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SCUOLA ALTA FORMAZIONE

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STUDENT: **SILVIA ANTONINI** CYCLE: **XXIX** YEAR: **2013-2014**

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Background

Free oxygen radicals or, more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. They are highly reactive molecules and they include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), anion hydroxide (OH^-),hypochlorite (OCl^-), nitroxyl anion, nitrosonium cation, higher oxides of nitrogen, S-nitrosothiols, and dinitrosyl iron complexes. ROS and RNS are well recognised for playing a dual role both as detrimental and beneficial species, since they can be either harmful or beneficial for living systems (Valko et al., 2006). Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, in defence against infectious agents and in the function of a number of cellular signalling systems (Valko et al., 2007).

The damaging effect of free radicals occurs in biological systems when there is an imbalance between the production of ROS/RNS and the antioxidants systems. In fact, at high concentrations, ROS can be important mediators of damage to cell macromolecules, such as nucleic acids, lipids and proteins. Furthermore, some ROS act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruption in normal mechanisms of cellular signaling.

Defense mechanisms against free-radical induced oxidative stress involve enzymatic antioxidants (dismutase, glutathione peroxidase, catalase) and non-enzymatic antioxidants (ascorbic acid, α-tocopherol, glutathione, carotenoids, flavonoids).

In humans, oxidative stress is thought to be involved in the development of cancer (Van de Bittner et al., 2010), neurodegenerative diseases (Patel and Chu, 2011), cardiovascular diseases, diabetes, ischemia/reperfusion damage (Dalle-Donne et al., 2006), heart failure, myocardial infarction (Singh et al., 1995), other diseases and ageing.

In the heart susceptibility to oxidative stress is known to be higher than in other organs, because of the low levels of antioxidant enzymes (Dhalla et al., 2000). Thus, an increase in the antioxidants, either through exogenous administration or through the upregulation of the endogenous ones could protect hearts from oxidative stress associated with I/R injury and be an important therapeutic strategy to prevent ROS-mediated cardiac injury.

In the last decade, a growing number of publications has shown that heart is an organ that can self-renew constantly, albeit at a very low level (Bergmann et al., 2009). This property depends on the presence of stem cells commonly called cardiac progenitor cells (CPCs), that are able to provide the physiological turnover of myocytes, endothelial cells, and smooth muscle cells (Beltrami et al., 2001). CPCs express surface stemness markers such as c-kit, Sca-1, CD29, CD44, CD71, CD90, CD106, CD120a e CD124 (Augello et al., 2010; Forte et al., 2011), which allow their identification and isolation. The evidence that cardiac progenitor cells, upon activation, can move into the injured areas, where they can proliferate and differentiate, could pave the way towards new therapeutic strategies based on the regeneration of damaged myocardium. However, besides the fact that the level of proliferation of cardiac progenitor cells is insufficient, other causes also seem to have a role in the establishment of heart failure, such as ischemic heart disease, age or diabetes. Furthermore, stem cells distributed in the injured area generally are not able to survive in the ischemic environment and die by apoptosis and necrosis in the same way of myocytes and cells of coronary vessels. CPCs, consequently, are not able to oppose to death signals that are activated by ischemic damage, and they are not able to proliferate and regenerate the infarcted myocardium while the stromal component prevails due to a greater proliferative potential of fibroblasts. In addition, these

cells, cannot escape replicative senescence characterized by telomere shortening and activation of death programs (Kajstura et al.; Urbanek et al., 2005). Thus, as in other tissues, in which stem cells have a low replicative potential (e.g. nervous tissue), stem cells have minimal effect in restoring damaged tissue.

Preclinical and clinical studies have shown that stem cell-based therapies may have great potential in the repair of cardiac damage. Possible candidates for transplantation are the bone marrow derived stem cells (BMC), mesenchymal stem cells (MSCs), skeletal myoblasts, embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) and cardiac progenitor cells (CPC). The improvement of culture techniques has allowed to obtain in vitro expansion of human CPCs, obtained from endomyocardial biopsies and to use them for therapeutic purposes. These results allowed the development of two phase II trials. The results of these studies, called SCIPIO (cardiac Stem Cells Infusion in Patients with Ischemic Cardiomiopathy) and CADUCEUS, were reported recently(Bolli et al., 2011; Makkar et al., 2012). In both trials populations of cardiac stem cells were used for autologous intracoronary infusion in patients with a recent myocardial infarction arising from a short time. However, only in the study CADUCEUS, where they use CPCs cardiospheres, there were significant effects in the improvement of the left ventricular ejection fraction. Cardiospheres, in fact, after being implanted into the infarcted heart, have the capacity to differentiate into multiple cell lines and to confer functional benefits (Davis et al., 2009). Despite this evidence, the success of transplantation of adult stem cells, remains limited. Limiting factors are principally three: low survival, marginal proliferation and partial engraftment and migration within the damaged tissue. Implanted stem cells poor survival is affected by many factors such as inflammation, mechanic offense, ischemic and ischemic/reperfusion events and the origin and quality of the cells(Hodgetts et al.). Therefore, promoting the survival rate of the implanted cells is a necessary procedure to increase the therapeutic potential of transplantation.

The last decade has observed an increased interest on the use of plant-derived products, also known as phytochemicals or phytoceuticals as preventive and therapeutic agents against a wide range of diseases. Polyphenols, ubiquitously present in fruits and vegetables, have been traditionally viewed as antioxidant molecules, mainly because of their well established in vitro ability to scavenge free radicals and other reactive oxygen species (ROS).

Polyphenols are molecules whose structure contains one or more benzene rings to which at least two hydroxyl groups are attached. The term also applies, however, to some simple phenols where only one free hydroxyl group is attached to a single benzene ring. Dietary polyphenols are found in nature especially as secondary metabolites of edible plants(Sandoval-Acuña et al., 2014).

Cocoa (Theobroma cacao) represents the richest source of polyphenols in human diets. Since cocoa is extensively consumed all over the world, interest in the biological activities of cocoa polyphenols is increasing(Khan et al., 2014).

The caffeoylated amino acid clovamide is present in the antioxidant polyphenolic fraction of cocoa. clovamide is the natural amidic analogue of rosmarinic acid and could represents an interesting antiradical/antioxidant compound for its bioactive activity. Clovamide anti-inflammatory activity on human monocytes has been reported as well as its neuroprotective effects(Fallarini et al., 2009; Zeng et al., 2011).

Among the polyphenols, curcumin, the main constituent of the spice tumeric, has attracted a lot of attention due to its ability to exert beneficial effects in multiple pathological conditions. Curcumin has been shown to exert a potent scavenger activity for a variety of ROS such as O2-, OH-, nitrogen

dioxide radicals and non-free radical species such as $H,,_2O_2$. It has also been shown to enhance the activity of antioxidants enzymes and, in particular, to counteract the activity of ROS generating enzymes. Since increased oxidative stress is associated with various cardiovascular diseases, the inhibitory activity of curcumin on ROS generation coupled with its anti-inflammatory properties may contribute to its protective role in cardiovascular diseases (Kapakos et al., 2012).

α-(-)-bisabolol is a natural monocyclic sesquiterpene alcohol. It's present in the essential oil of German chamomile and Myoporum crassifolium have generated large interest in the chemical and pharmaceutical industries. α-bisabolol is known to have anti-irritant, anti-inflammatory and anti-microbial properties (Mohamed et al., 2013). α-bisabolol has recently been shown to induce apoptosis in models of leukemia (Cavalieri et al., 2011). Less is known about its antioxidant activity.

Quercetin, a type of polyphenolic compound found in various plant products, possesses anti-oxidant, anti-proliferative, anti-inflammatory and anti-histamine properties. Several reports have demonstrated that quercetin exerts protective effects and scavenge ROS on various cell, including myocytes, testes, renal cells and liver in ischemia and reperfusion injury (Chen et al., 2013).

Rice is one of the most produced and consumed food in the world. Pigmented rice bran contains high amounts of fiber and molecules, most of which are documented as bioactive compounds improving human health. Part of these compounds are flavonoids, which are responsible for the color of rice. This molecules have attracted considerable attention due essentially to their potential on free radicals scavenging, antioxidant activity, enhancement of immune systems and reduction of the risk of developing heart diseases and cancer (Bordiga et al., 2014).

Less is known about the mechanisms of action of these natural compound in mesenchymal stem cells induced to oxidative stress.

Project aim/objectives

In the laboratory of Histology we previously show that a short-term H_2O_2 treatment leads to oxidative stress and induces apoptosis in H9c2 cells, and that a monoclonal antibody, agonist of the Hepatocyte Growth Factor receptor, could reverse this effect (Pietronave et al., 2010).

We want now to extend this kind of studies to molecules from the vegetable world, which are known to display antioxidant and radical scavenging activities, such as clovamide, curcumin, α-bisabolol and black and red rice extractsIn particular we want to analyze their ability to inhibit the production and release of reactive oxygen species and can protect cells from oxidative stress-induced apoptosis.

To this purpose we will use different cellular models: the H9c2 rat cardiomyoblasts, the m17 ASC mouse adult stem cell line and human Cardiac Progenitors cells (hCPCs).

We choose H9c2 cell-line because this is the only available cardiomyoblast cell-line and it is useful for set the right condition of concentration and time. In the same way, m17 ASC represent a good model of spontaneously immortalized adult mesenchymal stem cells (Zamperone et al). which indeed can also be induced to acquire differentiation traits of cardiomyocytes.

Once the experimental conditions are settled, we will use this information to carry on experiments on hCPCs, obtained from biopsies provided by the Department of Cardiac Surgery of the "Clinica San Gaudenzio" (Novara, Italy) from patients undergoing cardiac surgery after signing a written informed consent according to a protocol approved by the Institutional Review Board (IRB) of Novara (Italy).

On all these cells we will evaluate the protective effects of the different pure polyphenols and plants extracts on the H2O2-induced oxidative stress using different tests.Moreover aim of this project will be also to establish pathways and mechanisms of action involved using Real-time PCR and Western Blot analysis.

These are the aims for my three year PhD program. In the first year I have examined the effects of clovamide on H9c2 rat cardiomyoblasts. We have demonstrated that clovamide protects cardiomyoblasts H9c2 cell line from H2O2-induced stress by inhibiting ROS production, apoptosis and caspases activation and reducing DNA fragmentation (Zamperone et al., 2014).

Experimental plan and methods

CHEMICALS

Clovamide ([(-)-N-[3'-4'-dihydroxy-(E)-cinnamoyl]-dihydroxyphenylalanine]) was provided by Prof. Arlorio, who synthetized and characterized it, as already described(Arlorio et al., 2009). Rice extracts were obtained from Professor Arlorio, Drug and Food Biotechnology Center. Gelatin, dimethyl sulfoxide (DMSO), ethylenediaminetetracetic acid (EDTA), Claycomb Medium, curcumin, α-bisabolol and quercetin were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and L-Glutamine were from Lonza Group Ltd (Verviers, Belgium). F-12 K Nut Mix medium and Slow Fade Gold were obtained from Gibco Life Technologies Corporations (New York, U.S.A.). The tissue culture plates were from Orange Scientific (Braine-l'Alleud, Belgium). Water was obtained by Milli-Q instrument (Millipore Corp., Billerica, MA). Coversplips were purchased from Marienfeld (Lauda-Konigshofen, Germany). The Total ROS Detection kit, provided by Enzo Life Sciences®(Lausen, Switzerland), was used to evaluate oxigen and nitrogen reactive species production. The Image-iT™ LIVE Red Poly Caspases Detection Kit was purchased from Molecular Probes (Eugene, OR). Annexin V-FITC and Propidio iodide were from Alexis (Lausen, Switzerland).

CELL CULTURE

H9c2 cell line was purchased from America Type Culture Collection (Manassas, VA). Cell were cultured in DMEM containing 1500 mg/l $NaHCO_2$, 10% FBS, 50 U per ml penicillin and 50 µg/ml streptomycin. M17 ASC clone was obtained in our laboratory as described by Zamperone and colleagues (Zamperone et al., 2013). Cells were cultured in Claycomb Medium containing 1500 mg/l $NaHCO_2$, 10% FBS, 50 U per ml penicillin and 50 µg/ml streptomycin. Cells were usually passaged and used for the experiments when 90% confluent.

INDUCTION OF OXIDATIVE STRESS

Oxidative stress was induced as described by Pietronave and colleagues(Pietronave et al., 2010) with few modifications. Cells were washed twice in PBS and then incubated in serum-free medium containing 400 µM H_2O_2; the medium was then changed and cells were incubated in presence or not of 3,3 µM clovamide and different concentration of rosmarinic acid and epicatechin from 3 to 24 hours according to the assay.

REACTIVE OXYGEN SPECIES (ROS) PRODUCTION

To reveal reactive oxygen and nitrogen species production (ROS/RNS) within the cells the "Total ROS Detection Kit" was used. Cells were plated on 12 mm^2 glass coverslips (1×10^4) coated with o,1 % gelatin 24 hours before the experiment. Cells were induced to apoptosis, and then treated with clovamide 3,3 µM, rosmarinic acid for 6 hours as described above. At the end of the treatments cells were processed following the kit manufacturer's instructions and mounted, without been fixed, on microscope glass slides with Slow Fade Gold. The kit contained a non-fluorescent, cell permeable dye that react directly with a wide range of reactive species, yielding a green fluorescence. The fluorescent products (excitation at 490 nm, emission at 525 nm) were visualized using a Leica DMI 6000B fluorescence microscope. For each condition (n=3-5) twenty consecutive

fields and, in any case, not less than 150 cells were analyzed. Quantitative analysis was conducted by evaluating cells' relative fluorescence unit (RFU) with the graphic software ImageJ using the ROI Manager function of the Multi Measure plug-in.

ANNEXIN V / PROPIDIUM IODIDE

At least 50,000 cells, induced to apoptosis, were treated or not with clovamide for 24 hours as described above. Cells were then detached with 5 mM EDTA, washed twice with PBS and incubated for 15 minutes at room temperature with Annexin V – FITC (100 Nm) and Propidium Iodide (50 µg/ml) both diluted in a Hepes/NaOH 10 mM , NaCl 140 mM, CaCl2 2,5 mM (pH 7.4) buffer. Cells were then washed twice with ice cold PBS and fixed in buffered 1% paraformaldehyde, 2% FBS for 15 minutes at 4°C and analyzed with FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 488-nm argon laser.

DAPI AND TUNEL STAINING

Apoptotic cells were revealed, 6 hours after H_2O_2 withdrawal in a serum-free medium, by in situ TUNEL assay performed with the ApopTag® Fluorescein Direct In Situ Apoptosis Detection Kit according to the manufacturer's instructions. The cell nuclei were labelled with the DNA-labelling fluorescent dye 40,6-diamidino-2-phenylindole dihydrochloride (DAPI; 50 mg/ml) in PBS, 0.1% Triton X-100, and 4% FBS. The cells were visualized under a Leica DMI 6000B fluorescence microscope equipped with a UV lamp for DAPI detection. Twenty consecutive fields for each sample, and in any case not less than 150 cells, were scored in a double-blind manner, and the ratio of TUNEL-positive cells versus the total number (DAPI-positive figures) in the untreated samples was given a value of 1, to which the values similarly obtained in the treated samples were referred.

POLYCASPASES ACTIVITY ASSAY

Cells were plated on glass coverslips one day before the experiment. Caspases activity was measured after 15 minutes transient H_2O_2 treatment and a further 6 hours treatment with serum-free medium containing or not 3.3 µM clovamide. hCPCs cells were stained using the Image-iT™ LIVE Red Poly Caspases Detection Kit following the manufacturer's instructions and mounted on microscope glass slides. Cells were visualized under a Leica DMI 6000B fluorescence microscope equipped with appropriate bandpass filters and an UV lamp for the detection of all three fluorochromes. Twenty consecutive fields for each sample and in any case not less than 150 cells were scored in a double-blind manner.

STATISTICAL ANALYSIS

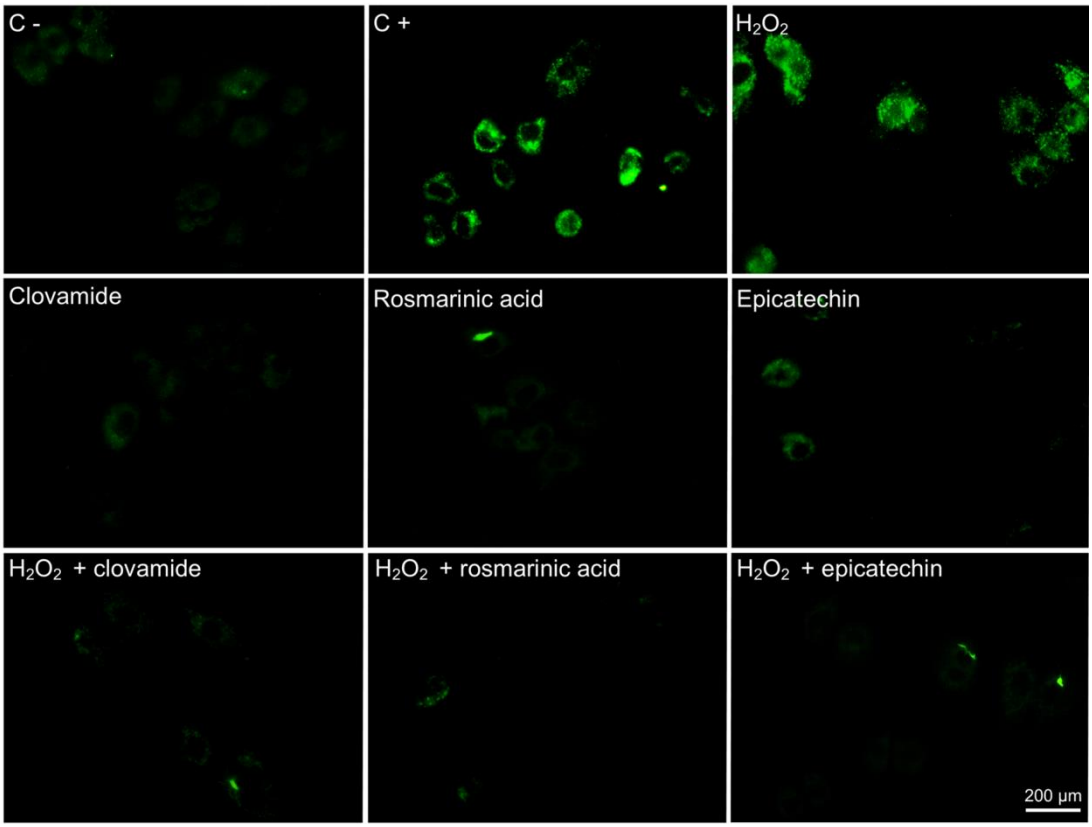
Quantitative analysis are presented as mean + standard deviation (SD) and differences between samples were determined by Student's t-test. One-way ANOVA and Bonferroni post-test analyses on selected pairs of groups were also performed with Prism (GrapPad software Inc., USA, version 4.03). Values with a $p<0.05$ or $p<0.01$ were considered as statistically significant.

Results

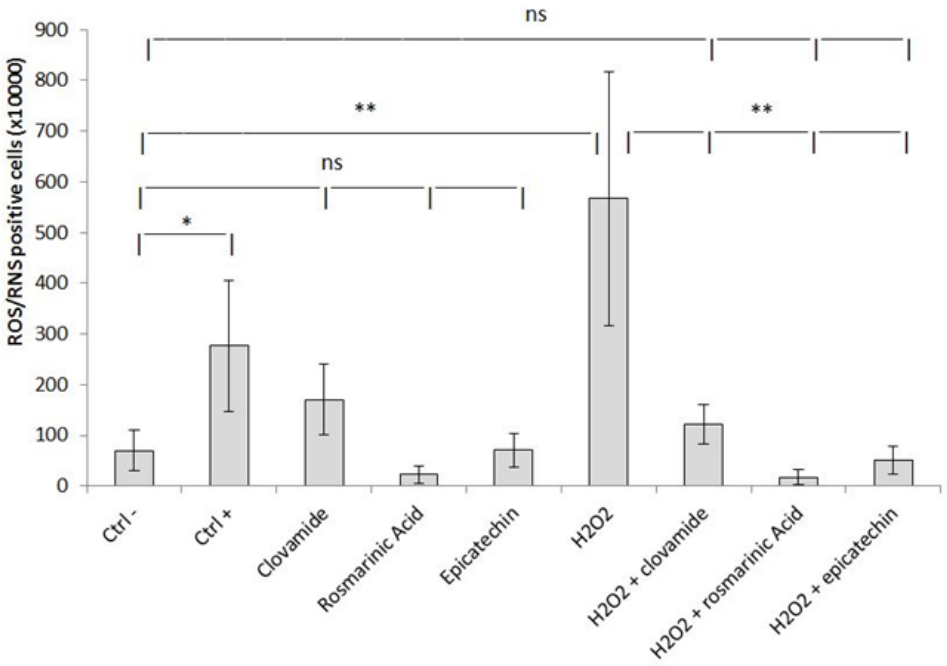
CLOVAMIDE INHIBITS ROS PRODUCTION IN H9C2 SUBJECTED TO HYDROGEN PEROXIDE OXIDATIVE STRESS TREATMENT

A short-term H_2O_2 treatment was previously shown to induce apoptosis in rat cardiomyoblasts (H9c2 cell line) and the treatment with a monoclonal antibody agonist of the Hepatocyte Growth Factor receptor was shown to be able to reverse this effect (Pietronave et al., 2010). I have now extended these studies by examining the biological activity of clovamide on H9c2. More precisely I have examinated whether clovamide was able to inhibit the production and release of reactive oxygen species (ROS) and counteract apoptosis directly within the cells. In parallel I tested rosmarinic acid and (-)-epicatechin, molecules with known antioxidant and radical scavenging activities. To this purpose cells, after short-term treatment with 200 µM H_2O_2 for 15 minutes and its subsequent withdrawal, received 3,3 µM clovamide. ROS production was evaluated by an assay which traces ROS production (including NO, which is not revealed by the previous assay) directly within the cells. The three molecules were able to reduce the green intracellular signals, associated with the presence of the ROS induced by H_2O_2 treatment, to the basal levels (Fig. 1A, lower row as compared to the top right panel). The ability of clovamide to inhibit ROS production was evaluated with another assay in which was revealed as the spectrophotometric absorbance of SOD-inhibitable cytochrome C reduction, that was markedly suppressed by clovamide addition in the range of 30 nM– 3.3 µM 2 hours after hydrogen peroxide treatment in a dose dependent manner (Fig. 2C). As expected, the other two molecules used in this study, rosmarinic acid and epicatechin, were also able to inhibit ROS production from the H_2O_2-treated cells. Clovamide displayed a somehow lower activity, but these three molecules appeared more efficient than Trolox, a water soluble analogue of vitamin E, commonly used as a standard reference for antioxidant activity, which was used at a 50 mM concentration.

A


C -
C +
H_2O_2
Clovamide
Rosmarinic acid
Epicatechin
H_2O_2 + clovamide
H_2O_2 + rosmarinic acid
H_2O_2 + epicatechin
200 µm

B


ROS/RNS positive cells (x10000)
900
800
700
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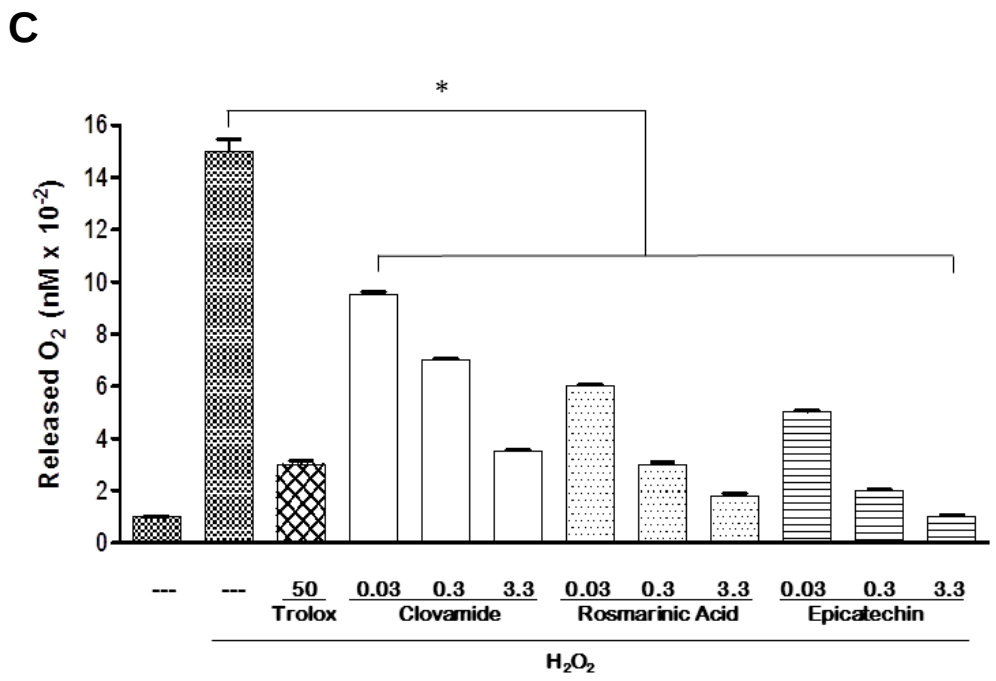
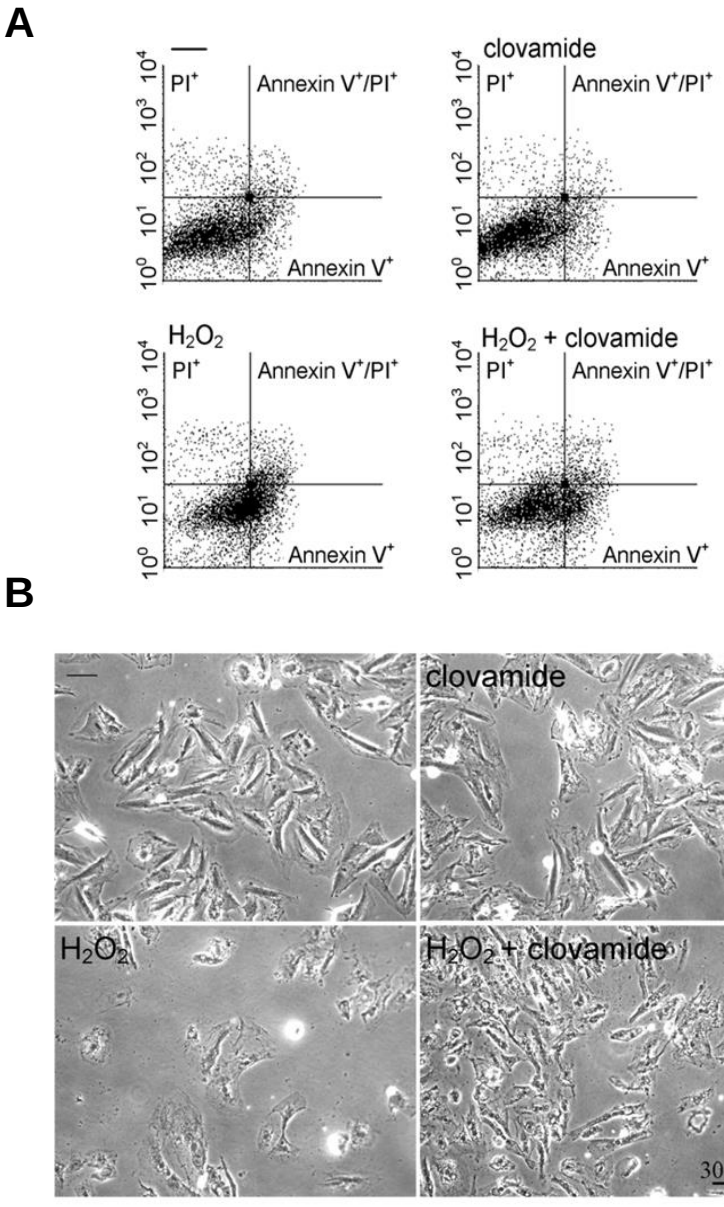


Fig. 1: Clovamide inhibits the triggering of ROS production in cardiomyoblasts subjected to hydrogen peroxide oxidative stress treatment. (A) H9c2 cells were plated on glass coverslips and incubated with 200 µM H_2O_2 for 15 min in a serum-free medium, which was then replaced with a fresh phenol-free and serum free medium containing clovamide, rosmarinic acid or epicatechin at different concentrations, all expressed in µM. At the end of 2 h incubation in the presence of 3.3 µM clovamide, 300 nM epicatechin or rosmarinic acid, reactive oxygen and nitrogen species were traced by the addition of a green fluorescent probe. Pyocyanin and N-acetyl-L-cysteine were used as positive (C+) and negative (C) controls, respectively, according to the manufacturer's protocol. A representative experiment out of the three performed is shown. In both types of experiments, the three molecules significantly reduced the ROS induced by H_2O_2 treatment. (B) Quantitative description of Fig. 2A. Data were obtained by counting 20 consecutive fields and,
in any case, never less than 100 cells, detected in optic microscopy, in each of the 3 experiments performed. Quantitative analyses were carried out by calculating the cells' relative fluorescence unit (RFU) normalized by the same background with the graphic software ImageJ (ROI Manager
function of the Multi Measure plug-in). Clovamide alone, as well as rosmarinic acid and epicatechin, induced no significant production of ROS/RNS. In contrast, the three molecules reduced the amount of ROS/RNS induced by H2O2 treatment to their basal levels.
(C) H9c2 cells were plated and treated as above. After 2 h of further incubation, the SOD-inhibitable cytochrome C reduction was analyzed in a chromogenic assay (550 nm). Trolox was used as a reference anti-oxidant control. The experiments were repeated at least three times. A couple of conditions were analyzed with Bonferroni post-test after one-way ANOVA (*$p < 0.001$).

CLOVAMIDE PROTECTS CARDIOMYOBLASTS FROM OXIDATIVE STRESS INDUCED APOPTOSIS

Apoptosis was induced with the same short-term 200 mM H_2O_2 treatment, followed by replacement with a fresh serum-free medium, since this protocol mirrors the frequently encountered situation of acute myocardial ischemia-reperfusion, and incubation for further 24 h with Clovamide 3,3 µM. In this system, clovamide was able to reverse the pro-apoptotic effect of H_2O_2 (Fig. 2A), as shown in a representative cytogram where apoptotic cells were stained with annexin V. Upon this treatment, the level of necrotic cells, labelled with PI, also decreased. A microscopic examination confirmed that the highly toxic H_2O_2 treatment, which induced cell shrinkage, membrane blebbing and apoptotic bodies, was efficiently counteracted by clovamide (Fig. 2B). Clovamide, as well as rosmarinic acid and epicatechin, significantly reduced the H_2O_2-induced apoptosis, as shown in Fig. 2C. It can, therefore, be concluded that clovamide displays bioactivity on H9c2 cardiomyoblasts by protecting them from H_2O_2-induced apoptosis.



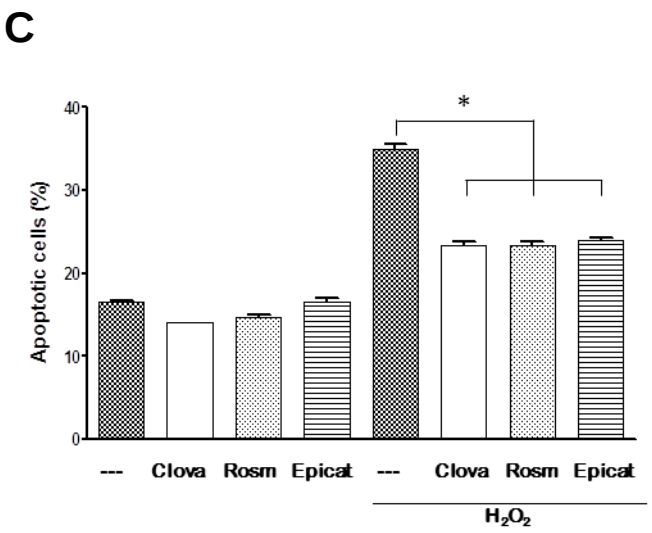


Fig. 2 Clovamide protects cardiomyoblasts from oxidative stress induced apoptosis. Cells were incubated with H_2O_2 for 15 min in a serum-free medium, which was then replaced with a serum-free medium containing 3.3 µM clovamide, 300 nM rosmarinic acid or epicatechin. After further 24 hours of incubation, cell apoptosis and necrosis were evaluated by labelling with FITC-annexin V and propidium iodide (PI), respectively. (A) Representative cytograms of such an experiment performed using 3.3 µM clovamide. (B) Morphological aspects of control untreated cells, cells treated with hydrogen peroxide, or clovamide alone, and cells treated with both H_2O_2 and clovamide. (C) Graphical representation of the percentage of apoptotic cells (annexin V-labelled) upon H_2O_2 treatment and subsequent treatments with different polyphenols. Statistical analyses were performed by comparing the cells treated with a protective agent (clovamide, rosmarinic acid or epicatechin) vs. untreated cells (*$p < 0.001$). All the experiments were repeated at least three times in triplicates. The three molecules were able to significantly reduce apoptosis induced by H_2O_2 treatment.

CLOVAMIDE REDUCES DNA FRAGMENTATION INDUCED BY OXIDATIVE STRESS

The anti-apoptotic activity of clovamide was also tested for its ability to inhibit DNA fragmentation using the TUNEL/DAPI co-staining assay six hours after H_2O_2 withdrawal. The H_2O_2 treatment induced a six-fold increase of TUNEL-positive cells in comparison to the untreated cells. Incubation with 3.3 µM clovamide reduced the number of TUNEL positive cells to about 20%. Furthermore, rosmarinic acid used at a concentration of 300 nM was able to decrease the number of positive cells reaching the same percentage (Fig. 3A).
Are also reported representative photographs of the experiments, showing the co-staining of DAPI-labelled nuclei with TUNEL-positive shrink led nuclei of cells undergoing apoptosis (Fig. 3B).

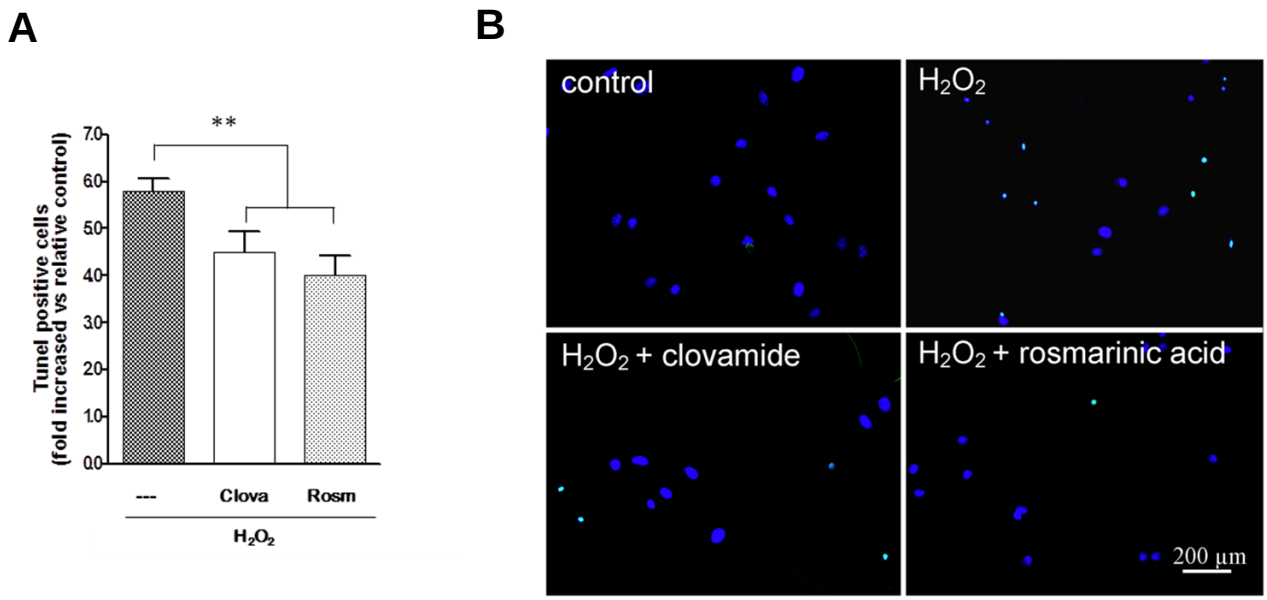


Fig. 3 Clovamide reduces DNA fragmentation induced by oxidative stress. H9c2 cells plated on glass coverslips underwent transient oxidative stress and further incubation in a FBS-free medium in the absence or presence of 3.3 µM clovamide or 300 nM rosmarinic acid for 6 hours. After incubation, cells were fixed with paraformaldehyde, permeabilized, and nuclei co-stained with DAPI (blue) and TUNEL (green, cells undergoing apoptosis). (A) Twenty fields and at least 150 cells were scored in each experimental condition. Statistical analyses were performed by comparing the cells treated with protective agents, clovamide or rosmarinic acid, versus H_2O_2-untreated cells without molecules (**$p < 0.05$). The assays were repeated three times in triplicates. (B) Representative photographs of an experiment. The two molecules were able to significantly inhibit H_2O_2-induced DNA fragmentation.

CLOVAMIDE INHIBITS THE ACTIVATION OF CASPASES INDUCED BY HYDROGEN PEROXIDE TREATMENT

Apoptosis usually results from the activation of caspases by the offending factor. We thus investigated the ability of clovamide to inhibit caspases activation in H9c2 cells 6h after H_2O_2 withdrawal. In this assay, show on picture 4A, caspases activation is visualized by the red-fluorescence staining. Hydrogen peroxide treatment increases the percentage of cells in which caspases were activated compared to the control. Clovamide was able to reduce the number of cells in which caspases were activated, after H_2O_2 treatment. This value was comparable to the control. Thus, in general, Clovamide is able to interfere with the activation of caspases, which are involved in the apoptotic process.

Hoechst 33342 fluorescence microscopy was used to qualitatively observe apoptosis of cells. Images, at the phase contrast microscope, revealed, after hydrogen peroxide treatment, clear morphological signals of apoptosis, namely cell shrinkage, membrane blebbings and apoptotic bodies. Labelling of cells with SYTOX®, a green nuclear dye able to reveal cells with damaged cell membranes, and thus tracing necrotic cells or cells in the late steps of apoptosis, gave negative result.

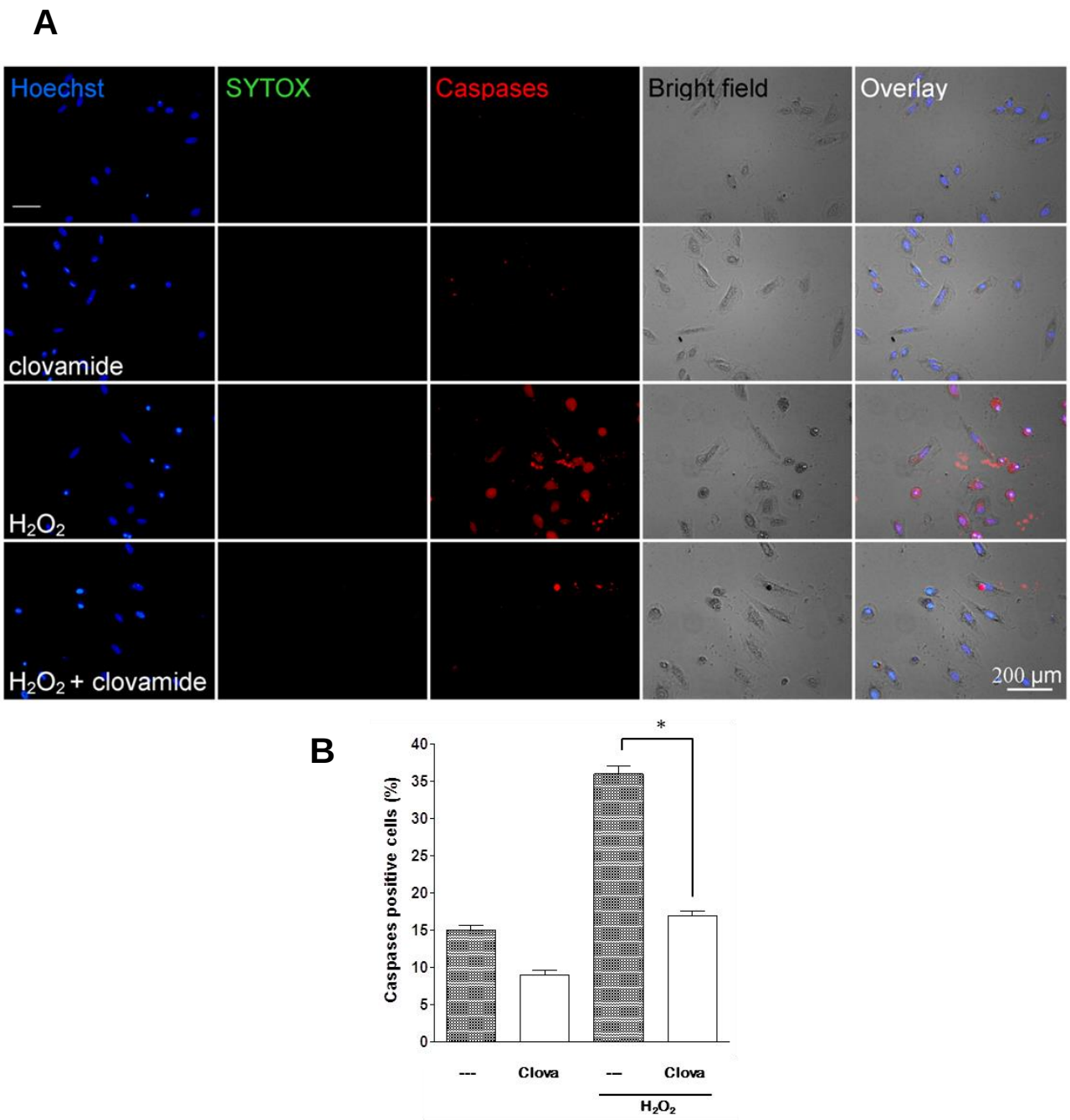


Fig. 4 Clovamide inhibits the activation of caspases induced by hydrogen peroxide treatment. H9c2 cells plated on glass coverslips underwent transient oxidative stress for 15 minutes and further incubation in a FBS-free medium in the presence or absence of 3.3 µM clovamide. (A) After 6 h, the cells were fixed with paraformaldehyde and stained with Hoechst (left column), SYTOX green (second column), and SR-DEVD-FMK red poly caspases reagent (third column). Micrographs were merged (fifth column) with bright field images (fourth column). (B) Graphical representation of the percentage of cells positive for caspases activation vs. total cells. Values were obtained by means of eight images from three separate experiments. Statistical differences among the groups were determined (*$p < 0.001$).

Discussion

Cardiovascular diseases are the leading cause of morbidity and mortality in the industrialized countries (Gräsner and Bossaert, 2013). Several studies have demonstrated how apoptosis is involved in both acute and chronic cases and is one of the prevalent phenomenon during myocardial infarction, This data suggests an important role of apoptosis during the acute phase of heart attack. Moreover, apoptotic phenomenons have been shown during later phase suggesting an involvement in the heart remodeling.

Short term treatment with H_2O_2 induces apoptosis in H9c2 cardiomyoblasts, as already reported (Han et al., 2004). This sentence is confirmed by the acquisition of features typical of this event, namely, positivity to annexin V, activation of caspases, and fragmentation of genomic DNA. This treatment also induces an increase in the cellular levels of ROS, thus establishing a cause–effect relationship in promoting apoptosis. Similar data were also reported in the case of oxidative stress induced in these cells by other molecules. For the first time, the present study demonstrates that clovamide, a minor constituent of cocoa, is able to protect cardiomyoblasts from apoptosis induced by oxidative stress by counteracting the effects induced by ROS. We have shown that clovamide, as well as its isostere (rosmarinic acid) and the main monomeric flavan-3-ol from cocoa ((-)-epicatechin) interfere with and reduce apoptosis triggered by the H_2O_2 treatment in H9c2 cardiomyoblasts by decreasing the levels of intracellular ROS. The analyses for annexin V positivity, DNA fragmentation, and caspase release and activation provided significant evidence on the direct protective efficacy of clovamide. This work, thus, confirms and strengthens the radical scavenging properties of these polyphenols and their anti-apoptotic activity.

These data will be the basis for the same kind of experiments to be performed on hCPCs, which, being cells obtained from patients undergoing cardiac surgery, are more difficult and time-consuming to be prepared. Moreover, other natural compounds, such as curcumin, α-bisabolol, quercetin and rice extracts, will also be tested in this context for their potential protective effects on H_2O_2-induced apoptosis.

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Publications (articles, posters, abstracts)

- **Articles**

Protective effects of clovamide against H2O2-induced stress in rat cardiomyoblasts H9c2 cell line.
Zamperone Andrea, Pietronave Stefano, Colangelo Donato, Antonini Silvia, Locatelli, Monica, Travaglia Fabiano, Coïsson Jean Daniel, Arlorio Marco and Prat Maria (2014). *Food & Function*, *5*(10), 2542–51. doi:10.1039/c4fo00195h

- **Posters**

Generation and characterization of implantable spheroids made of human cardiac progenitors cells by a novel methylcellulose hydrogel-based system
Francesca Oltolina, Andrea Zamperone, Luca Gregoletto, Silvia Antonini, Eugenio Novelli, Marco Diena, Carmine Nicoletti, Antonio Musarò and Maria Prat
TERMIS – EU 2014 Chapter Meeting ; 10 – 13 Giugno 2014; Genova

Quick generation of spherical aggregates of human cardiac progenitor cells for cardiac scaffold-less tissue engineering by means of a smart methylcellulose hydrogel
Francesca Oltolina, Andrea Zamperone, Silvia Antonini, Luca Gregoletto, Eugenio Novelli, Marco Diena, Carmine Nicoletti, Antonio Musarò, Maria Prat
Stem Cell, Development and Regenerative Medicine (SCDRM); 6-7 Giugno 2014; Salerno

Attended seminars

- 20/10/2014 - Famà Rosella - "*The Krϋppel-like factor 2 transcription factor is a novel tumor suppressor gene recurrently mutated in Splenic Marginal Zone Lymphoma*"

- 1/10/2014 - Ciardullo Carmela - "*Clonal evolution and clinical relevance of subclonal mutations in chronic lymphocyticleukemia"*

- Steven R Ellis – "*The Erbolario Sessions*"

  - 8/09/2014
    10:00 Clinical case – Skin as an organ
    11:00 Layers of skin, cell types, developmental origins

  - 9/09/2014
    10:00 Cell-Cell Interactions – anchoring junctions
    11:00 Cell-Cell Interactions – occluding junctions, tight junctions

  - 10/09/2014
    10:00 Cell Matrix Interactions – basal lamina
    11:00 Epithelial-mesenchymal transition

- 11/09/2014
  10:00 Angiogenesis
  11:00 Innervation

- 15/09/2014
  10:00 Basal layer stem cells, symmetric versus asymmetric divisions, transient amplifying cells
  11:00 Solar radiation, nucleotide excision repair

- 16/09/2014
  10:00 Basal and squamous cell carcinomas
  11:00 Melanoma – biology

- 17/09/2014
  10:00 Melanoma - treatment
  11:00 Contact dermatitis

- 22/09/2014
  10:00 Other skin disorders
  11:00 Other components of skin

- 21/7/14 - Dr Maria Giuseppina Miano - "*A functional link between Arx and Kdm5c genes linked to neuronal diseases defines a crucial epigenetic path*"
- 16/07/2014 - Prof. John F. McDonald - "*The potential of small regulatory RNAs for the treatment of ovarian cancer*"
- 15/07/2014 - Prof.ssa Follenzi - "*Gene therapy application*"
- 30/06/2014 - Dott. Cotella - "*The C-value paradox, junk DNA and ENCODE*''
- 27/06/2014 - Manuela Sironi – "*Has nature done the experiment for us? Evolutionary insights into infection susceptibility and autoimmunity*"
- 26/06/2014 - Prof Gianni Del Sal - "*Disarming mutant P53 in cancer*"
- 19/06/2014 - Prof.ssa Follenzi - ''*Advance in Gene Therapy*''
- 12/06/2014 - Gianni Cesareni - "*Metformin rewires the signaling network of breast cancer cells and changes their sensitivity to growth and apoptotic stimuli*"
- 11/06/14 - Prof. Fabrizio Loreni - "*Ribosome alteration in cancer: effect or cause?*"
- 9/06/2014 - Dott. Iacopo Baussano - "*Assessment of cervical cancer control in Rwanda and Bhutan*''
- 5/05/2014 - Prof. Vittorio Colombo e Dr. Matteo Gherardi - ''*Atmospheric pressure plasma sources ad processes for biomedical and surface treatment applications*''

- 19/03/2014 - Prof Emilio Hirsch - ‘‘*Role of phosphoinositides-3-kinase C2-alpha Class II PI 3-kinase, in development and cancer*’’
- 19/02/2014 - Prof. Salvatore Oliviero - ‘‘*Epigenetic modifications that control stem cell differentiation*’’