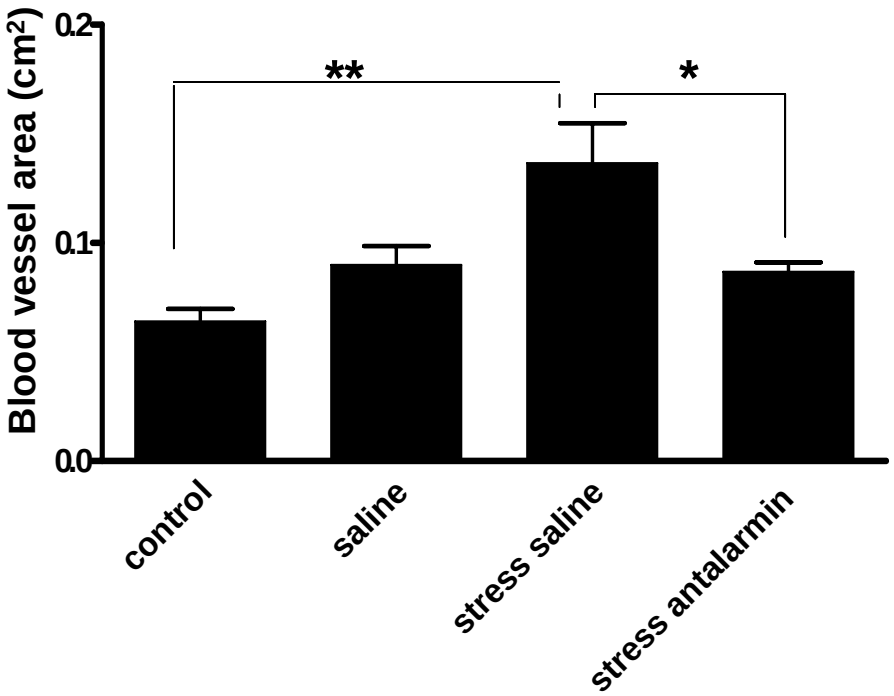


**Figure 21.** Area of blood vessels that are surrounding the mammary gland, representing the extent of angiogenesis. *p<0.05, **p<0.01