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Role of Reactive Oxygen Species in signalling and oxidative stress

Presentata da: **Benedetta Rizzo**

Coordinatore Dottorato **Relatore**

Prof. Mario Santi Spampinato **Prof.ssa Silvana Hrelia**

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Chapter 1- Introduction

1.1. The physiological roles of Reactive Oxidizing Species – ROS

The presence of free radicals in biological materials was discovered less than 50 years ago (Commoner, Townsend et al. 1954). Immediately after, Denham Harman hypothesized that oxygen radicals may be formed as by-products of enzymatic reactions *in vivo*. In 1956, he described free radicals as a Pandora's box, that may account for cellular damages, mutagenesis, cancer, and degenerative process of biological aging (Harman 1981). Present day evidence shows that reactive oxygen-, nitrogen- and chlorine-derived species, now collectively called "Reactive Oxidizing Species" (ROS), have a crucial role in human physiological and pathological processes (Vina, Borras et al. 2013).

1.1.1 Reactive Oxygen Species

From the univalent reduction of molecular oxygen, the superoxide radical anion ($O_2^{-}\bullet$) is formed. This process is mediated *in vivo* by enzymes such as NAD(P)H oxidases, xanthine oxidase, by the electron leakage from mitochondrial respiratory complexes, or by redox reactive compounds. Superoxide dismutase (SOD) catalyzes the spontaneous dismutation of superoxide into hydrogen peroxide (H_2O_2) (Deby and Goutier 1990). In the presence of reduced transition metals (*i.e.*, ferrous or cuprous ions), hydrogen peroxide can be converted into the highly reactive hydroxyl radical (•OH) (Chance, Sies et al. 1979). More frequently, hydrogen peroxide is converted into water by the enzymes catalase or glutathione peroxidase. In the glutathione peroxidase reaction, glutathione (GSH) is oxidized to glutathione disulphide (GS-SG), which can be converted back to glutathione by glutathione reductase in an NADPH-consuming process.

1.1.2 Reactive Nitrogen Species

The nitric oxide radical (NO) is enzymatically produced by nitric oxide synthase (NOS) in higher organisms through the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine. Depending on the microenvironment, NO can be converted into various other reactive nitrogen species, such as nitrosonium cation (NO^{+}), nitroxyl anion (NO^{-}) or peroxynitrite ($ONOO^{-}$) (Stamler, Singel et al. 1992). Some of its physiological effects may be mediated through the intermediate formation of *S*-nitroso-cysteine or *S*-nitroso-glutathione (Gow and Stamler 1998).

1.1.3 ROS in signal transduction and the revisited free radical theory of aging

By the turn of the century, the free radical theory of aging appeared to be established. Much of the evidence gathered in favour of this theory was based on the correlation between high levels of antioxidant defences and increase in life-span. But other data have shown that in some cases, an

increased oxidative stress provoked longevity and metabolic health. In other words, evidence was produced either in favour or against the free radical theory of aging. The final knockdown for this theory came from epidemiological studies showing that antioxidant supplementation did not lower the incidence of many age-related diseases but, in some cases, increased the risk of death. Moreover, recent molecular evidence shows that, sometimes, an increased ROS formation, increases longevity. This is not surprising, since in the last decades it has become clear that cells generate ROS at lower levels to serve as signalling functions, that are crucial for cellular homeostasis. These oxidizing species function in signal transduction by reversible oxidative modifications of proteins, leading to phosphorylation cascades and altered gene transcription. ROS-mediated signalling can promote cell proliferation and survival, but also can regulate programmed cell death, or necrosis, or, as recently demonstrated, autophagy, thereby playing a key role in the modulation of cell turnover (Fisher 2009).

A fine balance between ROS generation and antioxidant levels allows the control of fundamental intracellular functions. If the cell can cope with the stress caused by relatively mild action of ROS, adaptation takes place and damages do not occur. If, however, ROS formation is dysregulated and surpasses antioxidant defences, oxidative stress is induced and subcellular damages and aging will take place (Fig. 1).

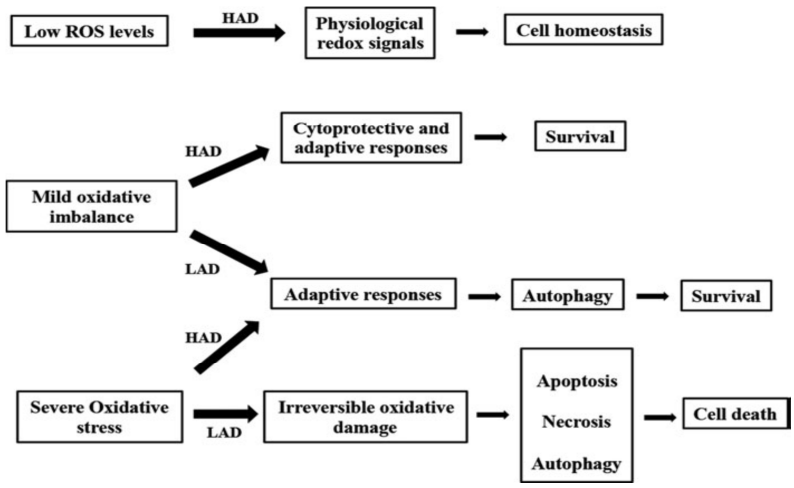


Fig. 1. ROS and antioxidant defences in the mechanisms of cell survival or death.

In physiological conditions, low ROS levels together with HAD (highly efficient and unaltered antioxidant defences) allow the generation of intracellular signals leading to cell homeostasis. In conditions of mild increase of ROS formation (mild oxidative stress), the HAD promote cell survival through the activation of cytoprotective and adaptive responses. When antioxidant defences are exhausted, LAD (low antioxidant defences), autophagy can be triggered. Under severe oxidative stress conditions, necrotic, apoptotic, or autophagic signalling pathways can be activated, which lead to cell death.

From: Pietraforte D. and Malorni W, 2014 (Pietraforte and Malorni 2014).

The observation that ROS, which normally act as physiological signalling molecules, could activate intracellular pathways leading either to activation of survival mechanisms or to the triggering of the cell death program, earned them the epithet of “double edge sword” (Pietraforte and Malorni 2014). Thus, Viña and coworkers proposed “The cell signalling disruption theory of aging”, which postulates that aging is caused by a disruption of the whole signalling network involving ROS, and that, in general terms, it is better to increase endogenous defences by nutritional or physiological manipulations than administering antioxidant compounds or vitamins (D'Autreaux and Toledano 2007).

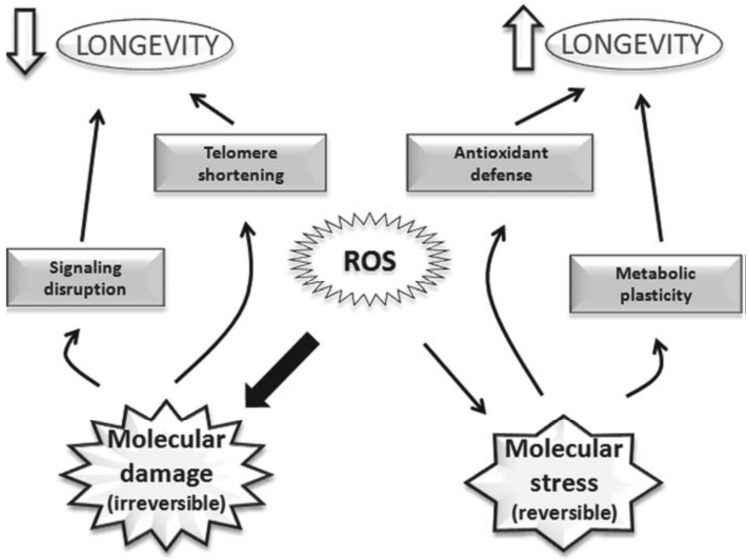


Fig. 2. The double edge sword of free radicals and longevity.

When radicals cause severe effects on biomolecules, it causes damage (irreversible alterations) and shortens life-span, whereas when the aggression is mild, endogenous antioxidant defences are produced, which extends the organism longevity.

From: Vina J, 2013 (Vina, Borras et al. 2013).

1.1.4 The NADPH oxidases: Nox family

Mitochondria were considered for a long time the main source of intracellular ROS as a part of aerobic respiration, but the demonstration that ROS are produced in response to growth factors, cytokines, or G protein-coupled receptor agonists, raised the hypothesis of the existence of other intracellular ROS sources, where oxidant production occurs nearby to the intended target. In fact, ROS production in the precise subcellular compartment after receptor activation is essential for specific redox signalling. Therefore, in order to act as efficient second messengers, ROS must satisfy the stringent criteria required for the signal transduction modulation, that is, the oxidation

should be a) mild, fast and reversible, b) regulated and compartmentalized, c) specific for target proteins and d) able to induce a distinct cellular biochemical outcome (Pietraforte and Malorni 2014).

The discovery of NADPH oxidases isoforms widely distributed among many non-phagocytic cell types, has brought to the notion that these membrane-bound enzymes appeared to be the major source of intracellular ROS (Ushio-Fukai and Urao 2009).

These regulated enzyme, now recognized to have specific subcellular localization, are the best candidate for localized and programmed ROS production, which activates specific redox signalling pathways mediating various cell functions. Moreover, the NADPH oxidase subunits strictly interact with targeting proteins.

These enzyme complexes contain a homologous catalytic subunit, Nox, for this reason they form the Nox family, which differs primarily in the flavocytochrome component. Five Nox proteins have been described, along with two additional enzymes called DUOX, which contain a peroxidase-like domain (Altenhofer, Radermacher et al.).

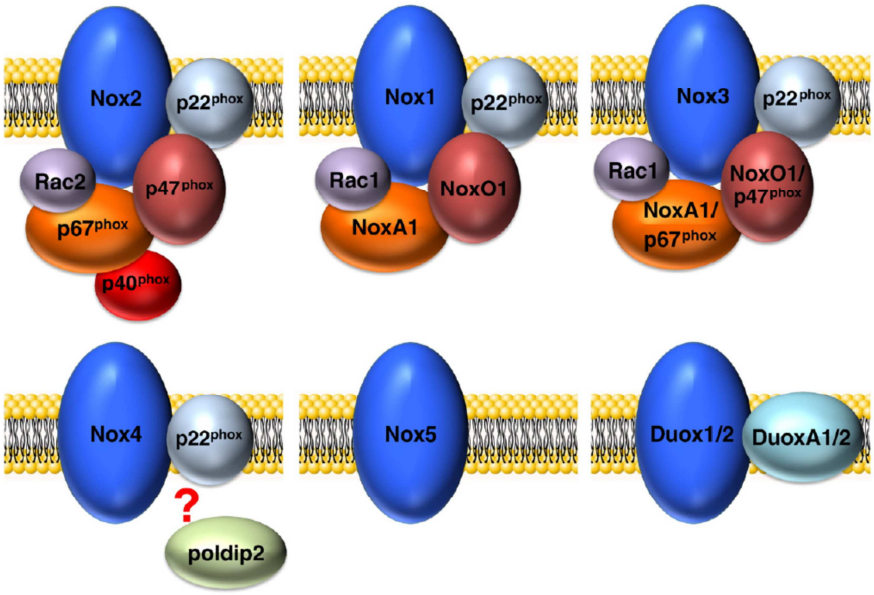


Fig. 3. Current model of the active Nox complexes.

Each of the seven members of Nox family requires a different set of conditions and protein associations. The cytosolic subunits that are believed to assemble onto each Nox isoform in the active oxidase state are shown. *From: I. A. Ghouleh et al., (Al Ghouleh and Magder 2011).*

All the seven Nox members contain two iron-heme prosthetic groups, as well as a flavin adenine dinucleotide (FAD) and an NAPDH-binding domain in the cytosolic c-terminus. FAD is constitutively bound to the enzyme, whereas NADPH serves as its substrate. Nox2 is the membrane oxidase isoform identical to the phagocytic NADPH oxidase complex, nevertheless the rate of

superoxide generation by Nox2 in non-phagocytic cells is lower than the levels required for microbicidal function, but is sufficient for cell signalling (Fisher 2009). Nox enzymes catalyze the electron transport across plasma membrane from NADPH to molecular oxygen outside the cell, generating the superoxide anion in the extracellular space. Here, the neo-formed superoxide anion undergoes to spontaneous or catalysed dismutation reaction, giving rise to hydrogen peroxide, H_2O_2.

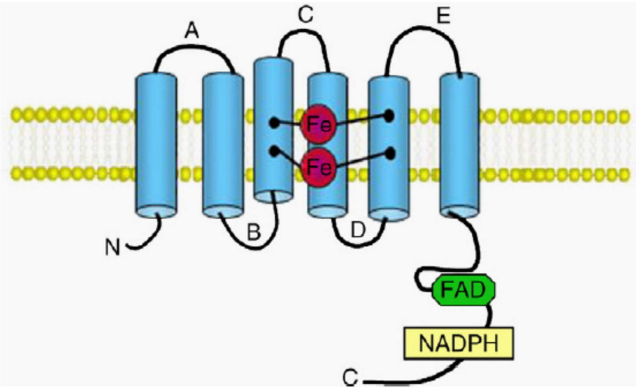


Fig. 4. Schematic representation of common Nox features.

Shown here are the key features in common for all Nox family members, along with the heme-binding site (Fe) and the FAD- and NADPH-binding domains.

From: I. A. Ghouleh et al.,(Al Ghouleh and Magder 2011).

It is generally believed that, once generated, H_2O_2 could simply diffuse into the cell across the plasma membrane for signalling purposes, but recent evidence demonstrates that hydrogen peroxide might preferentially enter the cell through specific plasma membrane aquaporin channels (Bienert and Chaumont 2014).

The hypothesis that H_2O_2 represents the signal molecule is compatible with the properties of this peroxide, that coincide with the theoretic requirements for a second messenger function: it is produced by a regulated enzyme in a specific site and its concentration is regulated through rapid removal by various enzymes with specificity for it (Fisher 2009).

Depending on the cell type and on the different subcellular localization, Nox enzymes activate specific, context-dependent signalling pathways *via* localized production of ROS following numerous stimuli, including growth factors, cytokines and physical or mechanical stresses. On this regard, it has been recently demonstrated that specialized plasma membrane microdomains called caveolae/lipid rafts, rich in cholesterol and sphingolipids, concentrate multiple signalling molecules, thus forming signalling platforms (Ushio-Fukai 2009). The assembly of functional active NADPH

oxidase and subsequent ROS production has been demonstrated to be dependent on caveolae/lipid rafts in many cell types, such as, for example, in vascular and endothelial cells (Hilenski, Clempus et al. 2004). As far as the Nox stimulation is concerned, it has been reported that Nox1 expressed in vascular cells and Nox2 in endothelial cells are activated by AngII, thrombin, prostaglandin F, PDGF and VEGF, whereas in the same cells Nox4 is regulated by shear stress, mitochondrial dysfunction, hypoxia and transforming growth factors (Hilenski, Clempus et al. 2004).

Thus, recognition of the cellular context is essential to an appreciation of Nox-mediated signal transduction; the cell type, the functional endpoint, the complement of Nox proteins expressed in the cell, and the tissue environment must be considered.

1.1.5 The Aquaporin family

Aquaporins (AQPs) are integral, transmembrane, small hydrophobic water channel proteins extensively expressed in many organs and tissues, where they play a prominent role in regulating physiological functions, particularly those involved in fluid transport.

The first characterization of AQP, now named as AQP1, was performed in 1992 by Peter Agre, who demonstrated that the presence of a transmembrane channel protein could explain the high membrane water permeability of erythrocytes and other cells, which could not be justified only by simple passive diffusion of water molecules across the lipid bilayers (Agre 2004). Currently, it is known that AQP expression on cell plasma membrane increases osmotic membrane water permeability up to about 50-fold compared to the lipid bilayer (Verkman 2012).

From a structural point of view, AQPs usually possess a tetrameric organization in membranes. Each monomer is quite small, about 30 kDa, and comprises six transmembrane α-helical segments, two half helices and five connecting loops of variable length, with the N- and C-terminal domains sited in the cytoplasm. The mechanism allowing AQPs to facilitate water (or glycerol) transport involves an aqueous pore; however, the conveyance of protons is avoided, thus hindering the dissipation of proton gradients (Verkman 2012).

To date, the family consists of 13 members, highly conserved across the plant and animal kingdoms and it could be divided in two groups, the mainly water-permeable aquaporins and the mainly glycerol-permeable aquaglyceroporins.

The major AQP function is to facilitate the transport of water or glycerol over cell plasma membranes; some members of AQP family are also able to transport other small molecules, such as urea, CO_2, ammonia, nitric oxide.

In the last decade, it has been demonstrated that also H_2O_2 can be carried by some AQP members in several living organisms (Bienert, Moller et al. 2007).

Only specific AQP isoforms are able to facilitate the passive diffusion of H_2O_2 across biological membranes, thereby having a crucially impact on the membrane permeability of H_2O_2. These AQP isoforms are AQP1, AQP3 and AQP8 and they were named "peroxoporins" by Henzler and Steudle (Henzler and Steudle 2000).

Their transport ability was ascertained through experiments in which AQP inhibitors, such as mercuric chlorid, silver nitrate, or phloretin, were found to significantly inhibit the transmembrane flux of H_2O_2.

This regulated entry of H_2O_2 provides another potential mechanism through which oxidants could be channelled to an intended target and thereby achieve some measure of overall signalling specificity (Finkel 2011).

1.1.6 Downstream targets of H_2O_2 signalling

Transmission of a redox signal initiated by H_2O_2 to a protein occurs through the oxidative modification of some amino acid side chains, such as, in decreasing order of reactivity and biological reversibility, cysteine, methionine, proline, histidine and tryptophan. In particular, thiol modification is a key factor in H_2O_2 sensing and perception in proteins (Sies 2014).

Growth factors, through H_2O_2 production, induce downstream effects on tyrosine phosphorylation acting by different mechanisms, such as the inactivation of protein phosphatases, thereby increasing the level of protein phosphorylation. Alternatively, direct modification of the growth factor receptor by H_2O_2 at a critical active site cysteine was shown to enhance tyrosine kinase activity.

Insulin signalling was probably the first transduction chain in which H_2O_2 was invoked as a second messenger, thus this peroxide was called an "insulin-mimetic" (Heffetz, Bushkin et al. 1990).

It is well known that insulin signals through the PI3K/Akt signalling pathway, and it has been shown that activation of this pathway supports survival of cells exposed to hydrogen peroxide, in agreement with the insulin-mimetic function of H_2O_2. The major functions of the PI3K/Akt signalling pathway are to block apoptotic processes and to regulate fuel metabolism. Hence, the activation of this pathway by ROS leads to interference with cellular proliferation, survival and energy metabolism.

Regarding non-receptor kinases, signal-mediated H_2O_2 production increases Akt activation and many studies documented the H_2O_2-induced activation of MAPK pathway.

In Fig. 5 it is reported a possible mechanism of a growth factor signal transduction: after the ligand-receptor binding, the PI3K/*rac* pathway is activated, leading to the Nox activation and the consequent superoxide production outside the cell. In parallel, a *src* family member switches to the active form thanks to a phosphorylation reaction occurring near the receptor tyrosine kinase

domain. In the active form, the *src* kinase is able to inactivate the main intracellular peroxide scavenger, peroxiredoxin (Prx-1), thus allowing for the local accumulation of hydrogen peroxide. When H_2O_2 reaches sufficient levels, target molecules such as protein phosphatases can be reversibly oxidized on their cysteine residues, increasing the level of protein phosphorylation.

The oxidation of critical thiols are centrally involved also in the activation of essential switches in defense reactions, such as the Nrf2/Keap1 system, important in chemopreventive and cytoprotective functions (Sies 2014).

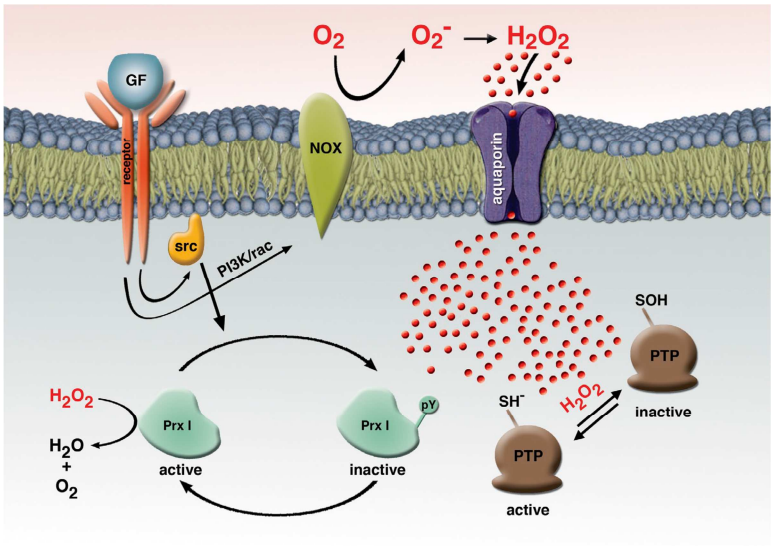


Fig. 5. Growth factor signalling through H_2O_2 as second messenger.

The signalling pathway resulting after growth factor (GF) stimulation is shown. Ligand binding to its receptor stimulates, through a PI3K/Rac-dependent process, superoxide production from an isoform of the Nox family. Extracellularly produced H_2O_2 is channeled back into the cell through specific plasma membrane aquaporins. Although hydrogen peroxide (red circles) can be rapidly and efficiently degraded by intracellular antioxidants, Src family members stimulated by growth factors appear to phosphorylate and subsequently inactivate the main intracellular peroxide scavenger, peroxiredoxin (Prx I). This Prx1 inactivation only occurs in the region surrounding the stimulated growth factor, thus allowing for the local accumulation of hydrogen peroxide. When the ROS reach sufficient levels, target molecules such as protein phosphatases (PTP) can be reversibly oxidized.

From: T. Finkel, (Finkel 2011).

1.2 ROS in diseases

Given the essential role played by redox physiological signalling, it is not surprising that alteration or dysfunction of this system contribute to the development of many disease processes. Therefore, ROS are involved in a wide range of pathologies. Nonetheless, the precise role played by ROS remains controversial, since it is not clear whether a rise in ROS causes or merely correlates with the disease states.

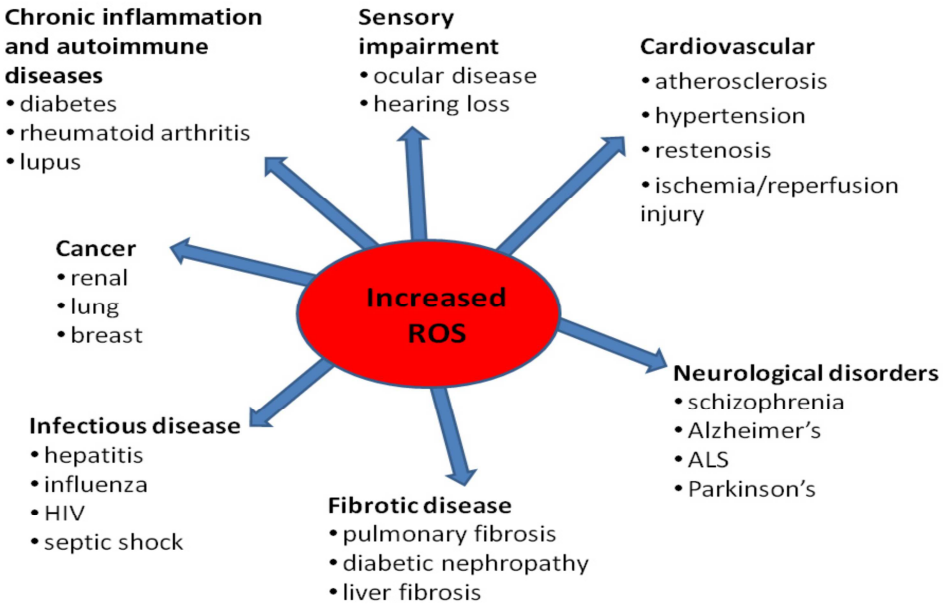


Fig. 6. ROS involvement in many diseases.

From: Brieger et al., (Brieger, Schiavone et al. 2012).

1.2.1 Cancer

High levels of ROS have been detected in several cancers, such as in breast, ovarian, liver, colon, or tumour epithelial cells (Pereira, Chaves et al. 2013).

In cancer cells, the constitutive and uncontrolled activation of growth factor pathways leading to cellular growth, survival and proliferation, changes the course of normal cell machinery. This allows cancer cells to take up abundant nutrients, survive stress and continuously proliferate. This unusually high metabolism rate causes abundant ROS generation from mitochondria, endoplasmic reticulum and, above all, from NADPH oxidases (Cairns, Harris et al. 2011). In fact, cancer cells generate higher ROS levels than their non-transformed counterparts. For example, leukaemia cells freshly isolated from blood samples of patients with chronic lymphocytic leukaemia, showed increased ROS production compared with normal lymphocytes (Zhou, Hileman et al. 2003). At an advanced stage, cancer cells exhibit genomic instability and show an even more higher increase in

ROS generation, due in part to a “vicious cycle”, in which ROS induce gene mutations, leading to further metabolic malfunctions and ROS generation (Trachootham, Alexandre et al. 2009).

As above stated, a part of the high ROS content in tumour cells arises from mitochondrial dysfunction coupled with the metabolic readjustment to generate ATP. The highly proliferative rate exhibited by tumour cells requires high levels of energy and building blocks for biomass production. Despite this high energy requirement, cancer cells shift their oxidative metabolism to glycolysis, a process known as the “Warburg effect”, which is observed regardless the oxygenation levels (Warburg 1956). The mitochondrial dysfunction probably causes both the shift of the oxidative metabolism toward glycolysis and the leakage of electrons giving rise to oxygen radical species owing to the compromised efficiency of the electron transport chain. Malignant, rapidly growing tumor cells, typically have glycolytic rates up to 200 times higher than those of their normal tissues of origin and this occurs even if oxygen is plentiful. This metabolic strategy is also useful in conditions of reduced oxygen tension (hypoxia), which often occurs during tumour initiation and development. Under hypoxic conditions, cells undergo a shift from aerobic to anaerobic metabolism and cells activate signalling pathways which regulate proliferation, angiogenesis and death. The adaptation of tumour cells to hypoxia contributes to the malignant phenotype and to aggressive tumour progression (Arnold, Shi et al. 2001).

Another part of the high ROS content in tumor cells originates from the Nox proteins, whose expression has been reported to increase in many cancer cell types; furthermore, activated NADPH oxidase seems to be required for the control of proliferation and the maintenance of the transformed state (Meitzler, Antony et al. 2014).

In particular, Nox isoforms have been described to be altered in leukaemia cells, and such changes have been specifically linked to sustained leukaemia cell viability, proliferation and migration, as well as altered drug susceptibility. It has been also shown that leukemic oncogenes (such as BCR/ABL, Flt3-ITD or c-Kit) are able to control Nox activation and assembly *via* the PI3K/Akt and MAPK pathways (Irwin, Rivera-Del Valle et al. 2013).

As above mentioned, both solid and liquid tumours are often under hypoxic conditions, thereby cells activate signalling pathways in order to adapt themselves to this environment, possibly leading to the angiogenic process. Indeed, ROS production within tumor cells dramatically promotes the release of paracrine growth factors such as VEGF and the expression of its receptors, VEGF receptor-1 and -2, which, in turn, stimulate proliferation, migration, and tube formation in nearby endothelial cells (Maraldi 2013).

NADPH oxidase within endothelial cells cooperates with growth factors, such as VEGF released by tumours, to stimulate endothelial cell proliferation and then angiogenesis. Thus, Nox isoforms as

ROS source in tumours and in endothelium may be considered novel targets for antiangiogenic treatment.

If increased ROS levels are essential to promote and reinforce proliferative signals, it conceivable to suggest that antioxidants could serve as tumour suppressors, reducing cellular ROS to levels that do not support proliferation. On the contrary, cancer cells that survive intrinsic oxidative stress may have mobilized a set of adaptive mechanisms, which consists in activating ROS–scavenging systems to cope with the stress and in the inhibition of apoptosis. Indeed, besides the generation of ROS, the same Nox activity is able to elevate the expression of the antioxidant heme oxygenase-1 (HO-1), thereby altering the balance of oxidative stress in the cells. There is also evidence of enhanced activation of the antioxidant program driven by the nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor, which has differential impacts on tumorigenesis (DeNicola, Karreth et al. 2011; Mateescu, Batista et al. 2011; Sporn and Liby 2012).

Therefore, at the same time of ROS production, cancer cells maintain a high level of antioxidant activity to prevent the ROS-induced cell death. This opens two ways to approach ROS therapy in cancer: should treatments focus on lowering ROS levels to prevent signalling or on increasing ROS to selectively kill cancer cells? A systematic review of randomized control studies with many antioxidants, i.e. β-carotene, vitamins A, E, and C concluded no significant benefits, on the contrary detrimental effects in both cancer prevention and therapy (Bjelakovic, Nikolova et al. 2007).

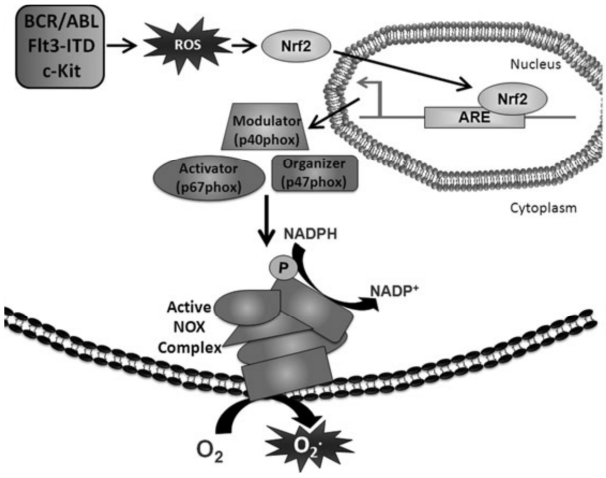


Fig. 7. Leukemic oncogenes control NOX assembly and activity in leukaemia cells.

Leukemic oncogenes, including BCR/ABL, Flt3-ITD, and c-Kit, increase ROS levels, leading to translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) to the nucleus, where it elevates mRNA expression of the NOX components p67phox, p47phox, and p40phox. Then they can interact to form the active NOX complex.

From: M.E. Irwin et al., (Irwin, Rivera-Del Valle et al. 2013).

1.2.2 Cardiovascular diseases

ROS at physiological levels are now appreciated to function as signalling molecules to regulate a wide range of processes in the cardiovascular system and to contribute to the maintenance of cardiovascular homeostasis (Droge 2002). In contrast, excessive and/or sustained increase in ROS generation plays a pivotal role in the initiation, progression and clinical consequences of cardiovascular disorders (CVD) (Sugiyama, Kugiyama et al. 2004; Touyz and Briones 2011; Csanyi, Yao et al. 2012). Although mechanisms need to be elucidated, it is clear that Nox enzymes have a crucial function. Numerous studies have examined the expression levels of Nox isoforms in the vascular wall in experimental models of hypertension, focusing in particular on the increase of Nox1, Nox2, Nox4 and, more recently, Nox5 (Guzik, Chen et al. 2008).

Most CDVs are a direct consequence of atherosclerosis. Free radicals and ROS, derived primarily from Nox and mitochondria, lead to the oxidation of low density lipoproteins, that strongly contribute to the atherosclerotic plaque formation and progression. Plaques and platelets are also involved in the onset of vascular inflammatory processes, through macrophage activation leading to the release of pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1, interferon-γ, AngII, and endothelin-1 (Massberg, Schurzinger et al. 2005).

An important risk factor for atherosclerosis and heart failure is hypertension, whose pathophysiology is strictly linked to both Nox-derived ROS *via* renin-angiotensin-aldosterone system (Feng, Yang et al. 2012) and mitochondrial ROS (Dikalova, Bikineyeva et al. 2010). Moreover, Nox system and mitochondria create a cross-talk that elicits a vicious cycle of "ROS catalyzed ROS production" (Chen and Keaney 2012).

It has been shown that H_2O_2 plasma production is higher in hypertensive patients than normotensive subjects. Moreover, patients with hypertension have decreased content of antioxidant enzymes (SOD, glutathione peroxidase, and catalase) and reduced levels of antioxidant vitamins A, C, and E (biblio). In addition, shear stress, vascular endothelial alterations and elevated blood pressure generate a loop of dysfunction that reduces vasodilation and increases contraction. Although there is still no firm evidence that oxidative stress causes hypertension in humans, a relationship between oxidative stress and hypertension has been demonstrated, so that circulating biomarkers of ROS act as oxidative stress markers able to predict cardiovascular diseases (Montezano and Touyz 2012).

Pathology like diabetes increases the level of advanced glycation end products (AGEs), that play a pivotal role in the development and progression of diabetic heart failure. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes.

A key to contrast the incidence of CVD could be represented by bioactive compounds found in natural food. Plant-derived nutraceutical compounds were largely investigated *in vivo* and *in vitro* in the prevention and/or treatment of CVD (Siow and Mann 2010). In particular, isoflavones (genistein, daidzein, and equol) increase eNOS and antioxidant gene expression in the vasculature (Mahn, Borras et al. 2005) and stimulate eNOS activity at basal cytosolic Ca^{2+} levels *via* ERK1/2 and PI3-kinase/Akt-dependent pathways (Siow, Li et al. 2007), resulting in reduced oxidative stress and increased NO bioavailability. The molecular mechanisms by which soy isoflavones and other natural compounds as sulforaphane counteract oxidative stress in CVD remain to be better investigated and nutraceutics could represent a prevention strategy against CVD.

1.2.3 Diabetes

Diabetes mellitus is a family of metabolic diseases characterized by hyperglycemia deriving from defects in insulin secretion, insulin action or both, and leading to long-term damage, dysfunction, and failure of various organs, primarily kidneys, eyes, nerves, heart, and blood vessels.

Type 1 diabetes originates from an autoimmune destruction of pancreatic β-cells causing an absolute deficiency of insulin secretion, completely insufficient to prevent hyperglycemia.

Type 2 diabetes mellitus is an endocrine disorder characterized by insulin resistance and relatively deficient production of insulin, provoking hyperlipidemia, elevating free fatty acids, increasing advanced glycation end-products and overproducing mitochondrial superoxide anion. Under physiological conditions, the activation of the insulin receptor by insulin stimulates signal transduction pathways: insulin receptor substrate (IRS1/2) induce the phosphorylation of phosphoinositide 3 kinase (PI3K), the conversion of PIP_2 to PIP_3 and the activation of pleckstrin homology (PH) domains to the cell surface. PH activates threonine protein kinases protein kinase B (PKB or Akt), which constitutively induce Glut4, and in a lesser extent, Glut1 translocation from their intracellular pool to the plasma membrane, and glucose transport into the cell (Leney and Tavare 2009). In particular, organs like skeletal muscle, liver, and adipose tissue enhance their uptake of glucose after the insulin stimulation (Pirola, Johnston et al. 2004). In addition to this recruitment, there is also evidence that insulin modifies the intrinsic activity of both Glut4 and Glut1 (Whitesell and Abumrad 1985). Glut1 is a facilitative glucose transporter which is expressed ubiquitously in human tissues and it is correlated to the promotion of cancer development and cancer's metabolism (Baer, George et al. 2002).

It has been shown that ROS are produced in various tissues under diabetic conditions (Baynes and Thorpe 1999). The aforementioned production of superoxide anion activates different pathways, for instance an increase of AGE formation, an activation of protein kinase C isoforms, a rise in the

activity of hexosamine pathway, or a growth of polyol pathway flux. Moreover, AGEs and insulin activate Nox enzymes, leading to further ROS production. The major risk factor for the progression of diabetic complications is oxidative stress; indeed, ROS induced under diabetic conditions are involved in the progression of pancreatic β-cell dysfunction and insulin resistance found in type 2 diabetes (Rains and Jain 2011). Oxidative stress plays a pivotal role in the development of diabetes because the permanent high glucose induces increase of mitochondrial ROS, causing microvascular and cardiovascular complications (Giacco and Brownlee 2010). Type 2 diabetes is also associated with obesity since adipose tissue produces a number of hormone-like compounds that can increase insulin resistance (Jackson and Ahima 2006).

There are different strategies to counteract the effects of diabetes, for example synthetic drugs (Rochette, Zeller et al. 2014) and natural compounds as resveratrol (Szkudelski and Szkudelska 2014) or steviol glycosides from *Stevia Rebaudiana* (Rizzo, Zambonin et al. 2013).

1.2.4 Neurological diseases

The brain is particularly vulnerable to oxidative stress because it is rich in polyunsaturated fatty acids, it consumes much more oxygen compared to other tissue, it has less antioxidant enzymes than other organs, and it has higher level of iron in certain regions. Oxidative stress may induce neuronal damage, modulate intracellular signalling, and may ultimately lead to neuronal death by apoptosis or necrosis, induce early event of Alzheimer and Parkinson diseases, increase inflammatory disorders. In the brain, even small redox imbalances can be deleterious (Hsieh and Yang 2013).

Moreover, the brain is exposed throughout life to excitatory amino acids (such as glutamate), whose metabolism produces ROS, thereby promoting citotoxicity.

Alzheimer implicates a loss of neurons, loss of memory, dementia and finally death (Selkoe 2004). ROS favourite lipid peroxidation, the deposition of amyloid and generate a protein cross-linking that aggregates the tau protein (Troncoso, Costello et al. 1993).

Parkinson is a disorder of motor system that starts with tremor up to decline of several motor centration functions (Sonntag, Carter et al. 2005). ROS are produced during the normal metabolism of dopamine, and they increase the level of polymerized oxidation products of dopamine, the toxic neuromelanin. Substantia nigra of Parkinson patients shows an impaired redox balance between GSH and GSSG: this could be a critical primary event, leading to a weakening or deficiency of the natural antioxidant cellular defence mechanisms thereby triggering degeneration of the nigral neurons.

Oxidative stress has been linked to other neurodegenerative diseases, such as cognitive impairment and dementia in the elderly, amyotrophic lateral sclerosis, schizophrenia and neuroleptic induced tardive dyskinesia, and perhaps Huntington's disease (Gilgun-Sherki, Melamed et al. 2001).

1.2.5 Aging

Aging is a physiological process, dynamic and irreversible, which occurs in the natural development of living organisms over time (Dziechciaz and Filip 2014).

In particular, aging is a progressive decline in the efficiency of biochemical and physiological processes (Rahman 2007). The aging's degeneration gives rise to several pathologies, such as sarcopenia, atherosclerosis and heart failure, osteoporosis, macular degeneration, pulmonary insufficiency, neurodegenerative diseases (such as Alzheimer's and Parkinson's diseases), cancer and many others. Many different mechanisms relate aging to oxidative stress, but since the free radical theory of aging appeared to be established, the link between ROS and aging is highly debated. Recent theories assert that mitochondrial respiration plays a major role in preserving longevity and it has been demonstrated that impaired mitochondrial functioning occurs with aging and leads to deterioration in the structure and function of tissues, implicating this process in the development of several age-related diseases (Martin-Montalvo and de Cabo 2013). The proposed causes of mitochondrial dysfunction involve the synthesis of nonfunctional complexes of the electron transport chain, damaged mitochondrial DNA and/or the accumulation of nonfunctional proteins by ROS-induced modifications.

1.3 Redox Homeostasis

1.3.1 Maintenance of redox homeostasis

ROS possess a multitude of biological functions, including host defence (direct and indirect killing of microorganisms), biosynthetic processes (for example, crosslinking of matrix proteins and iodination of thyroid hormones), and cell signalling.

The term *redox signalling* is widely used to describe a regulatory process in which the signal is delivered through redox chemistry. Redox signalling is used by a large range of organisms, including bacteria, to induce protective responses against oxidative damage and to reset the original state of "redox homeostasis" after temporary exposure to level of ROS higher than physiological one (Droge 2002).

The concentrations of free radicals and reactive nonradical species are determined by the balance between their rates of production and their rates of clearance by various antioxidant compounds and enzymes (Halliwell 1989). Antioxidants include the enzymes SOD, glutathione peroxidase (GPx), and catalase, as well as nonenzymic compounds such as α-tocopherol (vitamin E), β-carotene, ascorbate (vitamin C), Coenzyme Q, and glutathione.

1.3.2 What happens if oxidant-antioxidant balance is changed?

Living cells and tissues have several mechanisms for re-establishing the original redox state after a temporary exposure to increased ROS concentrations. Elevated ROS concentrations induce in many cells the expression of genes whose products exhibit antioxidant activity (Droge 2002). A major mechanism of redox homeostasis is based on the ROS-mediated induction of redox sensitive signal cascades which lead to increased expression of antioxidant enzymes or an increase in the cysteine transport system. Moreover, proteolysis contributes to the maintenance of redox homeostasis, since proteins provide less ROS scavenging activity than an equivalent amount of free amino acids. Cells or tissues are in a stable state if the rates of ROS production and scavenging capacity are essentially constant and in balance. Redox signalling requires that this balance is disturbed by an increase in ROS concentrations or a decrease in the activity of one or more antioxidant systems.

If the initial increase in ROS is relatively small, the antioxidant response may be sufficient to compensate for the increase in ROS and to reset the original balance between ROS production and ROS scavenging capacity. Pathological conditions may develop in more extreme cases of persistently high ROS levels (Arnold, Shi et al. 2001).

Natural human antioxidant defences are not always sufficient to maintain the proper ROS balance. Therefore, a normal process may become pathological if this failure persists for a long time.

Many epidemiological studies support the relationship between oxidative state and global health as the high consumption of foods rich in antioxidants is associated with lower disease rates or preventive protection (Wannamethee, Lowe et al. 2006).

1.3.3 Hormesis

Hormesis is a dose response phenomenon characterized by a low dose stimulation and a high dose inhibition. In other words, hormesis is an adaptive response to a negative stimulus. After stimulation by a negative stimulus, the cell/organism is more resistant to the future stimuli, that could act in a higher form: a toxin/stressor renders cells/organisms resistant to higher (and normally harmful) doses of the same stressing agent (Martins 2011).

There are several evidences that, after exposition of low concentration of hydrogen peroxide, cells activate numerous biochemical mechanisms that strengthen the antioxidant cell defences.

Superoxide anions and hydrogen peroxide are regulated by SOD that catalyzes the dismutation of superoxide anion to H_2O_2 and oxygen. H_2O_2 enhances SOD activity that decreases superoxide levels, thus creating a feedback regulatory mechanism that promotes hormesis effects by maintaining hydrogen peroxide at homoeostatic levels (Ludovico and Burhans 2014).

In human skin keratinocytes, low concentrations of hydrogen peroxide increase the replicative lifespan, together with telomere length (Yokoo, Furumoto et al. 2004).

Since in mammalian cells telomere elongation is inhibited by superoxide anions (Passos, Saretzki et al. 2007) and SOD activity enhances telomere maintenance (Serra, von Zglinicki et al. 2003), the extension in replicative lifespan could possibly be related to H_2O_2-induced SOD activity.

Different natural compounds with a particular chemical reactivity are able to activate adaptive cellular stress response pathways. For example, kinases and transcription factors induce the expression of genes encoding antioxidant enzymes, such as phase 2 enzymes, neurotrophic factors and other cytoprotective proteins (Calabrese, Cornelius et al. 2012). This adaptive response is very close to hormesis phenomena.

1.3.4 The Nrf2/Keap1/ARE pathway

The induction of many cytoprotective enzymes in response to reactive chemical stress is regulated primarily at the transcriptional level. This transcriptional response is mediated by elements termed Antioxidant Responses Elements, ARE, initially found in the promoters of genes encoding the two major detoxication enzymes, glutathione S-transferase A2 and NADPH:quinone oxidoreductase 1. Now, it has been demonstrated that ARE increase the synthesis of many ARE-dependent antioxidant enzymes in different cell systems, such as glutathione reductase, glutathione peroxidase,

glutaredoxin, thioredoxin reductase, heme oxygenase 1 (HO-1) and peroxiredoxin 1 (Prx-1). The ARE possesses structural and biological features that characterize its unique responsiveness to oxidative stress. Compounds that have the propensity to react with sulfhydryl groups such as diethyl maleate, isothiocyanates, and dithiothiones are also potent inducers of ARE activity. Thus, alteration of the cellular redox status due to elevated levels of ROS and electrophilic species and/or a reduced antioxidant capacity appears to be an important signal for triggering the transcriptional response mediated by this enhancer.

Activation of gene transcription through the ARE is mediated primarily by Nrf2. The Nrf2/Keap1 pathway is the most important regulator of cytoprotective responses to endogenous and exogenous stresses caused by reactive ROS and electrophiles (Kansanen, Jyrkkanen et al. 2012). Nrf2/Keap1/ARE system is mediated by some natural compounds (e.g. SF and resveratrol) but also by a change of electrophilic balance. Under basal conditions Keap1, a cytosolic protein rich in cysteine residues, binds Nrf2, thereby keeping this factor in the negative form. When the electrophilic balance is changed or due to the presence of compounds like SF, cysteine residues are oxidized and Nrf2 is released (Baird and Dinkova-Kostova 2011), turning on its active state. Then, Nrf2 can translocate into the nucleus, where it binds ARE in the regulatory regions of target genes (Baird and Dinkova-Kostova 2011).

Among the genes induced in response to oxidative stress, whose expression is regulated by the binding of Nrf2, the heme oxygenase HO-1 is of particular interest, since the protein product of this gene has antioxidant, anti-inflammatory and anti-apoptotic activity.

HO are rate-limiting enzymes in heme catabolism. Heme oxygenases catalyze the reduction of heme to biliverdin, carbon monoxide and ferrous ions in the presence of NADPH and oxygen, and the produced biliverdin is further reduced to bilirubin in the presence of biliverdin reductase (Takahashi, Takahashi et al. 1998). Both biliverdin and bilirubin are efficient ROS scavengers (Babusikova, Jesenak et al. 2008).

HO-1 plays several role in oxidative balance, in particular related to vasculature and diabetes (Siow, Sato et al. 1999); indeed HO-1 knockout mice exhibit an high susceptibility to hypertension (Wiesel, Patel et al. 2001).

In addition, HO-1 is a potential target for cancer therapy, because this enzyme gives survival and growth advantages to malignant cells by means of its anti-apoptotic activity. The modulation of HO-1 expression affects cellular growth or survival of myeloid leukaemia cells (Miyazaki, Kirino et al. 2010).

Another Nrf2-induced enzyme is represented by Prx-1. Peroxiredoxins are a class of thiol peroxidases that degrade hydroperoxides to water. Peroxiredoxin enzymes contain a conserved

cysteine residue in the N-terminal region that is the primary site of oxidation by H_2O_2. This family protects cellular components by removing the low levels of hydroperoxides and peroxinitrites produced as a result of normal cellular metabolism in the cytosol (Rhee, Chae et al. 2005). Several features of the Prxs make measurement of their redox state a valuable biomarker of oxidative stress, moreover the varied intracellular expression pattern of different Prxs enables exploration of redox disruption in different cellular compartments.

Nrf2 activates another fundamental pathway involved in the maintenance of the intracellular antioxidant defence enzymes in response to stress, the GSH system. Nrf2-deficient mice are more susceptible to liver damage due to diminished basal GSH levels and an impaired compensatory induction GSH synthesis (Lu 2009). Moreover, decreased level of GSH were observed in long-term exposure to high glucose in mouse endothelial cells that became more vulnerable to oxidative stress (Kline, Bassit et al. 2009).

1.3.5 Klotho

Klotho is a newly discovered anti-aging gene, found in 1997 by Kuro-o and his research group. The name is derived by one of three Fates of Greek mythology who regulates the life and the destiny of humanity.

Klotho protein exists in two forms, a transmembrane and a soluble secreted form.

The membrane protein (130 kDa) is mainly expressed in the kidney, parathyroid glands and choroid plexus of the brain; the extracellular domain of Klotho transmembrane form can be enzymatically cleaved off and released into the systemic circulation, where it acts as β-glucuronidase and as a hormone. The Klotho transmembrane family are formed by α-, β-, and γ-Klotho.

The second transcript is soluble Klotho, smaller than the membrane form, it may be found in the blood, cerebrospinal fluid, and in mammal urine. Klotho deficiency results in early appearance of multiple age-related disorders and premature death, whereas overexpression of Klotho exerts the opposite effect (Kuro-o 2010).

Studies have shown that the membrane and the secreted form have distinct functions, all related to regulatory effects on general metabolism.

Klotho regulates the level of Ca^{2+} in the blood: Klotho -/- mice show an increase of this cation in the blood and the urinary excretion. Moreover, absence of Klotho increases the vitamin D serum level. All these events induce the demineralisation of the bones (Chang, Hoefs et al. 2005).

Klotho also inhibits sodium-dependent phosphate co-transporters that mediate phosphate uptake (Hu, Kuro-o et al. 2010). In vascular smooth muscle cells, this inhibition prevents vascular

calcification events, since excessive phosphate influx in these cells promotes a cascade of events responsible for the calcium and phosphate mineralization in their interior (Lau, Festing et al. 2010).
Klotho -/- mice secrete less parathyroid hormone (PTH), therefore it is likely that Klotho regulates PTH secretion (Yoshida, Fujimori et al. 2002).
Klotho works with fibroblast growth factors 23 (FGF23) as a required co-factor/co-receptor for the activation of FGF receptor. Knockout mice for either Klotho or FGF23 show an inability to activate the receptor, resulting in increased expression of co-transporter sodium-phosphate, that causes a severe hyperphosphatemia (Kuro-o, Matsumura et al. 1997). Therefore Klotho participates in the regulation of the bone-kidney endocrine axis.
Klotho induces inhibition of insulin/insulin-like growth factor-1 (IGF-1) signalling pathway, which has been linked to an extended lifespan in a wide variety of species (Yamamoto, Clark et al. 2005). Moreover, the reduction of activation of insulin/IGF-1 signalling induces an increase of the forkhead transcription factors (FOXO), that regulate the expression of multiple target genes, including antioxidant enzymes such as catalase and mitochondrial manganese-superoxide dismutase (SOD2) (Kops, Dansen et al. 2002). The resulting decrement of ROS is linked to increased resistance to oxidative stress, which potentially contributes to the anti-aging properties of Klotho.
Klotho has protective cardiovascular effects and a correlation between plasma's Klotho level and CVD incidence in humans has been reported (Dermaku-Sopjani, Kolgeci et al. 2013). The role of Klotho is not only protective, but also predictive of cardiovascular risk. In *vitro* Klotho protects the vessels and the vascular smooth muscle cells (VSMCs) from calcification (Hu, Shi et al. 2011), moreover soluble Klotho protects the vascular endothelium (Saito, Nakamura et al. 2000).
On vascular endothelial cells, Klotho proteins have anti-apoptotic action (in Klotho-treated human umbilical vein endothelial cells, caspase-3 and caspase-9 activity decreases) and anti-aging activity (by mechanisms involving p53/p21) (Ikushima, Rakugi et al. 2006). The beneficial effect of soluble Klotho becomes more relevant when the natural aging process makes vascular endothelial cells more susceptible to stress-induced injury: Klotho has protective effect in HUVEC following TNF-α induced oxidative stress (Carracedo, Buendia et al. 2012).
This "anti-aging" molecule may influence various biological processes and the identification of a common basis for its pleiotropic function will be of particular importance in understanding its anti-aging properties.

1.3.6 Natural compounds

Many compounds present in plants and vegetables have the ability to contrast cancer, to drop the glucose blood level, to act like antioxidant scavenger and to increase the antioxidant indirect defences.

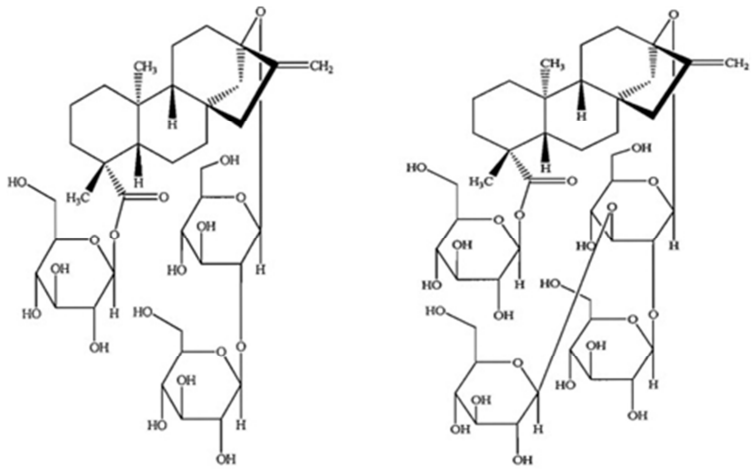
Polyphenols comprise a wide variety of compounds, divided into several classes (i.e., hydroxybenzoic acids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes and lignans), that occur in fruits and vegetables, wine and tea, chocolate and other cocoa products (Manach, Scalbert et al. 2004). Since phenolic compounds are present almost in all fruits, vegetables and other plant products, Mediterranean diet, rich of olive oil, fruits, vegetables, whole grains, legumes and nuts is the best helper against oxidative stress-related pathologies, in particular neurodegenerative and cardiovascular diseases (Widmer, Flammer et al. 2014).

The so-called "healthy food" owes its name to the presence of nutraceutical compounds, molecules rich of properties that may preserve and extend the quality of life. For example, the presence of omega-3 fatty acids in Mediterranean diet has positive effects on glucose/insulin homeostasis (Bedard, Corneau et al. 2014; Nosova, Conte et al. 2015), resveratrol and other polyphenols widely distributed in red wine increase the Glut4 expression (Sun, Zhang et al. 2007).

Broccoli sprouts and all the plants belonging to the *Cruciferous* family are rich of sulforaphane (SF). SF derives from enzymatic action of myrosinase on glucoraphanin contained in cruciferous vegetables (Fahey, Zhang et al. 1997). After 1.5 h consumption of broccoli and other cruciferous vegetables, the physiological concentrations of SF measured i*n vivo* range between 0.031 μM to 7.3 μM (Dinkova-Kostova and Kostov 2012). SF has several documented effects: neuroprotection in retina (Pan, He et al. 2014), anti-carcinogenic activity (Fimognari, Turrini et al. 2014), activation of cell survival pathways in cardiomyocytes (Leoncini, Malaguti et al. 2011), reduction of oxidative stress (Yoon, Kang et al. 2008) and insulin resistance in diabetic patients (Bahadoran, Tohidi et al. 2012). These effects share a common way of modulation: the Nrf2/Keap1/ARE pathway. In fact, it has been demonstrated that SF treatment induced an increase in Prx1 and HO-1 expression and an enhancement of the total GSH levels in many cell types. Taken together this evidence suggests that SF up-regulates antioxidant defences promoting an hormetic regulation (Cheng, Siow et al. 2011).

In the last years a "new" natural product has gained increasing interest from nutritional researchers and commercial area: *Stevia rebaudiana* Bertoni, a perennial shrub of *Asteraceae* (*Compositae*) family, native to subtropical regions of Brazil and Paraguay.

This plant has a very sweet taste thanks to glycosides such as stevioside, rebaudioside A, rebaudioside C and dulcoside. The leaves contains also β-carotene, thiamine, riboflavin, terpenes, and flavonoids (Yadav and Guleria 2012).



Stevioside **Rebaudioside A**

Fig. 8. Chemical structure of stevioside and rebaudioside A.

Since ancient times, the leaves of *Stevia rebaudiana* Bertoni were used as a sweetener in Guarani Indians of Paraguay and Brazil (Cekic, Vasovic et al. 2011) and for many other medicinal purposes, including treatment of diabetes. Nowadays this feature is of particular interest, since metabolic disorders, such as type 2 diabetes mellitus and obesity, have become major public health problems worldwide (DeFronzo 1988; Zimmet, McCarty et al. 1997).

A key strategy of glycemic control for prevention and control of diabetes includes monitoring and limitation of sugar intake. *Stevia* leaves and extracts can substitute sucrose, due to their taste approximately 200–400 times sweeter than sucrose (Hanson and De Oliveira 1993).

Results from *in vivo* animal studies and, at a lesser extent, in humans, suggest that steviosides and related compounds affect plasma glucose by modulating insulin secretion and sensitivity (Jeppesen, Gregersen et al. 2000; Lailerd, Saengsirisuwan et al. 2004). Moreover, steviosides inhibit gluconeogenesis in the liver of diabetic rats (Chan, Wong et al. 2003; Chen, Chen et al. 2005). These antihyperglycemic, insulinotropic, and glucagonostatic effects, particularly exhibited by rebaudioside A, seem to be largely dependent on plasma glucose level, requiring high glucose levels for accomplishment (Jeppesen, Gregersen et al. 2002; Abudula, Matchkov et al. 2008).

The literature lacks studies elucidating the cellular and molecular mechanisms underpinning these effects. It is possible to hypothesize that the compounds present in *Stevia* leaves could be involved in the same transduction pathway of insulin, the PI3K/Akt pathway. This pathway is a key

component of the insulin signalling cascade and it is essential for glucose transport into the cells. Upon the binding of insulin to its receptor, the PI3-K/Akt pathway is activated and, among the many cellular responses, Glut4 translocates from intracellular pools to the plasma membrane, thus enhancing glucose uptake into the cell (Dugani and Klip 2005).

Chapter 2- Aim of thesis

2 Aim

Physiological redox signaling refers to processes in which reactive oxygen-, nitrogen- and chlorine-derived species, collectively called “Reactive Oxidizing Species”, ROS, are produced and employed in the intracellular signaling as well as in physiological cross talk between cells, providing a redox sensing control of living cell functions. The mechanism at the basis of redox signaling implies the interaction of the oxidant species with specific targets, among which the most physiologically relevant are thiol/disulfides groups in proteins, with particular regard to those present within catalytic or regulatory sites, as well as in receptors, channels and transporters, transcription factors, kinases and phosphatases. In redox signaling the reaction of ROS with the target molecules acts as an on/off switch signal, resulting in the activation or inhibition of specific intracellular pathways.

A key point still unsolved is the implications of these cellular products in the regulation of both pro-survival and cell death pathways. In general terms, basal levels of ROS formation modulate the activities of different transcription factors and protein signaling cascades, maintaining proper cellular homeostasis and mediating cell proliferation and survival. In contrast, when antioxidant defenses are surpassed by ROS formation, an oxidative damage is not repaired by endogenous mechanisms, and oxidative stress leads to cell demise. In few words, the redox regulation seems to be at the cross road between different programs, regulating cell life and death, thus ROS can be considered a double-edged sword. This statement is particularly intriguing considering that several proteins, typically involved in physiological redox signaling, could play a key role in the determination of cell survival or death, contributing to the development of diseases such as cancer and degenerative disorders.

With this context in mind, three major problems have been investigated and here reported.

The first one concerns the role of ROS in biosignalling.

Data from the research group have previously demonstrated that Nox enzymes are a major source of ROS in acute leukaemia-derived cell lines and that Nox-derived ROS are required for proliferation and survival of these cells, being directly involved in maintaining the high glucose uptake essential for cellular aberrant growth (Prata, Maraldi et al. 2008; Maraldi, Prata et al. 2009).

Since Nox enzymes produce ROS outside the cells and specific aquaporin water channels (AQPs) have shown the ability of funnelling H_2O_2 across the plasma membrane, it was investigated whether AQPs could represent one potential way through which H_2O_2 moves inside these leukemic cells behaving like a signal molecule, thus contributing to cell survival and proliferation.

Other data previously obtained by the research group have shown that in the human leukemic B1647 cell line, constitutively producing VEGF, Nox-derived ROS are involved in the

autophosphorylation of VEGF receptor 2 (VEGFR-2) and in the modulation of glucose uptake, a cellular activity strictly bound to VEGF-induced leukemic cell proliferation (Maraldi, Prata et al. 2010).

Since caveolae/lipid rafts are involved in signal transduction and VEGFR-2 and Caveolin-1 (Cav-1) have been reported to be frequently co-localized in these domains, it was investigated whether, in B1647 cells, VEGFR-2 could be associated with Cav-1 in caveolae/lipid rafts, in order to verify that these domains could act as platforms for the modulation of VEGF redox signal transduction cascades, leading to glucose uptake and cell proliferation.

The comprehension of these complex mechanisms could indicate novel potential targets and new therapeutic strategies against blood cancer.

The second faced problem concerns the role of natural or physiological molecules in maintaining or altering the ROS balance inside the cells.

Doctor Siow's team, at King's College London, demonstrated that sulforaphane (an isothiocyanate present in cruciferous vegetables) mediates pre-activation of the redox sensitive transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) and its downstream target hemeoxygenase-1 (HO-1) in the cerebral vasculature, protecting the brain against stroke (Alfieri, Srivastava et al. 2013). Other data concerning the chemopreventive role of sulforaphane come from studies by our research group, which demonstrated that sulforaphane decreased intracellular ROS production, DNA fragmentation, and induced the expression of antioxidants and phase II detoxifying enzymes in cardiomyocytes, thus exerting a cardioprotective action (Angeloni, Leoncini et al. 2009).

Due to the pleiotropic role of sulforaphane in increasing the cellular defences against oxidative stress, it was investigated whether sulforaphane or Klotho (an endogenous protein endowed with multiple pleiotropic effects associated with anti-ageing properties) (Kuro-o 2010) could increase antioxidant defences in cultured human aortic smooth muscle cell subjected to high glucose conditions mimicking hyperglycaemia.

This study contributes to elucidate the role of Klotho protein, whose deficiency results in rapid ageing in many animal models, and the action mechanism of sulforaphane, a nutraceutical antioxidant present in human diet.

Also the third problem deals with the ROS balance inside the cells, concerning the study of the antioxidant and antihyperglycemic properties of natural molecules, since it is well known that high blood glucose causes high ROS production, which can lead to diabetes development.

It has been reported that the leaves of perennial shrub named *Stevia rebaudiana* Bertoni have antioxidant activity both *in vitro* and *in vivo* (Shivanna, Naika et al. 2013; Tavarini and Angelini 2013) and that Rebaudioside A and stevioside (the two main steviol glycosides found in this plant)

are the two predominant derivatives used in high potency sweeteners alternative to sucrose. Besides sweetness, steviol glycosides have been shown to possess many beneficial effects on human health, and a number of animal studies have demonstrated that, in diabetic rats, steviosides exert antihyperglycemic and insulinotropic actions (Jeppesen, Gregersen et al. 2002).

The antioxidant activity of Rebaudioside A and other commercial *Stevia* extracts was therefore investigated in SH-SY5Y and HL-60 cells, furthermore, taking advantage of the group expertise in the study of glucose transport activity, the antihyperglycemic properties of these steviosides were also studied. The neuroblastoma SH-SY5Y and the promyelocytic leukemia HL-60 cell lines are good models for this study, since they both express the Glut1 isoform of glucose transporter, which is responsible for the basal glucose uptake, and Glut4, responsible for insulin-stimulated glucose uptake in peripheral tissues (Lord, Bunce et al. 1988; van der Heide, Ramakers et al. 2006). Furthermore, these cells express also the insulin-like growth factor-1 receptors (IGF-1) (Saltiel and Kahn 2001), thereby being susceptible to insulin action.

Whether the antihyperglycemic and insulinotropic properties of *Stevia* extracts will be confirmed in human cells, the use of these extracts could go beyond their sweetening power, offering also therapeutic benefits.

Part of the experiments concerning the role of Klotho and sulforaphane in cultured human aortic smooth muscle cell was performed at the King's College London (Cardiovascular Division, School of Medicine) under the supervision of Dr. Richard Siow and thanks to a Marco Polo grant for PhD Student’s mobility.

Chapter 3- Materials and Methods

3.1 Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), penicillin/streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), staurosporine, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, dichlorofluorescin diacetate), 2-deoxy-glucose (DOG), phloretin, CelLytic M, mammalian protease inhibitor mixture, primary antibody to β-actin (# A7816), sulforaphane (SF), hydrogen peroxide, bovine serum albumin (BSA), Caspase 3 Fluorimetric Assay Kit, methyl-β-cyclodextrin (CD), glutathione (GSH), Semaxinib (SU5416), and all other chemicals of the highest analytical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium (with Hepes, with L-glutamine) and Iscove's Modified Dulbecco's Medium (IMDM) (with Hepes, with L-glutamine), foetal bovine serum (FBS), human serum (HS) were purchased from PAA. 2-deoxy-D-[1,2-^{3}H]-glucose, [1,2-^{3}H]-cholesterol and Ultima Gold MV scintillation cocktail were from PerkinElmer. PhosSTOP, a phosphatase inhibitor cocktail, Bevacizumab were obtained from Roche Diagnostic. VEGF was from BioVision (Mountain View, CA, USA). Nitrocellulose membranes and Amersham ECL Advance Western Blotting Detection Reagents were from GE-Healthcare. Primary antibodies against phospho-Akt (Ser473) (#4058), total Akt (#9272), and horseradish peroxidase-conjugated secondary antibodies anti-rabbit (#7074) and anti-mouse (#7076) were purchased from Cell Signaling Technologies. Anti-GLUT1 (sc-1603), anti-GLUT4 (sc-1606), and anti-goat IgG conjugated to horseradish peroxidase (sc-2020) were obtained from Santa Cruz Biotechnology. Primary antibody anti-phospho-PI3 Kinase p85 pTyr458 /p55 pTyr199 (#PA5-17387) was from Thermo Scientific. Anti-PI3 Kinase (#06-195) antibody was purchased from Millipore. Anti-AQP1 (# TA502357-Origene), anti-AQP3 (# SC20811-Santa Cruz), anti-AQP8 (# WH0000343-Sigma-Aldrich), PageRuler Prestained protein ladder was from Fermentas – Thermo Fisher Scientific. Anti-caveolin 1 (# 610059) and anti-transferrin receptor (CD71) (# 612124) antibodies were provided by BD Biosciences (San Jose, CA, USA). Anti-Lyn antibody, anti-HO-1 and anti-Prx-1 antibodies were from Abcam (Cambridge, UK). Anti-VEGF receptor-2 (# 05-554) and anti-P-tyrosine (# 06–427) were from Millipore (Temecula, CA, USA). Anti-Glut1 (N-20) (# sc-1603) and fluorescent FITC-conjugated anti-goat IgG (# sc-2024) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant Human Klotho was from Peprotech (# 100-53). Peroxyfluor-1 (PF1) was synthesized according to (Miller, Albers et al. 2005).

Extracts from *Stevia rebaudiana* Bertoni were kindly supplied by Eridania Sadam SpA. According to FDA and EFSA (Goyal, Samsher et al.), total content of steviol glycosides in commercial *Stevia* extracts has to be at least 95% (w/w), and rebaudioside A plus stevioside must be at least 75%. The four extracts tested differ by the relative content of rebaudioside A and stevioside. In particular,

according to the certificates of analysis of each sweetener, Reb A (R97) contains 97-98% rebaudioside A, Stevia RA60 (R60) contains about 60% rebaudioside A and about 20% stevioside; Steviol Glycosides SG95 (SG) contains 50% rebaudioside A and at least 25% stevioside; Truvia (TRU) contains a mixture of steviol glycosides not analytically quantified.

3.2 Cell Culture

B1647 is a human acute myeloid leukemia (AML) erythro-megakaryocytic cell line cultured at density between 1×10^5 and 1×10^6 cells/mL in IMDM supplemented with 5% human serum (HS), 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin, at 37 °C in a humidified atmosphere maintained at 5% CO_2. When indicated, the experimental model employed 16–18 h serum-depleted cells, as these conditions were more apt for focusing experiments on self-produced VEGF and cholesterol roles, ruling out other growth factor effects.

HL-60, acute promyelocytic leukaemia cells, were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin, at 37 °C in a humidified atmosphere maintained at 5% CO_2. Cells were maintained at a density between 1×10^5 and 1×10^6 viable cells/mL, with medium renewal every 2 to 3 days.

HASMC are aortic smooth muscle cells and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum (FCS), 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified atmosphere maintained at 5% CO_2. Cells were split and sub-cultured upon reaching 80-90% confluence by trypsinization, using 1-1.5 mL (0.1%) trypsin / (0.02%) EDTA in phosphate buffered saline (PBS). Experiments were conducted between passages 4 and 6, in three different conditions: Normal Glucose, NG (5 mM glucose in the culture medium DMEM); High Glucose, HG (25 mM glucose in the culture medium DMEM, to mimic an hyperglycaemia condition); Mannitol, Man (5 mM glucose + 20 mM mannitol in the culture medium DMEM, as osmotic positive control with respect to HG).

SH-SY5Y, human neuroblastoma cells, were grown at 37 °C in a humidified incubator with 5% CO_2 in DMEM supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were split and sub-cultured upon reaching 80-90% confluence by trypsinization, using 1-1.5mL 1% trypsin in PBS. Experiments were conducted between passages 16-21.

All procedures, sub-culturing, treating of the cells and preparation of all treatment reagents were handled under sterile aseptic techniques in a Class 2 laminar flow safety cabinet.

Table 1. Cells were sub-cultured up in T75 flasks or in a Petri's dishes, after which they were seeded into 96-well plates, 24-well plates, 12-well plates, 6-well plates or T25 flasks for subsequent experiments.

Vessel	Cells at Confluency	Trypsin or lysis buffer vol (mL)	Growth Medium (mL)
Dishes			
Petri's Dishes	1×10^6	1	6
Culture Plates			
6-well	0.2×10^6	0.2	2
12-well	0.1×10^6	0.1	1
24-well	0.05×10^6	0.5	0.5
96-well	0.02×10^6	0.01	0.2
Flasks			
T-25	0.7×10^6		12
T-75	2.1×10^6		30
T-160	4.6×10^6		60

3.3 Cell Viability

Viable cells were evaluated by the Trypan blue exclusion test. Cell viability was also assayed by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.

The MTT assay developed by Mosmann is a quantitative colorimetric assay, which detects living cells, and the signal generated is dependent on the degree of mitochondrial dehydrogenase activity (Mosmann 1983). MTT can only be cleaved by active mitochondrial enzymes in living cells and the assay measures the activity of NAD-dependent dehydrogenase enzymes; it can also be used to assess the cytotoxic effects of drugs (Gerlier and Thomasset 1986).

Twenty-four hours after seeding adherent cells into the 96-well plate, they were subjected to the different treatments. Then, the medium was aspirated and replaced with 100 µL of MTT (0.5 mg/mL) in medium and cells were incubated at 37 °C and 5% CO_2 under sterile conditions for 2h. Then, DMSO (50 µL) was added to each well and the purple/brown crystals of formazan formed were dissolved by placing the plate on a shaker (room temperature) for approximately 10 min.

Suspended cells were centrifuged after treatments and re-suspended in a medium solution contained MTT (0.5mg/mL) for 2h, formazan salt crystal formed were dissolved by adding the solubilization solution (10% SDS-1% HCl).

The absorption at 570 nm was measured on a multiwell plate reader (Wallac Victor^2, PerkinElmer). The average absorbance reading of octuplicate treatments was calculated and analysis of cell viability was based on percentage change from the control for each treatment.

3.4 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity was assayed by a spectrophotometric method based on the reduction of pyruvate to lactic acid coupled to NADH oxidation. The decrease in absorbance at 340 nm was monitored at 37 °C. 100 µM H_2O_2 for 30 minutes was used as a positive control.

- SH-SY5Y and HL-60 cells were incubated with 1 mg/mL of each *Stevia* extract for 24 h. LDH release from cells was monitored by collecting aliquots of medium.

3.5 Assay for caspase 3 activity

Caspase 3 activity in the cell lysates was measured using a fluorimetric assay kit by following the instructions from the manufacturer (Sigma). After treatments, cells were collected and lysed using the lysis buffer provided in the kit (250 mM HEPES, pH 7.4 containing 25 mM CHAPS and 25 mM DTT). The assay was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC) by caspase 3, resulting in the release of free AMC moiety. The fluorescence of AMC was read using a multiwell plate reader (Wallac Victor^2, PerkinElmer); excitation and emission wavelengths were 360 nm and 460 nm, respectively.

The concentration of the AMC released was calculated using an AMC standard curve. Caspase 3 activity was expressed in nmole of AMC released per min per mL of cell lysate and normalised for total protein content in the lysate. Results are reported as percentage in respect to the control. Staurosporine (1 µg/mL) was used as an apoptosis inducer (positive control).

- SH-SY5Y and HL-60 cells were incubated with or without steviol glycosides (1 mg/mL) for 1, 6 or 24h. After, the samples were collected using the kit's buffer and processed as previously described.

3.6 Measurement of intracellular reactive oxidizing species (ROS) levels

ROS intracellular level was evaluated by using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). At the end of each time-point treatments, cells were washed twice in PBS and incubated with 5 µM H_2DCFD for 20 min at 37 °C. H_2DCFDA is a

small non-polar, non-fluorescent molecule that diffuses into the cells, where it is enzymatically deacetylated by intracellular esterases to a polar non-fluorescent compound, that is oxidised to the highly green fluorescent 2',7'-dichlorofluorescein (DCF). The fluorescence of oxidized probe was measured using a multiwell plate reader (Wallac Victor^2, PerkinElmer). Excitation wavelength was 485 nm and emission wavelength was 535 nm. Fluorescence values were reported as the percentage of intracellular ROS in respect to controls.

- B1647 were incubated with 5 μM $AgNO_3$ for 10 min, then 10 or 100 μM H_2O_2 was added to the cells. After the overexpression of AQP1, AQP3, AQP8 cells were treated or not with 100 μM H_2O_2 for 10 min.
- SH-SY5Y and HL-60 cells were incubated with 5 mg/mL of each *Stevia* extracts for 1 or 24h, then subjected or not to oxidative stress generated by 100 μM H_2O_2 for 30 min.

Hydrogen peroxide level was also determined in B1647 cells by using Peroxyfluor-1 (PF1). PF1 is a fluorescent boronate probe, selective for H_2O_2, although less sensitive than H_2DCFDA. The fluorescence of the oxidized probe was measured on a multiwell plate reader (Wallac Victor^2, Perkin Elmer) at excitation and emission of 485 nm and 535 nm, respectively. Fluorescence values were reported as the percentage of intracellular H_2O_2 with respect to controls.

- B1647 cells (1×10^6 cells/mL) were washed twice in HBSS and incubated with 10 μM PF1 for 20 min at 37 °C, in the dark. Then, the cells were washed three times in HBSS and pre-treated with 5 μM $AgNO_3$ for 10 min, then 100 or 200 μM H_2O_2 was added to the cells and the fluorescence was determined after 10 min.

3.7 Glucose transport assay

Glucose transport assay was performed as previously described (Hrelia, Fiorentini et al. 2002; Fiorentini, Prata et al. 2004). Adherent cells (SH-SY5Y) were seeded at density of 16.6×10^4. After treatments with different compounds, they were washed twice in PBS and treated for 10 min at 37 °C with a mixture (named as DOG mixture) of 2-deoxy-D-[1,2-^{3}H] glucose (0.8 μCi/assay) and 1.0 mM unlabeled glucose analogue; under these conditions the uptake was linear at least for 20 min (*zero-trans* conditions). The transport was stopped by adding phloretin (final concentration 0.3 mM), a potent inhibitor of glucose transport activity. Cells were scraped adding cold PBS and centrifuged at 4000 *g* for 1 min. The pellet was resuspended in 3 mL Ultima Gold MV scintillation cocktail and the radioactivity was measured by liquid scintillation counting (Tri-Carb® liquid scintillation analyser, PerkinElmer).

Suspended cell (HL-60 or B1647) were utilized at 4×10^6 cells/mL in PBS. They were incubated with different *stimuli* and/or inhibitors at 37 °C and then treated with a mixture (named as DOG

mixture) of 2-deoxy-D-[1,2-^{3}H] glucose (0.4 µCi/assay) and 1 mM unlabelled glucose analogue for 2 min at 37 °C. After this time, the uptake was stopped by adding phloretin (final concentration, 0.3 mM). Cell were washed with PBS and resuspended in 3 mL Ultima Gold MV scintillation cocktail. Sample radioactivity was measured by liquid scintillation counting (Tri-Carb® liquid scintillation analyser, PerkinElmer).

3.8 Cell transfection

The transfection was done using Cell Line Nucleofector™ Kit V (Amaxa® Biosystems, Cologne, Germany). 2×10^6 cells was resuspended in 100 µL Nucleofector® Solution at room-temperature, then 2 µg DNA or 2 µg pmaxGFP® Vector (positive control for overexpression) were added to the suspension, or 300 nM siRNA. This suspension was transferred into an Amaxa® certified cuvette (sample must cover the bottom of the cuvette without air bubbles). Cells was electroporated using appropriate Nucleofector® Program. Immediately, ~500 µL of culture medium, pre-equilibrated at 37 °C, were added to the cuvette and the sample was gently transferred into 6-well plate. Cells were maintained at 37 °C/5% CO_2 for 24 or 48h and after this period treatments were done.

- B1647 cells were nucleofected with Cell Line Nucleofector™ Kit V (Amaxa® Biosystems, Cologne, Germany) with Program T-019 following the manufacturer's instructions; plasmid vectors pCMV6-XL4 or pCMV6-XL5 for the overexpression of AQP1 (NM_198098), AQP3 (NM_004925) and AQP8 (NM_001169) were obtained from Origene (Rockville, USA).

For silencing, siRNA against AQP3 (Duplex sequence: GAGCAGAUCUGAGUGGGCA [dT][dT]; UGCCCACUCAGAUCUGCUC [dT][dT]), AQP8 (Duplex sequence: CUGCUCAUUAGGUGCUUCA [dT][dT]; UGAAGCACCUAAUGAGCAG [dT][dT]) and random RNA sequence (scrambled, as negative control) were obtained from Sigma-Aldrich. After 24 or 48h, cells were used for the experiments: cell viability assays, evaluation of AQP1, AQP3, and AQP8 expression by Western blotting, and transfection efficiency.

3.9 Immunoblotting analysis

- Preparation of whole cell lysates

On completion of a given treatment, cells were washed twice with cold non-sterile PBS and then lysed by the addition of buffer containing 2% sodium dodecyl sulphate (SDS), 10% glycerol, 50 mM Tris base, protease inhibitor cocktail (1:1000) and phosphatase inhibitors I and II (1:2000). Whole cell lysates were collected after 10 min incubation on ice and stored at -20°C for subsequent analysis.

- Preparation of cytosolic cell lysates

Following completion of the treatment period, cells were washed with ice-cold PBS and lysed on ice using CelLytic M containing mammalian protease and phosphatase inhibitor mixture.
The resulting lysed cells were left on ice to solubilize for 15 min. The lysates were centrifuged at 5000 *g* for 5 min at 4 °C to remove unbroken cell debris and nuclei and supernatants containing the cytosolic fraction were collected and stored at -80°C.

- Protein assay

Cell lysate protein concentration was colorimetrically determined by the Bio-Rad Bradford Coomassie Blue protein assay (Bio-Rad Laboratories) with bovine serum albumin (BSA) as standard (Bradford 1976). Since elevated concentration of detergents (such as Triton X-100 and SDS) interferes with Coomassie Blue reagent, protein concentration of fractions obtained from sucrose gradient or sample for Western blotting was determined by the Bio-Rad DC protein assay kit (Bio-Rad Laboratories), using BSA as a standard in the presence of appropriate concentration of Triton X-100 or SDS or lysis buffer. For the experiments carried out at King's College, bicinchoninic acid (BCA) protein concentration assay was used. This highly sensitive method was firstly described by Smith (Smith, Krohn et al. 1985). This colorimetric assay depends on the reduction of Cu^{2+} to Cu^{+} by peptide bonds in protein under alkaline conditions and can be used for determining protein concentrations of unknown samples by comparing absorbance at 562 nm relative to a standard curve of known protein concentrations. Standards and samples were prepared in triplicate. Lysis buffer was added to the standards at a 1:1 ratio and double-distilled water was added to each sample at 1:1 ratio. Once aliquoted into the 96-well plate, the BSA standards and samples were incubated with 200 μL of BCA reagents mixture (reagent A: reagent B = 50:1, Thermo Scientific, UK) at 37 °C for 25 min. The sample absorbance at 562 nm was then measured using a microplate spectrophotometer (Tecan Sunrise).

- SDS-PAGE and Western blot analysis

After determining protein concentration, the volume required to provide 10 μg of protein sample was determined and aliquoted into separate Eppendorf tubes containing reducing loading buffer (0.02% bromophenol blue + 2% 2-mercaptoethanol). The negatively charged protein extracts were denatured at 95 °C for 3 min prior to loading onto a sodium dodecyl sulfate – polyacrylamide gel (SDS-PAGE), 10% SDS-PAGE Mini-Protean® TGX™ precast gels using a Mini-Protean apparatus (Bio-Rad Laboratories). Samples were loaded alongside 4 μL PageRuler™ protein ladder (Fermentas, USA) to enable identification of proteins of interest and electrophoretically transferred to nitrocellulose membrane (Hybond-C; GE Healthcare) or PVDF membrane (for the experiments

set at King's College) in Tris-glycine buffer at 110 V for 90 min. Non-specific binding to membrane was blocked by incubating in Tris-buffered saline (TBS)/Tween, pH 8.0, containing 5% non-fat dried milk or BSA for 1 hour at room temperature. Blots were probed overnight at 4 °C with primary antibodies, washed with TBS/Tween and then incubated for 1 hour at room temperature with secondary horseradish peroxidase conjugates antibodies. Membranes were washed and the antigens were then visualized by addition of ECL Plus Western Blotting Detection Reagents. Images of the blots were obtained using a CCD imager (ChemiDoc MP System, Bio-Rad) and bands were acquired and analysed by using Image Lab analysis software; for the experiments set at King's College, membrane was detected using the Genesnap G-box and software Syngene, and data obtained was analysed using densitometry software Image J.

Table 2. Table of primary antibody.

Antigen	Species (antibody raised in)	Dilution	Supplier
α-tubulin	Rat	1:5000	Millipore
β-actin	Mouse	1:5000	Sigma-Aldrich
HO-1	Rabbit	1:500	Abcam
Prx-1	Rabbit	1:500	Abcam
flotillin-2	Rabbit	1:1000	Sigma-Aldrich
phospho-Akt	Rabbit	1:1000	Cell Signaling Technology
Akt	Rabbit	1:1000	Cell Signaling Technology
GLUT1 (C-20 and N-20)	Goat	1:1000	Santa Cruz
GLUT4	Goat	1:1000	Santa Cruz
PI3 Kinase	Rabbit	1:1000	Millipore
phospho-PI3K	Rabbit	1:1000	Millipore
AQP1	Rabbit	1:1000	Santa Cruz
AQP3	Rabbit	1:1000	Santa Cruz
AQP8	Rabbit	1:1000	Santa Cruz
caveolin 1	Rabbit	1:1000	BD Biosciences
transferrin receptor (CD71)	Rabbit	1:1000	BD Biosciences
Lyn	Rabbit	1:1000	Abcam
VEGF receptor-2	Rabbit	1:1000	Millipore
phospho-tyrosine	Rabbit	1:1000	Millipore

Table 3. Table of secondary antibody.

	Dilution	**Supplier**
Rabbit monoclonal HRP conjugated	1:10000	Cell Signaling Technology
Mouse monoclonal HRP conjugated	1:10000	Cell Signaling Technology
Donkey anti-goat HRP conjugated	1:5000	Santa Cruz
Donkey anti-goat FITC-conjugated	1:500	Santa Cruz
Rat monoclonal HRP conjugated	1:10000	Millipore

All antibody were diluted in 5% BSA or non-fat dried milk in PBS/0.1% Tween.

3.10 Cholesterol depletion

Cyclodextrins are cyclic oligomers of glucose that have the capacity to sequester lipophilic molecules in their hydrophobic core (Pitha, Irie et al. 1988). It has been shown that β-cyclodextrins remove cholesterol from cultured cells (Ohtani, Irie et al. 1989; Kilsdonk, Yancey et al. 1995), and among the different dextrin derivatives, methyl-β-cyclodextrin (CD) was shown to be the most efficient as acceptor of cellular cholesterol, and the most commonly used (Klein, Gimpl et al. 1995; Zidovetzki and Levitan 2007). Therefore, methyl-β-cyclodextrin was chosen to induce cholesterol depletion from plasma membrane of B1647 cells, and experiments were performed to set the desired conditions, as the degree of cholesterol depletion is a function of the β-cyclodextrin derivative concentration, incubation time, temperature and cell type subjected to the treatment (Christian, Haynes et al. 1997).

B1647 cells suspended in culture medium were incubated overnight with [^{3}H]-cholesterol (0.5 μCi/mL), then washed, suspended in PBS and exposed to different concentrations of CD (2.5 to 25 mM) for different incubation times (0–40 min). To measure the relative cholesterol content, cells were washed twice in PBS, pelleted at 4.000 *g* for 1 min and sample radioactivity was quantified by liquid-scintillation counting (Tri-Carb® liquid scintillation analyzer, PerkinElmer).

3.11 Isolation of membrane caveolae/lipid rafts

Caveolae/lipid rafts (DRM, detergent resistant membranes) and detergent-soluble membrane domains were separated by flotation assays adapted from previously described methods (Caliceti,

Zambonin et al. 2012). 200×10^6 B1647 cells (approx. 6 mg of protein) were washed twice with PBS, pelleted at 300 *g* for 7 min and left on ice for 10 min. The cell pellet was incubated at 4 °C in 1.2 mL of lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris and 5 mM EDTA supplemented with 0.1 mM PMSF, 0.1 mM TLCK, 0.1 mM TPCK, 1 mM orthovanadate and protease inhibitor cocktail, pH 8.0). In all subsequent steps, solutions and samples were kept at 4 °C. The lysates were then spun for 10 min at 6.000 *g* in an Eppendorf Microfuge and supernatants were homogenized in a Potter homogenizer with 20 strokes. For sucrose gradient separations, 1.0 mL of 80% sucrose prepared in PBS was mixed with an equal volume of homogenized sample and then overlaid with a 5–40% sucrose linear step gradient (1.3 mL each of 5%, 30% and 40% sucrose in PBS). After centrifugation in a SW50.1 Beckman rotor at 160.000 *g* for 18 h at 4 °C, nine 500 μL fractions were collected from the top of the gradient. Equal volume aliquots of each fraction was added with Laemmli buffer containing both mercaptoethanol and bromophenol blue and boiled for 3 min. Samples were then subjected to SDS-PAGE and immunoblotting.

This detergent-based fractionation on sucrose-density gradient isolate buoyant lipid rafts and caveolae (a subset of lipid rafts, flask-like invaginations of the plasma membrane with lipid composition similar to rafts and expressing a coat of caveolin proteins on the inner leaflet of the membrane bilayer (Razani, Woodman et al. 2002) but it is not able to discriminate between rafts and caveolae. Caveolin antibody-based immunoisolation of detergent-insoluble membranes selectively isolates caveolae and not lipid rafts, pulling down also the caveolar lipids, cholesterol, and associated proteins.

3.12 Immunoprecipitation

Following completion of the treatment period, cells were lysed using a lysis buffer (the same used for Western Blot). Lysates containing equal protein amounts were incubated overnight with the specific antibody for the protein chosen for the immunoprecipitation (VEGFR-2). Subsequently samples were incubated with protein G-Sepharose beads for 1.5 h at 4 °C and then pelleted. Pellets were washed four times with lysis buffer, treated with reducing buffer containing 4% 2-mercaptoethanol and boiled for 3 min. Samples were then subjected to SDS-PAGE and immunoblotting with primary antibodies (anti-caveolin-1, anti-phospho-tyrosine, or anti-VEGFR-2).

3.13 Immunofluorescence

B1647 cells (2×10^6) were incubated for 20 min with or without 10 mM CD, then pelleted, washed with PBS to remove culture medium, and fixed in 3% (w/v) paraformaldheyde for 15 min. Cells

were washed twice with HBSS, blocked with PBS/BSA 3% (w/v) for 1 hour at room temperature, washed 3 times, and then overnight incubated at 4 °C with 20 µg/mL of anti-Glut1 raised against a peptide within an extracellular domain of the human transporter protein (N-20). Cells were then treated for 1 hour at room temperature with fluorescent FITC-conjugated rabbit anti-goat IgG, mounted on slides and visualized using an Olympus IX50 microscope.

3.14 Real-time impedance-based used for detecting cell proliferation

Measurements of cell proliferation were performed using the iCELLigence Real-Time Cell Analyzer DP (Roche Applied Science, Indianapolis, IN) inside a cell culture incubator.

The iCELLigence system is a impedance-based system for cell-based assays, allowing for label-free and real-time monitoring of cellular processes such as cell growth, proliferation, cytotoxicity, adhesion, morphological dynamics (like contraction) and modulation of barrier function. The system is composed of an electronic analyzer that fits inside the cell culture incubator, and an iPad which runs the software and operates the entire system in a wireless mode. At the core of the system are electronic plates (E-Plates L8) that have integrated microelectrode sensors in the bottom of the wells. The presence or absence of cells sensitively and precisely affects the electronic and ionic exchange between cell culture media and the microelectrodes when an electric field is applied to the system. Changes in electrical impedance reflect the biological status of the cells, thus the system allows monitoring of time-dependent perturbation effects in a tissue culture environment, providing dynamic information and a unique approach to cell-based assays.

Cell index is the unit measure of cell impedance and normalized cell index is the cell index value at a time point divided by value of cell index at an earlier starting or reference point. Impedance was collected every 15 minutes for proliferation assays.

Cells are seeded at a density of 5×10^4 cells/well on an specific E-plate 8 (Roche Applied Science) in 10% FBS growth medium. All controls and treatments in these experiments were performed in duplicate.

- HASMC were seeded on E-plate 8 and treaded with 3 different Klotho concentrations (0.1; 0.5; 1 nM). Growth and proliferation were monitored for 48h.

3.15 Fluorescent assay for intracellular Glutathione (GSH) content

Glutathione (GSH) is a tri-peptide ubiquitously expressed in all cell types and it represents the major non-enzymatic regulator of intracellular redox homeostasis (Masella, Di Benedetto et al. 2005).

Cells were seeded in 24-well plates. After different treatments, cells were washed twice with ice-cold PBS, and cold 6.5% trichloroacetic acid (TCA) was added to the wells and left for 10 min on ice. TCA is widely used for the precipitation of proteins (Witt and Roskoski 1975), and in this case it was used for the extraction of GSH. After deproteinization, the supernatant was transferred into Eppendorf tubes; 7.5 μL was incubated with 277.5 μL of sodium phosphate buffer at pH 8.0 containing 5 mM EDTA and 15 μL of o-phthalaldehyde (OPA; 1 mg/mL in methanol) for 25 min at room temperature in a black 96-well plates. OPA is a fluorogenic reagent that reacts with primary amines and in the presence of thiol it yields an intense blue colored fluorescent product; the assay provides a rapid and very sensitive method for determining reduced GSH.

Fluorescence intensity was then measured at an excitation wavelength of 340 nm and an emission wavelength of 420 nm in a Hidex plate reader (Chameleon V, Hidex). The GSH content was calculated using a GSH standard curve, and expressed as nanomoles of GSH per milligram of protein.

After the supernatant removal from the 24-well plates, NaOH was added to each well to collect protein lysates for determination of the cellular protein concentration using the BCA assay (previously described).

- HASMC were plated into 24-well plates at a density of 5×10^4 cells/well. After 24 h growth, treatments were carried out. Total content was assessed in normal glucose, high glucose and mannitol conditions, after 12 and 24h treatment with 2.5 μM-5 μM sulforaphane or after 8 and 24h treatment with 0.1-0.5-1 nM Klotho.

3.16 Statistical analysis

Results are expressed as means ± SD or means ± SEM. Differences among the means were determined by Bonferroni's multiple comparison test following one-way ANOVA.

Chapter 4- Results

4.1 Redox Signalling in Human Leukaemia Cells: Role of Aquaporins

As previously stated, it has been shown that specific aquaporin isoforms facilitate the passive diffusion of H_2O_2 across biological membranes, controlling H_2O_2 membrane permeability and signaling in living organisms (Bienert and Chaumont 2014).

In particular, three AQP isoforms have been reported to channel H_2O_2 across the plasma membrane: AQP1, AQP3, widely expressed in blood cells, and AQP8, expressed in primitive erythroid cells (Vieceli Dalla Sega, Zambonin et al. 2014).

The aim of this study was to investigate the role of AQP isoforms in human cell lines derived from acute myeloid leukaemia, in order to verify whether, also in these cells, AQPs could represent one potential way through which H_2O_2, produced by Nox enzymes, moves inside the cells behaving like a signal molecule, thus contributing to cell survival and proliferation.

Our research group has previously reported that Nox enzymes are a major source of ROS in acute leukaemia-derived cell lines and that Nox-derived ROS are required for proliferation and survival of these cells, being directly involved in maintaining the high glucose uptake essential for cellular aberrant growth (Prata, Maraldi et al. 2008; Maraldi, Prata et al. 2010).

Experiments were performed in a erythro-megakaryocytic human cell line, B1647, focusing the attention on AQP1, AQP3 and AQP8.

4.1.1 Evaluation of the effect exerted by AQP inhibition on intracellular ROS level

Powerful tools to study the involvement of a protein in a biological process or disease are the pharmacological inhibition of its activity or the gene deletion/silencing. When an inhibitor is used, it should be characterized by high specificity and affinity, but unfortunately these compounds are often unspecific, due to several off-target effects or the inhibition of features that are not unique for the target enzyme. As far as AQP transporters are concerned, it has been reported that silver ions are potent and specific inhibitors of AQP activity, acting by binding to cysteine or histidine residues in the region of the channels (Vieceli Dalla Sega, Zambonin et al. 2014). Thus, the effect of $AgNO_3$ was investigated, and preliminary experiments were performed in order to test its potential cytotoxicity in B1647 cell line.

Cells were treated with different $AgNO_3$ concentrations for 10 min and the viability was evaluated by using MTT assay as reported in Material and Methods section (Fig.1A). The results show that treatment with $AgNO_3$ did not affect B1647 cell viability up to 10 μM, thus we chose 5 μM $AgNO_3$ for the subsequent experiments.

Cells treated with 5 μM $AgNO_3$ for 10 min were then exposed to 10 μM or 100 μM H_2O_2. Intracellular ROS level was evaluated spectrofluorimetrically by means of H_2DCFDA probe (as

reported in Material and Methods). As shown in Fig. 1B, cells treated with $AgNO_3$ exhibited a 22% decrease in the basal intracellular ROS level compared to control cells. The role of extracellular H_2O_2 in triggering DCF fluorescence is confirmed by the observation that the treatment with extracellular 5 mg/mL catalase abrogated the effect of AQP inhibition on the basal intracellular ROS level. When cells were externally added with 10 μM or 100 μM H_2O_2, a significant fluorescence increase was observed, while cells pre-incubated with AQP inhibitor exhibited a 35% and 19% decrease in fluorescence compared to the cells spiked with H_2O_2 10 μM or 100 μM, respectively.

In order to confirm that intracellular ROS level is mainly due to hydrogen peroxide, H_2O_2 level was also measured by using PF1, a fluorescent boronate dye endowed with high selectivity for H_2O_2 over similar ROS, such as superoxide anion and hypochlorite ion (Chang, Pralle et al. 2004). After the treatment with $AgNO_3$, cells were incubated with 100 μM or 200 μM H_2O_2, as shown in Fig. 1C. The trend of the experiment obtained with the PF1 probe is very similar to that obtained with the H_2DCFDA probe reported in Fig. 1B, confirming that H_2O_2 is the main intracellular ROS. Unfortunately, although PF1 is a useful tool owing to its selectivity towards H_2O_2, it is not sensitive enough to evaluate possible changes in basal intracellular H_2O_2 level (Miller and Chang 2007).

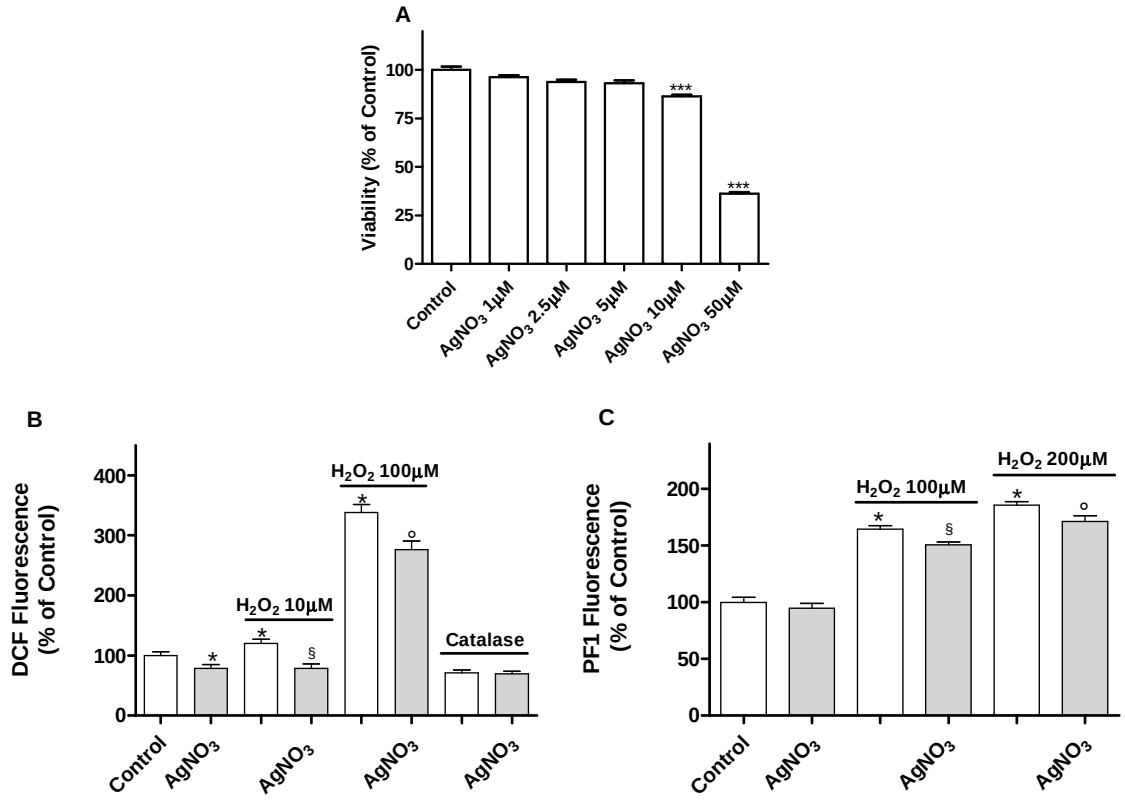


Fig. 8. Effect of $AgNO_3$ on viability and on intracellular ROS level in B1647 cells.

(A) Cells were incubated with different concentrations of $AgNO_3$ (1-50 μM) and their viability was evaluated by using MTT assay as reported in Material and Methods. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: ***p <

0.001 significantly different from Control. (B) Cells were incubated with 5 μM $AgNO_3$ and then treated with 10 or 100 μM H_2O_2. Intracellular ROS level was evaluated as H_2DCFDA fluorescence as reported in Material and Methods. In the experiment with 5 mg/mL catalase, the enzyme was added prior to $AgNO_3$ treatment. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: *$p < 0.05$ significantly different from Control; §$p < 0.05$ significantly different from cells treated with 10 μM H_2O_2; °$p < 0.05$ significantly different from cells treated with 100 μM H_2O_2. (C) Cells were incubated with 5 μM $AgNO_3$ and then treated with 100 or 200 μM H_2O_2. Intracellular ROS level was evaluated as PF1 fluorescence as reported in Material and Methods. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: *$p < 0.05$ significantly different from Control; §$p < 0.05$ significantly different from cells treated with 100 μM H_2O_2; °$p < 0.05$ significantly different from cells treated with 200 μM H_2O_2.

4.1.2 Study of the correlation between Nox-generated ROS and AQP activity

The single or combined effect of Nox and AQP inhibition could help to understand their mechanism of action and to investigate the correlation between their functions. Several small molecules have been and are still being used as direct NADPH oxidase inhibitors. The most frequently used Nox inhibitors are diphenylene iodonium (DPI) and apocynin, although they have been proved to be unspecific. DPI acts as a general flavoprotein inhibitor and, therefore, also inhibits eNOS, xanthine oxidase, and proteins of the mitochondrial electron transport chain. Apocynin shows intrinsic antioxidant activity, that is, ROS-scavenging properties (Altenhofer, Radermacher et al. 2014). Since many previously data in B1647 cell line were obtained with DPI, this inhibitor was chosen to perform the subsequent experiments.

B1647 cells were pre-incubated with DPI and then treated with $AgNO_3$. Intracellular ROS level was evaluated spectrofluorimetrically by means of H_2DCFDA probe (as reported in Materials and Methods section). As shown in Fig. 2A, a decrease in intracellular ROS level was observed after the pre-treatment with 10 μM DPI for 30 min. The treatment with both the inhibitors, $AgNO_3$ and DPI, caused a decrease in intracellular ROS level, but their effect was not additive, suggesting that they act by two different ways.

Evidence so far described allows to speculate that, in B1647 cell line, Nox-produced H_2O_2 outside the cell could cross the plasma membrane *via* AQP channel, thus acting as signal molecule. Therefore, the measure of a cellular activity linked to this signalling pathway could strengthen this hypothesis.

Data obtained in our lab have previously demonstrated that H_2O_2 mimics the growth factor stimulation of glucose uptake in leukaemia cell lines, suggesting a key role for ROS in the signalling leading to Glut1 regulation (Maraldi, Fiorentini et al. 2007). In B1647 cells, VEGF is supposed to utilize ROS as messenger intermediates downstream of the VEGF-receptor 2, and

glucose uptake is considered a model of cellular activity strictly bound to VEGF-induced cell proliferation. In this cell type, antioxidants and Nox inhibitors (DPI and apocynin) induce a similar decrease in the glucose uptake rate (Maraldi, Prata et al. 2007). Therefore, the effect of AQP inhibition on glucose transport in B1647 cells was assessed by means of a radioisotopic assay as reported in Material and Methods section. Cells were incubated with 5 μM $AgNO_3$ for 10 min and the results are shown in Fig. 2B. It can be seen that $AgNO_3$ treatment caused a 32% decrease in glucose transport compared to the control cells. In agreement with data on intracellular ROS level, the simultaneous treatment with DPI and $AgNO_3$ did not cause an additive effect on glucose uptake (Fig. 2B).

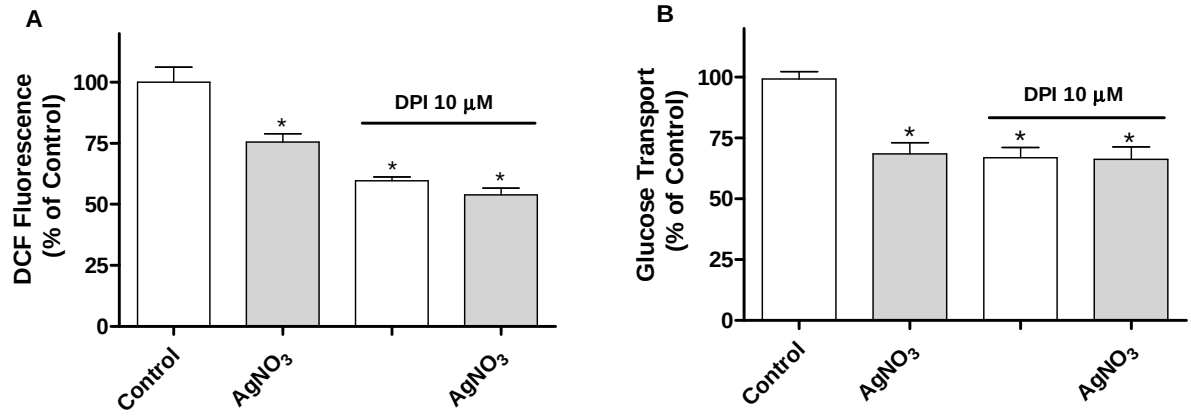


Fig. 9. Effect of AQP and Nox inhibition on intracellular ROS level and glucose transport in B1647 cells.
(A) Cells were incubated with 5 μM $AgNO_3$ and then treated with 10 μM DPI for 30 min. Intracellular ROS level was evaluated as DCF fluorescence as reported in Material and Methods. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: *$p < 0.05$ significantly different from Control. (B) Cells were incubated with $AgNO_3$ and then treated with 10 μM DPI for 30 min. Glucose uptake was evaluated by means of a radioisotopic assay as reported in Material and Methods. Data are expressed as % of Control and represent means ± SD of at least three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: *$p < 0.05$ significantly different from Control.

4.1.3 Evaluation of the effect exerted by AQP silencing on intracellular ROS level

Gene silencing is considered a gene knockdown mechanism since the methods used to silence genes generally reduce the expression of a gene but do not completely eliminate it. Gene silencing is usually more specific than the use of inhibitors, therefore this technique was chosen to support data obtained with $AgNO_3$.

Specific siRNA were used to silence AQP1, AQP3 and AQP8 isoforms, as described in Materials and Methods section.

First of all, constitutive expression of the target isoforms was established by Western Blotting analysis, using specific antibodies against AQP1, AQP3 and AQP8. As it can be seen in Fig. 3A, B1647 cell line does not express AQP1, while the constitutive AQP3 and AQP8 isoforms are clearly evident. For this reason, only AQP3 and AQP8 isoforms were silenced.

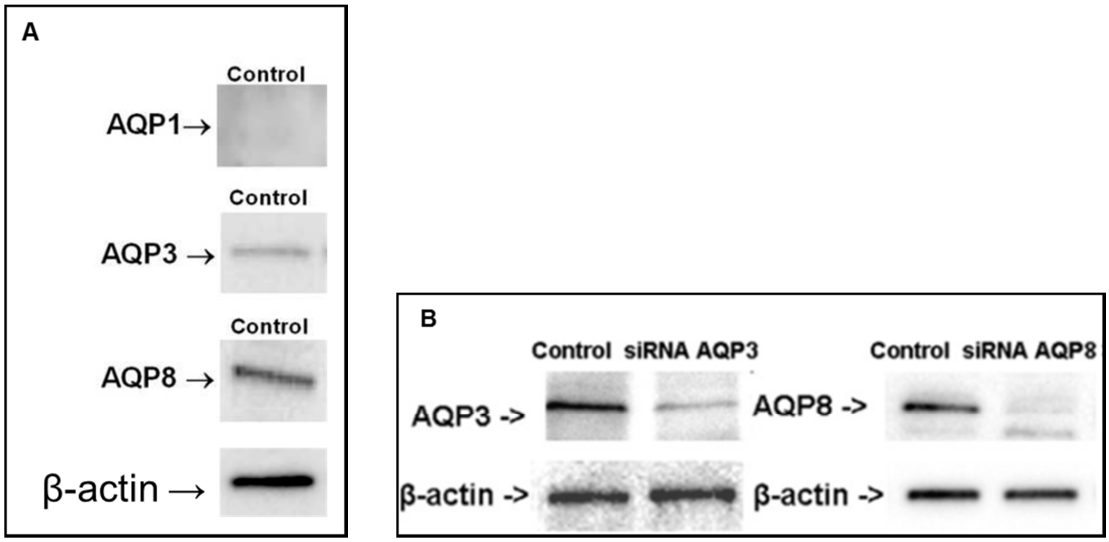


Fig. 10. Expression of AQP isoforms in B1647 cells.

(A) Constitutive expression of AQP isoforms in B1647 cells. The images show a representative experiment out of three independent analyses. (B) B1647 cells were transfected by electroporation with siRNA against AQP3, AQP8 or a random RNA sequence (scrambled) as negative control as described in Materials and Methods. Effect of RNA interference on the target isoforms was confirmed by Western blot with specific antibodies against AQP3 or AQP8. The images show a representative experiment out of three independent analyses.

B1647 cells were then transfected by electroporation with siRNA against AQP3, AQP8 or a random RNA sequence (scrambled) as negative control and the effect of this procedure was confirmed by Western Blotting analysis, as shown in Fig. 3B. The viability of the transfected cells was assayed by means of both the Trypan Blue exclusion test and the MTT test, indicating that this treatment does not affect cell viability (data not shown).

Then the effect of AQP3 and AQP8 silencing was evaluated on intracellular ROS levels (Fig. 4).

The results in Fig. 4A show that basal ROS level was significantly reduced only by silenced AQP8. On the other hand, when cells were stimulated with the addition of H_2O_2, both the silenced isoforms were able to cause a significant decrease in DCF fluorescence, although AQP3 siRNA in a lesser extent compared to AQP8 siRNA.

In order to corroborate also the data obtained in condition of Nox inhibition, the effect of DPI on intracellular ROS level was evaluated after AQP3 and AQP8 silencing, as shown in Fig. 4B.

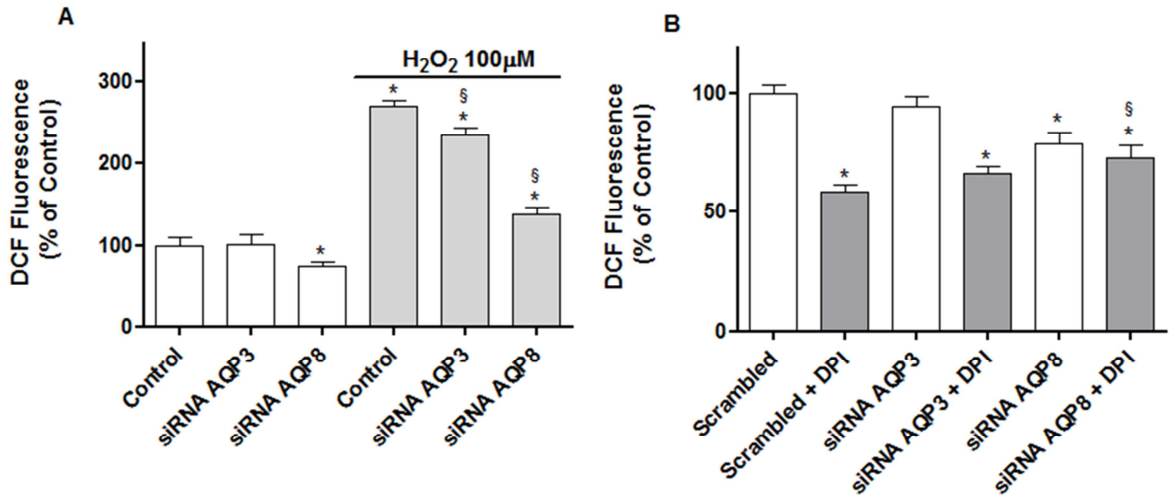


Fig. 11. Effect of AQP3 and AQP8 silencing on intracellular ROS level in B1647 cells.

(A) 24 h after transfection with siRNA, cells were treated with 100 µM H_2O_2, then intracellular ROS level was evaluated as DCF fluorescence as described in Materials and Methods. Data are expressed as % of control and represent means ± SD of, at least, three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: *$p < 0.05$ significantly different from control; $^{§}p < 0.05$ significantly different from cells treated with 100 µM H_2O_2. (B) 24 h after transfection with siRNA, cells were treated with 10 µM DPI, then intracellular ROS level was evaluated as DCF fluorescence. Data are expressed as % of control and represent means ± SD of, at least, three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: *$p < 0.05$ significantly different from control; $^{§}p < 0.05$ significantly different from cells treated with 10 µM DPI.

From the comparison of Figs. 1B and 2A with Figs. 4A and 4B, it can be seen that the inhibition profile of ROS formation obtained by the use of AQP and Nox inhibitors or by AQP silencing are similar. From the data obtained by the silencing technique it was possible to observe that AQP8 isoform, rather than AQP3, is able to transport H_2O_2 through the plasma membrane of B1647 cell line.

4.1.4 Evaluation of the effect exerted by AQP overexpression on intracellular ROS level

As well as the inhibition of an enzyme activity could be helpful in the understanding of its involvement in a biological process, the overexpression of its level amplifies its effect, corroborating the evidence obtained. Furthermore, overexpressed levels of AQPs *in vivo* are used as marker for several cancer types, thus this technique can mimic a pathological condition.

B1647 cells were transfected by electroporation with different plasmids designed for the overexpression of AQP1, AQP3 or AQP8, and in this case the overexpression of AQP1 was considered as a positive control. The transfection efficiency was estimated as fluorescent/non-

fluorescent cell ratio 24 hours after the electroporation with a GFP vector (see Materials and Methods section), and resulted to be about 70%.

Fig. 5A shows the Western blotting analysis of the target isoforms overexpression compared to their constitutive expression.

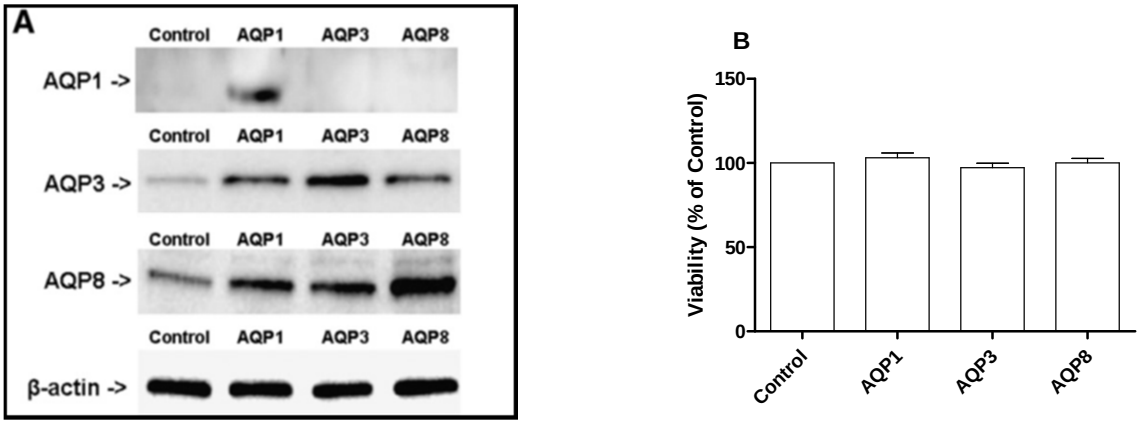


Fig. 12. AQP1, AQP3 and AQP8 overexpression and its effect on cell viability/proliferation in B1647 cells.

(A) B1647 cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA and lysed after 24 h. Overexpression of target isoforms was confirmed by Western blot with specific antibodies against AQP1, AQP3 or AQP8. The images show a representative experiment out of three independent analyses.

(B) B1647 cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA. After 24 h the cells were lysed, then viability/proliferation were evaluated by MTT assay as described in Materials and Methods, and compared to Control. Data are expressed as % of control and represent means ± SD of, at least, three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test. No significant differences were revealed.

Densitometric analysis normalized by actin content shows that AQP3 overexpression causes a 3.5-fold protein increase and AQP8 overexpression produces a 4.1-fold protein increase.

Trypan blue exclusion test (not shown) and MTT assay were performed 24 and 48 hours (not shown) after transfection, and data reported in Fig. 5B showed no significant differences in the viability of the cells transfected with the three plasmidic vectors compared to the control cells electroporated without DNA.

Twenty-four hours after transfection, intracellular ROS level was measured by means of DCF fluorescence either in basal condition or after 100 μM H_2O_2 addition.

As it can be seen in Fig.6A only AQP8 overexpression caused a little but significant increase in ROS basal level, while all of the three overexpressed isoforms were able to significantly enhance the intracellular ROS level in the presence of H_2O_2 (Fig. 6B).

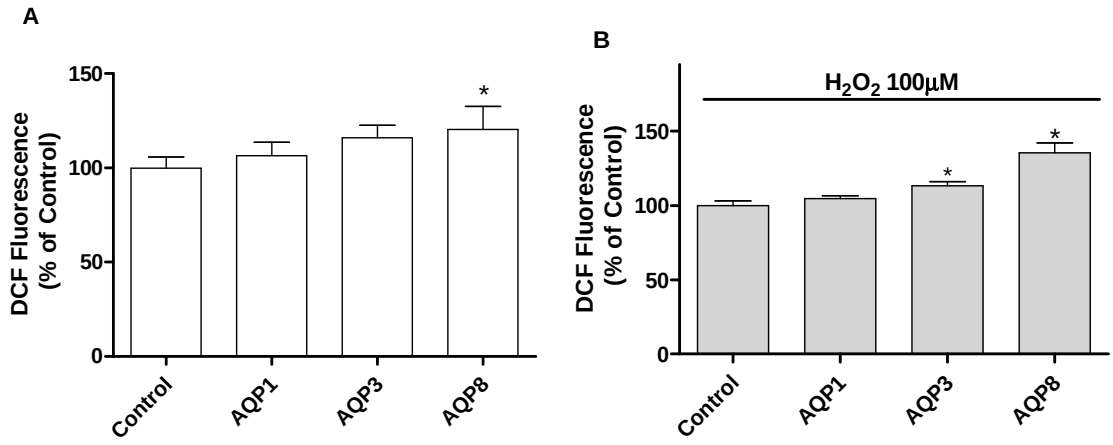


Fig. 13. Effect of AQP overexpression on intracellular ROS level in B1647 cells.

B1647 cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA. (A) 24 h after transfection, the intracellular ROS level of cells overexpressing different AQP isoforms was evaluated as DCF fluorescence as reported in Material and Methods. Data are expressed as % of control and represent means ± SD of three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: *$p < 0.05$ significantly different from control. (B) 24 h after transfection, cells overexpressing different AQP isoforms were treated with 100 µM H_2O_2, then intracellular ROS level was evaluated as DCF fluorescence. Data are expressed as % of control and represent means ± SD of at least three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: *$p < 0.05$ significantly different from control.

These results, along with that obtained in AQP3 and AQP8 silencing conditions, strongly support the notion that AQP8 and, in a lesser extent, AQP3 are able to funnel H_2O_2 across the plasma membrane in the acute leukemia B1647 cell line.

4.1.5 Effect of AQP isoforms on VEGF-dependent modulation of ROS intracellular level

The angiogenic growth factor VEGF exerts proliferative and migratory effects on endothelium, thus tyrosine kinase receptor VEGF receptor 2 (VEGFR-2) was thought to be exclusively expressed by adult endothelial cells. Since endothelial and hematopoietic cells share a common progenitor, known as hemangioblast, it has been shown that VEGF and VEGFR-2 are also expressed in a subset of multipotent hematopoietic stem cells (Ziegler, Valtieri et al. 1999) and in many leukemia cells (Fiedler, Graeven et al. 1997).

Therefore, coinciding expression of VEGF/VEGF receptors may result in the generation of an autocrine loop supporting the proliferation and survival of leukemia cells.

It has been shown that B1647 cells constitutively produces VEGF and express its VEGFR-2 receptor (Bonsi, Pierdomenico et al. 2005), thus VEGF/VEGFR-2 signalling pathway ensures the viability and the fast proliferation of these cells. In particular, the research group demonstrated that Nox-derived ROS provoke VEGFR-2 autophosphorylation, which causes the modulation of many

cellular activities, among which glucose uptake, strictly bound to VEGF-induced leukemic cell survival and proliferation (Maraldi, Prata et al. 2010).

Therefore, since it has been demonstrated that AQP isoforms mediate H_2O_2 transfer across the plasma membrane in B1647 cells, changes in AQP activity could be linked to VEGF signal proceeding through Nox proteins, thereby modulating the intracellular ROS level. In order to test this hypothesis, overexpressing and silenced AQPs cells were exposed to exogenous VEGF and assayed for intracellular ROS production.

To better evaluate the VEGF role, keeping out the effect of other growth factors, AQP1, AQP3 or AQP8 overexpressing cells and AQP3 or AQP8 silenced cells were serum starved for 16 hours. Cells were then stimulated with 50 ng/mL exogenous VEGF for 30 min and assayed for intracellular ROS level, as reported in Fig. 7.

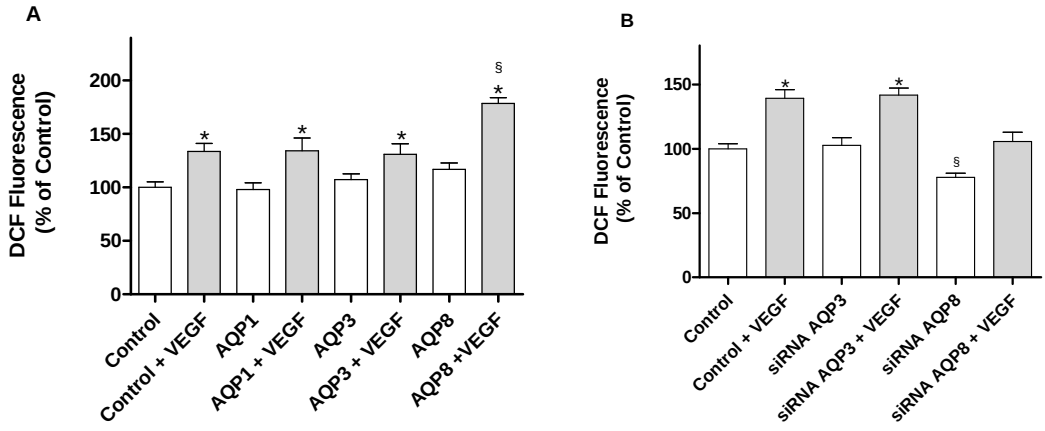


Fig. 14. Effect of VEGF on intracellular ROS level of AQP overexpressing or AQP silencing B1647 cells.

(A) B1647 cells were transfected by electroporation with plasmids designed for the overexpression of AQP1, AQP3, AQP8 or electroporated without DNA. After 24 h from transfection, control and overexpressing AQP1, AQP3 or AQP8 cells were serum starved for 16 h and then stimulated for 30 min with 50 ng/mL VEGF. The intracellular ROS level was evaluated as DCF fluorescence as reported in Material and Methods. Data are expressed as % of control and represent means ± SD of at least three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: $*p < 0.05$ significantly different from corresponding VEGF untreated cells; $^{\S}p < 0.05$ significantly different from VEGF treated control cells. (B) cells were transfected by electroporation with specific siRNA against AQP3, AQP8 or a random RNA sequence (scrambled) as control. After 24 h from transfection, control, AQP3 or AQP8 silenced cells were serum starved for 16 h and then stimulated for 30 min with 50 ng/mL VEGF. Then, intracellular ROS level was evaluated as DCF fluorescence. Data are expressed as % of control and represent means ± SD of at least three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: $*p < 0.05$ significantly different from corresponding VEGF untreated cells; $^{\S}p < 0.05$ significantly different from control cells.

Treatment with exogenous VEGF caused an increase in intracellular ROS level at the same extent in control, AQP1 and AQP3 overexpressing cells, while the VEGF-induced ROS increase in AQP8 overexpressing cells was significantly higher (Fig. 7A).

As it can be seen in Fig. 7B, VEGF-stimulated ROS production was not affected in AQP3 silenced cells, while the intracellular ROS content decreased significantly in AQP8 silenced cells, both in control and VEGF-treated cells.

These data confirm the above hypothesis and corroborate the importance of AQP8 constitutive expression in modulating VEGF-induced ROS generation.

4.2 Redox Signalling in Human Leukaemia Cells: Role of VEGF/VEGFR

In recent studies, the expression of VEGF/VEGFR in acute myeloid leukaemia patients has been detected and the increased level of plasma VEGF has been correlated with reduced survival (Aguayo, Kantarjian et al. 2002). Moreover, leukaemia cells have been associated with angiogenesis, upon the demonstration that leukaemia progression is accompanied by an increase in bone marrow vascularization (Perez-Atayde, Sallan et al. 1997). Therefore, the elucidation of the mechanism underlying VEGF/VEGFR activity in leukaemia cells is necessary for the development of new therapeutic agents.

Caveolae and lipid rafts are ordered structures of membrane microdomains, characterized by high concentration of cholesterol and glycosphingolipids, which are involved in fundamental cellular functions such as endocytosis, protein trafficking and signal transduction (Patel and Insel 2009). In endothelial cells, VEGFR-2 is present in caveolae through association with Caveolin-1 (Cav-1), which negatively regulates the receptor activity in basal state. Dissociation of VEGFR-2 from caveolae/Cav-1 seems to be essential for VEGFR-2 autophosphorylation and activation of downstream signalling events (Labrecque, Royal et al. 2003). Recent experimental evidence shows that, in endothelial cells, VEGF promotes the release of VEGFR-2 from caveolae/lipid rafts, thereby inducing its activation (Ushio-Fukai 2007). With the present experiments, the potential involvement of plasma membrane caveolae/lipid rafts in VEGF-mediated redox signaling was investigated in the human leukaemia B1647 cell line.

4.2.1 Effect of methyl-β-cyclodextrin on plasma membrane cholesterol depletion

The maintenance of cholesterol levels is essential for functional caveolae and depends, in part, on the interaction of cholesterol with Cav-1, the major structural component of caveolae (Labrecque, Royal et al. 2003). As well as the use of inhibitors is helpful in understanding the biological role of

a protein, disassembling of caveolae/lipid rafts can be useful to clarify the functions they are involved in.

It has been shown that cyclodextrins remove cholesterol from cultured cells (Ohtani, Irie et al. 1989; Kilsdonk, Yancey et al. 1995; Klein, Gimpl et al. 1995), since they are cyclic glucose oligomers able to sequester lipophilic molecules in their hydrophobic core (Pitha, Irie et al. 1988). Among the different cyclodextrin derivatives, methyl-β-cyclodextrin was shown to be the most efficient as acceptor of cellular cholesterol, and the most commonly used (Klein, Gimpl et al. 1995; Zidovetzki and Levitan 2007). Therefore, methyl-β-cyclodextrin (CD) was chosen in the subsequent experiments to induce cholesterol depletion from plasma membrane of B1647 cell line, thereby disrupting caveolae/lipid rafts.

First of all, experiments were performed to set the desired conditions, as the degree of cholesterol depletion is a function of the CD concentration, incubation time, temperature and cell type subjected to the treatment (Christian, Haynes et al. 1997). The purpose was to achieve a lipid environment alteration associated with membrane integrity and viable cells.

B1647 cells suspended in culture medium were incubated overnight with [^{3}H]-cholesterol (0.5 mCi/mL), then washed and exposed to different CD concentrations (2.5-25 mM) for 20-40 min in order to find the best working conditions (not shown).

Fig. 8A shows the time course of CD effect on cholesterol level, evidencing that cell treatment with 5 mM CD for 20 min was able to remove about 60% cholesterol. In these conditions cell viability was affected only about 10%, as resulted by Trypan Blue exclusion test (Fig. 8B) and MTT assay (not shown). Higher CD concentrations caused an higher decrease in cell viability, therefore 5 mM CD for 20 min was the treatment selected for the following experiments.

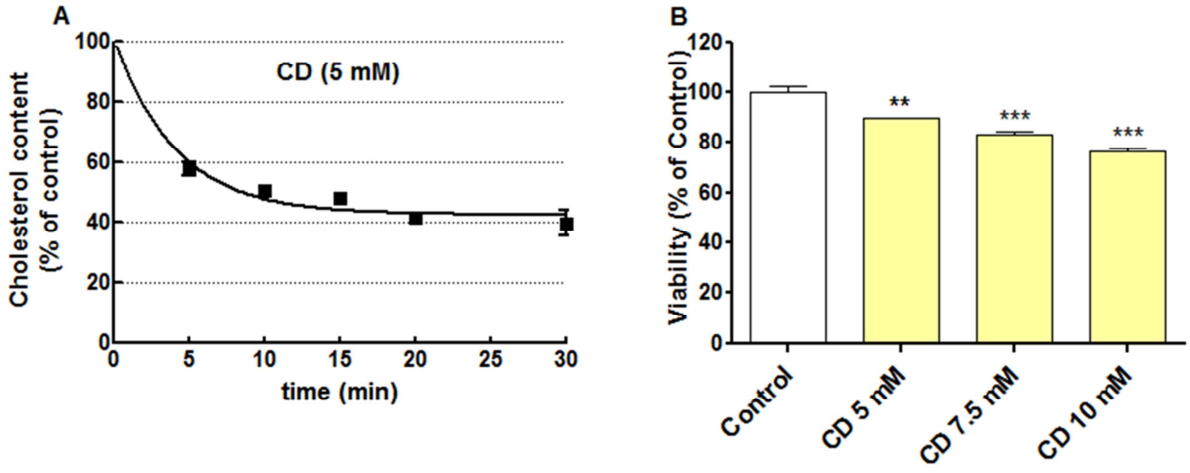


Fig. 15. Effect of CD concentration and incubation time on cholesterol content and cell viability in B1647 cells.

(A) B1647 cells were incubated with [^{3}H]-cholesterol (0.5 µCi/mL) in cell culture medium for 16 h at 37 °C, washed, re-suspended in PBS and treated with 5 mM CD for different time periods (0-30 min). Cell suspensions were washed with PBS, then [^{3}H]-cholesterol content was estimated by liquid scintillation counting. (B) The viability of the cells

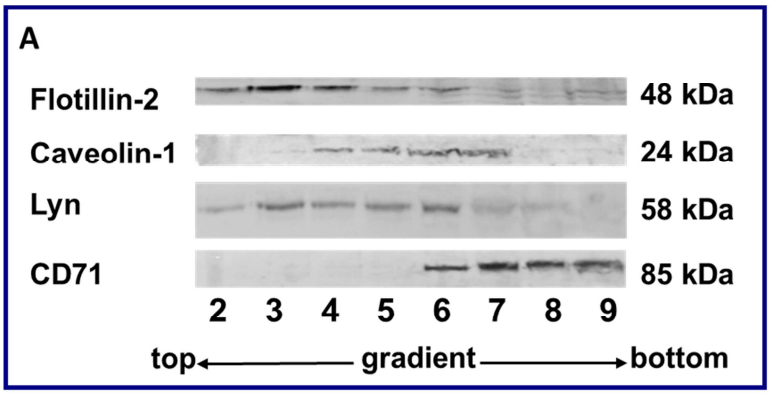
treated with different CD concentrations (5-10 mM) for 20 min was evaluated by Trypan Blue exclusion test as described in the Materials and Methods. Data are expressed as % of control and represent means ± SD of three independent experiments, each performed in triplicate. Data were analysed by one-way ANOVA followed by Bonferroni's test :**$p < 0.005$, significantly different from control cells; ***$p < 0.0005$, significantly different from control cells.

4.2.2 Isolation and identification of membrane caveolae/lipid rafts by detergent extraction and sucrose gradient centrifugation in B1647 cells

The isolation and identification of membrane caveolae/lipid rafts from the other portions of B1647 plasma membranes is the first step requested to investigate VEGFR-2 localization in this cell type.

Historically, caveolae/lipid rafts have been defined functionally by their low density and insolubility in cold 1% Triton X-100, thereby forming the Detergent Resistant Membrane fraction (DRM), and other membrane fractions that are solubilised by TX-100. Then caveolae/lipid rafts are isolated by flotation in a linear sucrose density gradient, where they distribute in the top few fractions of the gradient. This procedure yields a fairly consistent product that is enriched in cholesterol and raft marker proteins (L. J. Pike Journal of Lipid Research vol. 44, pp. 655-667, 2003).

B1647 cells were lysed using TX-100, centrifuged and the supernatant was collected and subsequently subjected to sucrose density-gradient (5-40%) centrifugation as described in the Materials and Methods section and the results are shown in Fig. 9A. Nine fractions were collected and analysed by SDS-PAGE followed by Western blotting using anti-flotillin-2 (48 kDa), anti-caveolin-1 (24 kDa) and anti-Lyn (58 kDa) antibodies as protein markers for DRM fractions. Indeed, flotillin-2 and Lyn are known to be associated with caveolae/lipid rafts in different cell lines (Barnes, Ingram et al. 2004), as well as Cav-1. The transferrin receptor (CD71), an integral membrane protein, was selected as a marker for detergent-soluble non-raft membrane fractions.



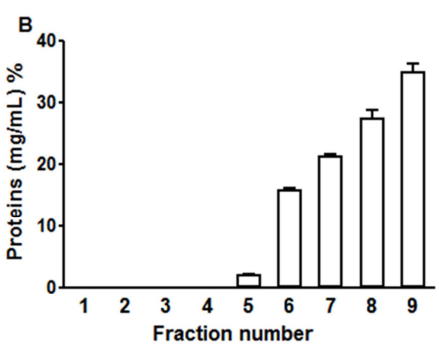


Fig. 16. Isolation and identification of membrane caveolae/lipid rafts by detergent extraction and sucrose gradient centrifugation in B1647 cell line.

(A) Cells were lysed with 1% Triton X-100 at 4 °C and separated by sucrose density-gradient ultracentrifugation as described in Materials and Methods. Equal aliquots of each fraction were subjected to SDS-PAGE and Western blotting. Flotillin-2, caveolin-1 and Lyn were used as markers for caveolae/lipid raft fractions; CD71 for non lipid raft fractions. (B) Typical profile of protein concentrations in gradient fractions after ultracentrifugation. Protein content was determined as described in Materials and Methods. Data represent means ± SD of three independent experiments, each performed in triplicate. Data were analysed by one-way ANOVA followed by Bonferroni's test: °$p < 0.01$, significantly different from each other.

As it can be seen in Fig. 9A, flotillin-2, Lyn and Cav-1 are localized in the low-density region of the gradient (fractions 2-6), where caveolae/lipid rafts are supposed to be, instead, CD71 is located in the fractions 6-9, corresponding to non-raft membrane fractions localization.

Fig. 9B shows the protein content of the different gradient fractions. It can be seen that the amount of proteins present in the DRM fractions is undetectable with this procedure (although evident in the Western blot), and the main part of B1647 proteins was found in the high-density region of the sucrose gradient.

4.2.3 Effect of cholesterol depletion on VEGFR-2 localization in B1647 cells

In order to investigate the effect of disrupting caveolae/lipid rafts on VEGFR-2 localization, B1647 cells, deprived or not of human serum (HS), were subjected to 5 mM CD treatment for 20 min, lysed by using TX-100, and separated by sucrose density-gradient centrifugation as previously described.

Also in this case nine fractions were collected and analysed by SDS-PAGE followed by Western blotting using anti-VEGFR-2 antibody, in order to observe VEGFR-2 localization.

As reported in Fig. 10, VEGFR-2 distribution differs between serum-starved (- HS) or serum added (+ HS) cells. In serum starved cells VEGFR-2 is present both in raft and non raft regions, whereas in serum added cells VEGFR-2 content increases in the non-raft fractions, suggesting that VEGF is able to link to its receptor causing a shift of the VEGF/VEGFR-2 complex towards non-caveolar membrane regions.

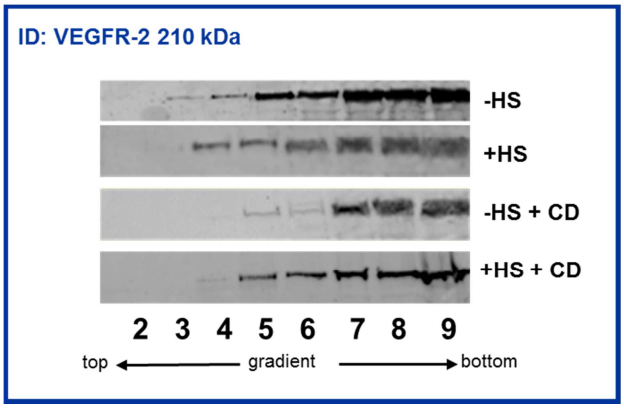


Fig. 17. Effect of methyl-β-cyclodextrin on VEGFR-2 localization in B1647 cells.

B1647 cells in the presence or absence of human serum (HS) pre-treated or not with 5 mM CD for 20 min were lysed with 1% Triton X-100 at 4 °C and separated by sucrose density-gradient ultracentrifugation as described in the Materials and Methods. Equal aliquots of each fraction were subjected to SDS-PAGE, Western blotting and revealed for anti-VEGFR-2 (210 kDa). A representative blot is shown.

It can be seen also that cell treatment with CD resulted in the redistribution of VEGFR-2 from caveolae/lipid rafts to non-caveolar fractions both in the presence and in the absence of HS, owing to the caveolae/lipid raft disruption.

Since it has been shown that CD is capable of removing cholesterol from both raft and non raft fractions (Gaus, Chklovskaia et al. 2005), experiments were performed to ensure that the efficiency of cholesterol removal was higher in DRM fractions The results (not shown) confirmed that fractions 2-6, corresponding to caveolae/lipid rafts in our experimental conditions, exhibited the higher cholesterol depletion compared to other fractions.

These data demonstrated that, as well as in endothelial cells, also in a human leukemia cell line VEGFR-2 is partially localized in caveolae/lipid rafts and that the VEGF/VEGFR-2 interaction displaces the receptor from raft to non raft regions.

4.2.4 Effect of cholesterol depletion from plasma membrane on ROS generation and glucose transport activity in B1647 cells

Since it has been demonstrated that CD treatment provokes a partial movement of VEGFR-2 from caveolae/lipid rafts towards non-caveolar membrane regions, it is interesting to investigate whether this receptor redistribution could correspond to its activation.

Therefore, experiments were performed in order to examine the effect of CD (in the presence or absence of HS) on intracellular ROS level and glucose uptake, two downstream events linked to VEGF/VEGFR-2 signaling pathway.

Figs. 11A and B show that, when HS is present, the CD effect is negligible on both ROS production (Fig. 11A) and glucose uptake (Fig. 11B), since in this condition VEGF-VEGFR-2 pathway is partially already activated. On the other hand, when cells were serum starved, CD treatment significantly enhanced both parameters, probably because VEGF-VEGFR-2 interaction is facilitated by the partial caveolae/lipid raft disruption, thereby triggering the signal transduction pathway which leads to an increased ROS generation and glucose uptake.

Moreover, analyses of the images obtained by immunofluorescence microscopy of cells labelled with an anti-Glut1 antibody against an extracellular domain of Glut1 revealed that incubation with CD, in condition of serum starvation, greatly enhances the staining for the transporter at the cell surface (Fig. 11C). These results indicate that CD treatment increases glucose uptake through Glut1 recruitment into the plasma membrane from intracellular pool.

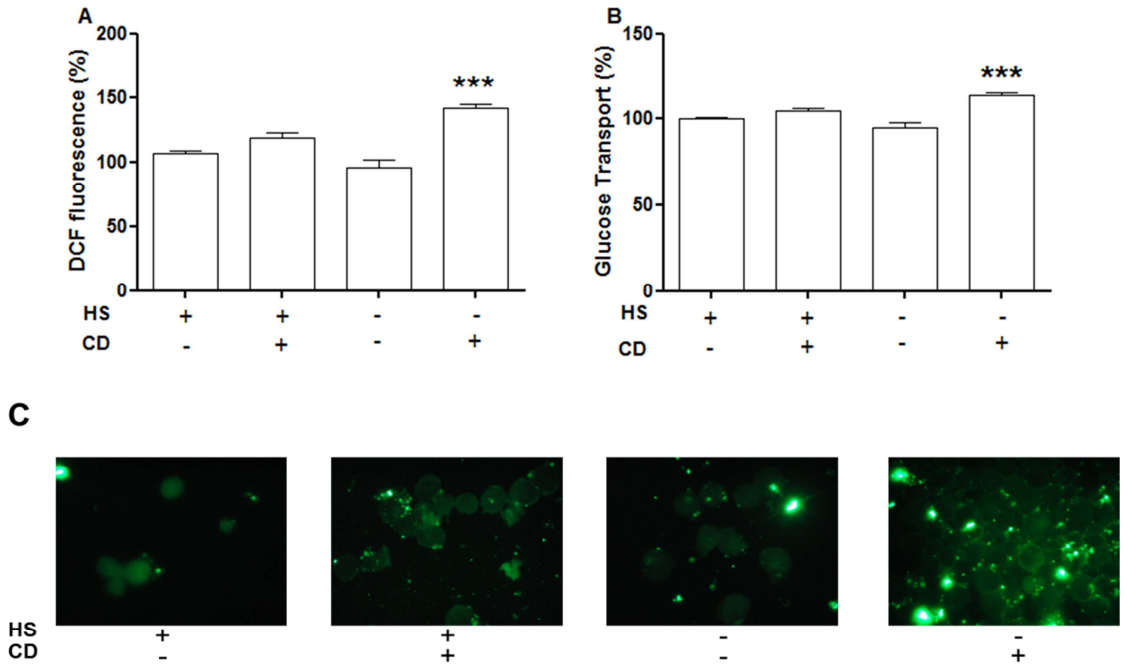


Fig. 18. Effect of methyl-β-cyclodextrin on ROS generation, glucose transport activity and Glut1 translocation in B1647 cells.

(A) B1647 cells in the presence or absence of human serum (± HS) and pretreated or not with 5 mM CD for 20 min (± CD) were incubated with 5µM DCFH-DA, then ROS intracellular level was measured spectrofluorimetrically as described in the Materials and Methods. (B) Cells in the presence or absence of human serum (± HS) and pretreated or not with 5 mM CD for 20 min (± CD) were incubated with DOG mixture, then glucose uptake was assayed by liquid scintillation counting as described in the Materials and Methods. Data in panel A and B are expressed as % of control and represent means ± SD of three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: ***$p < 0.001$, significantly different from control cells (+ HS, - CD). (C) Cells in the presence or absence of human serum (± HS) and incubated (or not) in PBS at 37 °C with 5 mM CD for 20 min (± CD) were fixed in 3% (w/v) paraformaldheyde for 15 min. Cells were then immunolabelled with anti-Glut1 (N-20) antibody (raised against an extracellular domain of Glut1, therefore evidencing Glut1 present on the cell surface), treated with fluorescent FITC-conjugated secondary antibody and visualized using immunofluorescence microscopy.

These results confirm that the disrupting effect caused by CD on B1647 plasma membrane caveolae/lipid rafts leads to VEGFR-2 redistribution to a non caveolar region, implying its consequent activation.

4.2.5 The relationship between VEGFR-2 and Caveolin-1

As previously stated, an association between VEGFR-2 and Cav-1 has been reported in endothelial cells, which negatively regulates the receptor activity in basal state. To investigate whether an interaction/co-localization between VEGFR-2 and Cav-1 also exists in B1647 cells and, if so, whether this relationship could affect the VEGFR-2 activation, the following immunoprecipitation experiments were performed.

B1647 cells were serum starved or not for 18h, then immunoprecipitated for anti-VEGFR-2. The samples were then subjected to SDS-PAGE and Western blotting analysis, using anti-Cav-1 and anti-phospho-tyrosine antibodies.

As reported in Fig. 12, in serum starved condition (- HS) Cav-1 and VEGFR-2 colocalize, and VEGFR-2 is slightly phosphorylated. On the other hand, when serum is present, the association between VEGFR-2 and Cav-1 is significantly less evident, whereas VEGFR-2 phosphorylation is markedly increased. These results are in accordance with those obtained in endothelial cells, where Cav-1 acts as negative regulator of VEGFR-2 activity, as previously stated.

Fig. 19. Association of VEGFR-2 with Caveolin-1 in B1647 cells.

Cells deprived or not of serum (± HS) were subjected to immunoprecipitation with anti-VEGFR-2 as described in Materials and Methods. Samples were electrophoresed, immunoblotted and revealed for anti-Cav-1, anti-phospho-tyrosine or with anti-VEGFR-2 as control. A representative blot is shown. Results were obtained considering three independent Western blot experiments. Relative amounts were determined by scanning densitometry and are expressed in arbitrary units. *$p < 0.05$, significantly different from control cells (+ HS).

4.2.6 The modulation of the VEGF/VEGFR-2 interaction on ROS intracellular level and glucose uptake

As above stated, the elucidation of the mechanism underlying VEGF/VEGFR activity in leukemia cells could be very useful for the development of new therapeutic strategies and agents.

To this purpose, the effect of different compounds able to inhibit VEGF/VEGFR-2 interaction by different mechanisms was investigated in B1647 cell line.

Bevacizumab was selected as a monoclonal antibody against VEGF since this drug is the most commonly used against VEGF-sustained cancers (Yang, Zhang et al. 2013). Semaxinib was chosen for its ability to inhibit VEGFR-2 tyrosine kinase activity, although the evaluation of its effect on ROS intracellular level is prevented by its autofluorescence (Vajkoczy, Menger et al. 1999). Finally, to deeply investigate the role of Cav-1 in B1647 cell model, the Cav-1 scaffolding domain (CSD), representing the portion of the protein (residues 82 to 101) essential for Cav-1 interaction with other proteins (Couet, Li et al. 1997), was used.

First of all, the influence of these compounds on B1647 cell viability was evaluated and the results were reported in Fig. 13. It can be seen that the three treatments, protracted until 2 hours, did not affect B1647 cell viability.

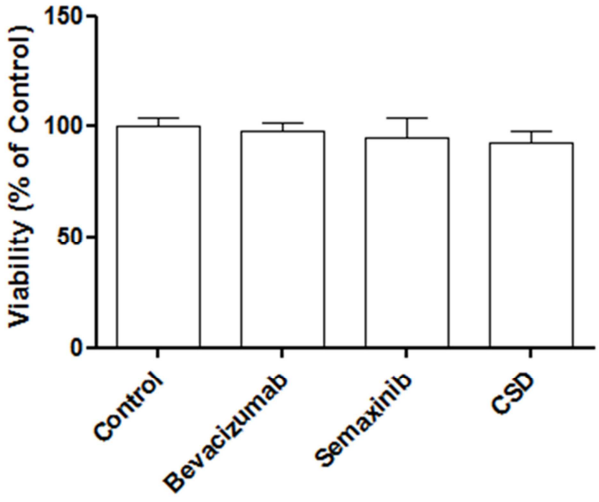


Fig. 20. Effect of Bevacizumab, Semaxinib or CSD on B1647 cell viability.

B1647 cells were incubated for 2 hours with 3.4 nM Bevacizumab, or 20 µM Semaxinib, or 5 µM CDS. Viability/proliferation was evaluated by MTT assay as described in Materials and Methods, and compared to control. Results are expressed as means ± SD of three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test. No significant differences were revealed.

Subsequently, B1647 cells in the presence or absence of human serum (± HS), were assayed for ROS intracellular level after pre-treatment with Bevacizumab (30 min) and CSD (2h) and the results are shown in Fig. 14A. In agreement with previous data, it can be seen that, in serum

cultured cells, the VEGF treatment is unable to induce ROS generation, since in this basal condition VEGF/VEGFR-2 signalling pathway is already activated, on the contrary 50 ng/mL VEGF treatment in serum-starved cells caused a significant increase in ROS level, reaching about the same ROS content of control cells. As far as the treatment with Bevacizumab or CSD is concerned, it can be seen that it was able to cause a significant decrease of intracellular ROS level independently on the presence of HS or VEGF.

Cells subjected to the same treatments and incubated with the different inhibitors were analysed for glucose transport (Fig. 14B), obtaining results very similar to that reported in Fig. 14A.

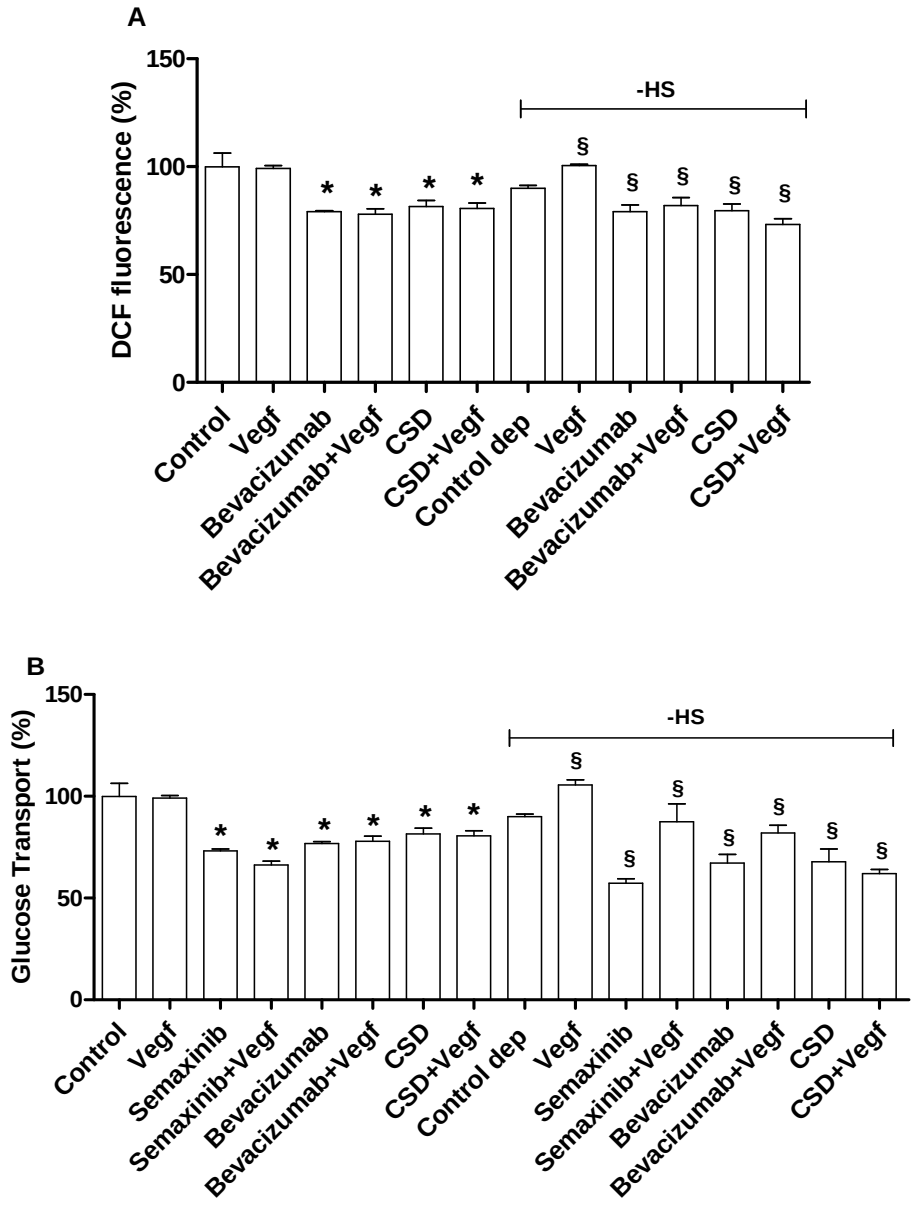


Fig. 21. Effect of different inhibitors of VEGF/VEGFR-2 interaction on intracellular ROS level and glucose uptake in B1647 cells.

(A) B1647 cells, control and serum-starved (-HS, “Control dep”) were treated with 500 µg/mL Bevacizumab (3.4 nM) for 30 min or 5 µM Cav-1 Scaffolding Domain (CSD) for 2h, in the presence or absence of 50 ng/mL VEGF; then the intracellular ROS level was spectrofluorimetrically measured by means of DCFH-DA, as described in Materials and Methods. Data are expressed as % of control and represent means ± SD of three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni’s test: $*p < 0.05$, significantly different from control cells; $^{§}p < 0.05$, significantly different from serum deprived control cells (- HS, Control dep).
(B) B1647 cells, control and serum-starved (- HS, “Control dep”) were treated with 20 µM Semaxinib for 2 h, or 500 µg/mL Bevacizumab (3.4 nM) for 30 min or 5 µM Cav-1 Scaffolding Domain (CSD) for 2h, in the presence or absence of 50 ng/mL VEGF; then glucose uptake was assayed by liquid scintillation counting as described in Materials and Methods. Data are expressed as % of control and represent means ± SD of three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni’s test: $*p < 0.05$, significantly different from control cells; $^{§}p < 0.05$, significantly different from serum deprived control cells (- HS, Control dep).

The results obtained with the three compounds able to inhibit VEGF/VEGFR-2 interaction confirm the importance of this molecular complex in maintaining the redox loop leading to high intracellular ROS level and glucose uptake in B1647 cells.

4.3 The maintenance of ROS balance inside the cells: Role of natural or physiological molecules

When ROS formation is dysregulated and goes beyond antioxidant defences, oxidative stress takes place, and when oxidative stress exceeds the ability of cell to repair biomolecule oxidation, oxidative damage occurs. During oxidative stress, the resulting cellular response is likely to involve both redox signalling and oxidative damages, whose contribution will depend on the concentration and nature of the ROS involved. When a redox imbalance occurs, a variety of cellular responses is activated, in order to minimize the potential damages and to re-establish the correct redox homeostasis.

The role of natural or physiological molecules in maintaining or altering the ROS balance inside the cells is, therefore, of extreme interest and intensively explored. Recent epidemiological studies demonstrated that increased consumption of cruciferous vegetables rich in organosulfur compounds is associated with reduced incidence of human cancer as well as atherosclerotic coronary heart diseases, inflammatory and oxidative stress-related disorders (Xue, Qian et al. 2008).

Sulforaphane (SF) [1-isothiocyanate-(4R)-(methylsulfinyl) butane] is one of the most abundant molecule found in these vegetables, and its chemopreventive role has been demonstrated in many cellular or animal models (Shapiro, Fahey et al. 2001). In particular, its mechanism seems to rely on the induction of phase II enzymes and antioxidants in cell lines and in animals (Fahey and Talalay 1999), as demonstrated also in cardiomyocytes by our group (Angeloni, Leoncini et al. 2009).

Cellular redox response mechanism is realized also by the transcription of a variety of antioxidant genes through sequences known as antioxidant response elements (ARE). The relationship between oxidative stress, activation of these ARE sequences and subsequent gene transcription is often mediated by specific transcription factors, such as the Nrf2 (nuclear factor erythroid 2-related factor 2), which plays a key role in a number of physiological and disease processes (Chen and Kong 2004). In response to oxidative stress, Nrf2 up-regulates phase II gene products aimed at detoxification, cytoprotection, and clearance of potential carcinogens as well as at the reduction of excessive ROS accumulation (Wilson, Kerns et al. 2013).

Among the many genes and gene products up-regulated by Nrf2 there are heme oxygenase 1 (HO-1), a protein having antioxidant, anti-inflammatory and antiapoptotic activity, or peroxiredoxin-1, (Prx-1), which is essential for eliminating H_2O_2 generated by a variety of cellular stresses and pro-inflammatory cytokines and has a protective role against oxidative stress-induced apoptosis.

In recent years, another protein, whose name is Klotho, has gained a great attention. Discovered in 1997, the Klotho gene has immediately attracted increasing interest, since its overexpression in small animals extends lifespan, whereas defective Klotho expression results in rapid aging and

early death. It seems that the same pathways might work in humans. The Klotho protein exists in two forms: membrane Klotho, which functions as a receptor, and secreted Klotho, which functions as a humoral factor with an enzymatic activity (Kuro-o 2011). Both the Klotho forms have regulatory effects on general metabolism.

Part of the experiments concerning the study of the role of natural and physiological molecules in the maintenance of redox balance was performed at the King's College London (Cardiovascular Division, School of Medicine) under the supervision of Dr. Richard Siow and Dr. Giovanni Mann.

Dr. Mann's team is interested in the role of antioxidant genes in vascular protection. Therefore, in his lab it has been studied the effect of a variety of molecules able to modulate the expression of antioxidant genes thus contributing to the protection of vascular dysfunction in atherosclerosis, diabetes and hypoxia condition. Among these natural molecules, the role of sulforaphane in mediating the pre-activation of Nrf2 and its downstream target HO-1 in the cerebral vasculature was studied by Dr. Mann's group (Alfieri, Srivastava et al. 2013).

For these reasons, the effects of sulforaphane (SF) or Klotho (Kl) on HO-1 and Prx-1 expression and GSH intracellular level were studied in cultured human aortic smooth muscle cells (HASMC) subjected to high glucose conditions mimicking hyperglycaemia. In fact, it has been shown that several pathologies such as dislipidaemia, hypertension, diabetes and metabolic syndrome can induce an increased oxidative stress, leading to cardiovascular disease or increased risk factors (Hopps, Noto et al. 2010).

4.3.1 Effect of sulforaphane on HO-1 and Prx-1 expression in HASMC

As far as SF toxicity is concerned, it has been shown that the consumption of broccoli and other cruciferous vegetables produces SF plasma concentrations ranging between 0.031-7.3 μM, reaching a maximum at 1.5-2h (Dinkova-Kostova & Kostov, 2012). These concentrations guarantee safety and efficacy of SF (Dinkova-Kostova & Kostov, 2012). For this reason the non toxic concentrations of 2.5 μM and 5 μM SF were chosen for the following experiments.

All the experiments were performed in HASMC maintained in three different conditions for 24 hours:

- "Normal Glucose, NG": 5 mM glucose in the colture medium DMEM;
- "High Glucose, HG": 25 mM glucose in the colture medium DMEM, to mimic an hyperglycaemia condition;
- "Mannitol, Man": 5 mM glucose + 20 mM mannitol in the colture medium DMEM, as osmotic positive control with respect to HG.

After this period, HASMC were treated with 2.5 µM or 5 µM SF for 12 or 24 hours. The whole HASMC lysates were then subjected to SDS-PAGE and Western blotting analysis, using anti-HO-1 and anti-Prx-1 antibodies and the results are shown in Fig. 15A. Fig. 15B shows that SF treatment causes a significant increase in HO-1 expression, which is strongly proportional to SF concentration. Also Prx-1 expression is augmented in HASMC following SF treatments, but in this case the increase is significant only with 5 µM SF and particularly in mannitol conditions (Fig. 15C).

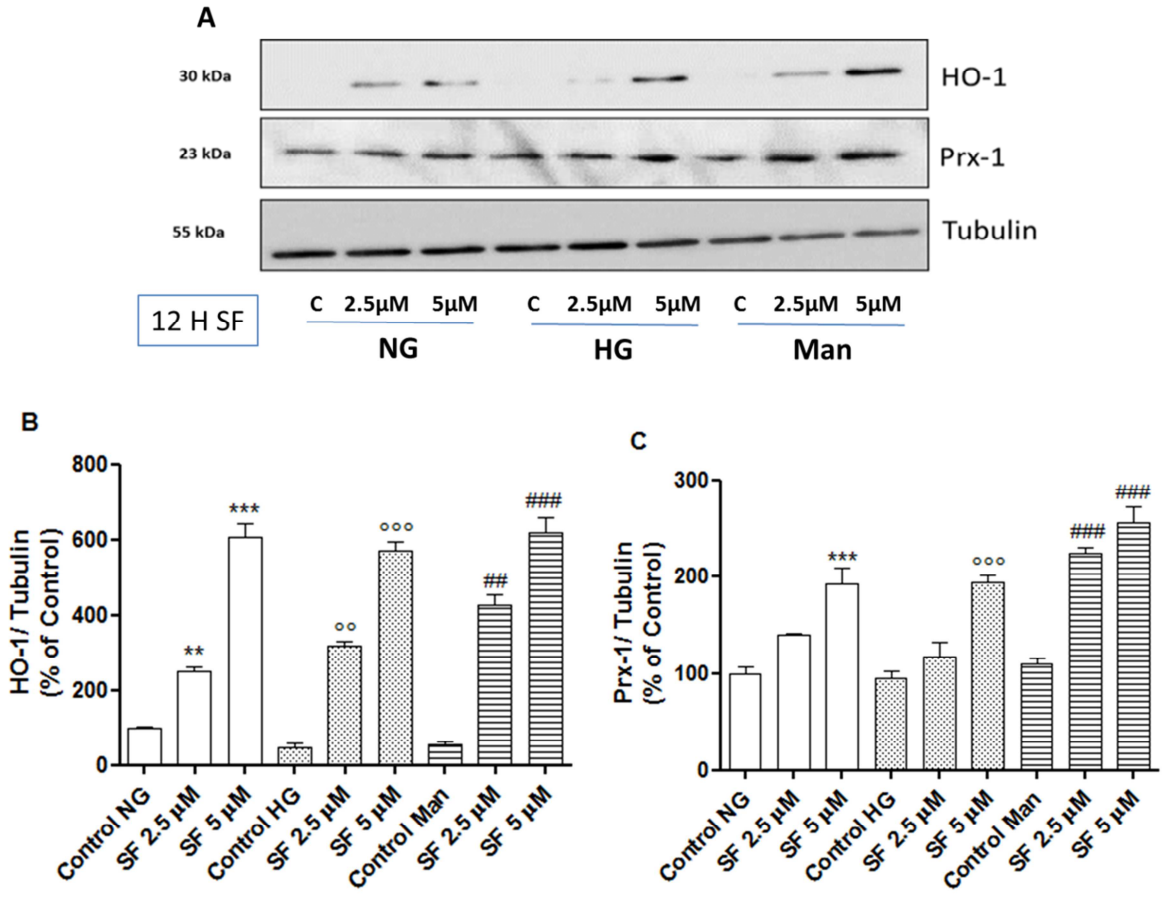


Fig. 22. Effect of 12 hours sulforaphane treatment on HO-1 and Prx-1 induction in HASMC maintained in Normal Glucose, High Glucose, or Mannitol conditions.

(A) Confluent HASMC were maintained in NG, HG and Man for 24 hours in DMEM, then treated with vehicle (Control; DMSO, 0.01% v/v) or 2.5 and 5 µM SF for 12 hours. Whole cell lysates were collected, electrophoresed, immunoblotted and revealed for anti-HO-1, anti-Prx-1 and anti-α-tubulin as control. A representative blot is shown. (B and C) Densitometric analysis of HO-1 and Prx-1 expression relative to α-tubulin in HASMC maintained in NG, HG and Man for 24 hours in DMEM, then treated with 2.5 and 5 µM SF for 12 hours. Data are expressed as means of six independent experiments and were analysed by one-way ANOVA followed by Bonferroni's test: $^{**}p < 0.05$ significantly different from control NG, $^{***}p < 0.001$ significantly different from control NG; $^{00}p < 0.01$ significantly different from control HG, $^{000}p < 0.001$ significantly different from control HG; $\#\#p < 0.01$ significantly different from control Man, $\#\#\#p < 0.001$ significantly different from control Man.

The same experiment was repeated treating HASMC with 2.5 µM or 5 µM SF for 24 hours and the results are shown in Fig. 16. From Figs. 16B and 16C it can be seen that after 24 hours the increased expression of both the proteins are less dependent on the SF concentration than data obtained after 12 hours, particularly in the case of Prx-1 (Fig. 16C).

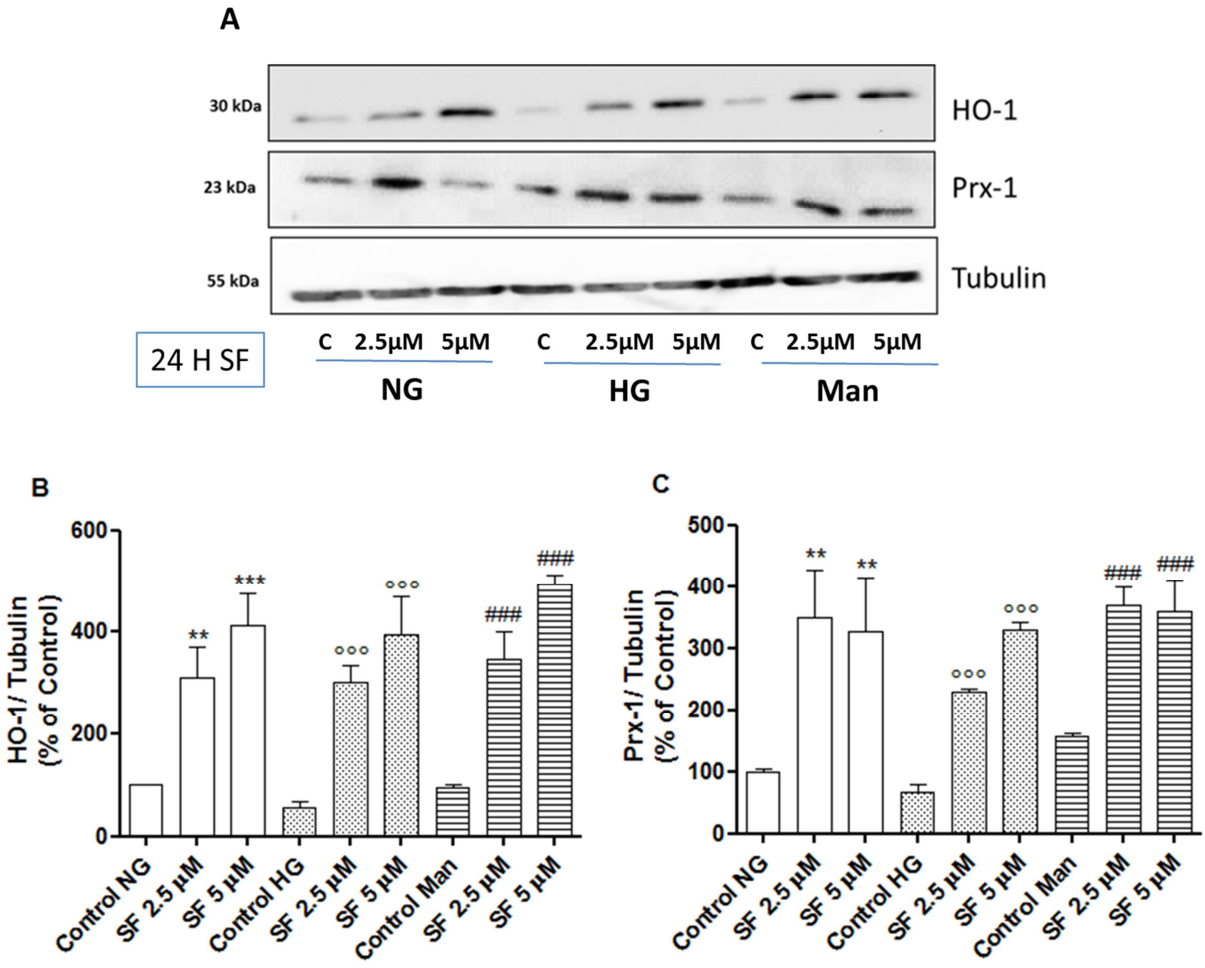


Fig. 23. Effect of 24 hours sulforaphane treatment on HO-1 and Prx-1 induction in HASMC maintained in Normal Glucose, High Glucose, or Mannitol conditions.

(A) Confluent HASMC were maintained in NG, HG and Man for 24 hours in DMEM, then treated with vehicle (Control; DMSO, 0.01% v/v) or 2.5 and 5 µM SF for 24 hours. Whole cell lysates were collected, electrophoresed, immunoblotted and revealed for anti-HO-1, anti-Prx-1 and anti-α-tubulin as control. A representative blot is shown. (B and C) Densitometric analysis of HO-1 and Prx-1 expression relative to α-tubulin in HASMC maintained in NG, HG and Man for 24 hours in DMEM, then treated with 2.5 and 5 µM SF for 24 hours. Data are expressed as means of three independent experiments and were analysed by one-way ANOVA followed by Bonferroni's test: $^{**}p < 0.01$ significantly different from control NG, $^{***}p < 0.001$ significantly different from control NG; $^{000}p < 0.001$ significantly different from control HG; $^{###}p < 0.001$ significantly different from control Man.

4.3.2 Effect of Klotho on HO-1 and Prx-1 expression in HASMC

Klotho is a molecule of recent discovering, so the effect of 0.1, 0.5, 1 nM Klotho Human Recombinant protein addition on HASMC viability and proliferation was analysed by using the xCell System iCELLigence Real-Time Cell Analyzer DP inside a cell culture incubator. This real-time cell system continuously monitors changes in impedance over time, represented arbitrarily as the cell index, as described in the Materials and Methods section. For proliferation assay, impedance was collected every 15 minutes up to 24 hours. No changes in vitality/ proliferation status of the cells were observed, as reported in Figs. 17A and 17B.

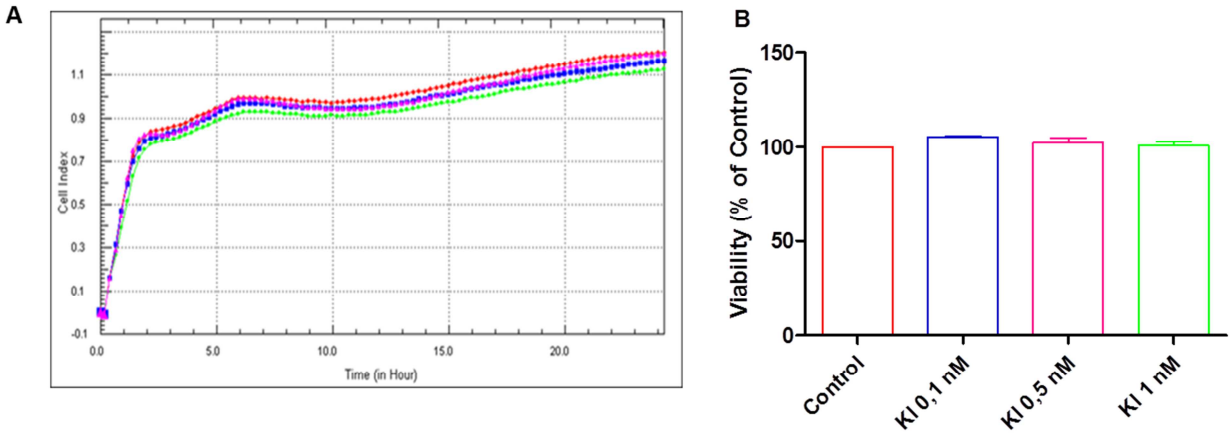


Fig. 24. Effect of different Klotho concentrations on HASMC viability/proliferation.

(A) HASM cells were treated for 24 hours with different concentrations of Kl. Red = control, Blue = 0.1 nM, Purple = 0.5 nM, Green = 1 nM. Viability/proliferation was evaluated by xCell System iCELLigence Real-Time Cell Analyzer DP as described in Materials and Methods. (B) Histogram representation of the results shown in Panel A. Data are expressed as % of control and represent means ± SD of three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's test: no significant differences were observed.

Confluent HASMC were maintained in the three above described conditions (NG, HG, Man) for 24 hours. After this period, HASMC were treated with 0.1, 0.5 or 1 nM Klotho for 24 hours. The whole HASMC lysates were then subjected to SDS-PAGE and Western blotting analysis, using anti-HO-1 and anti-Prx-1 antibodies and the results are shown in Fig. 18A.

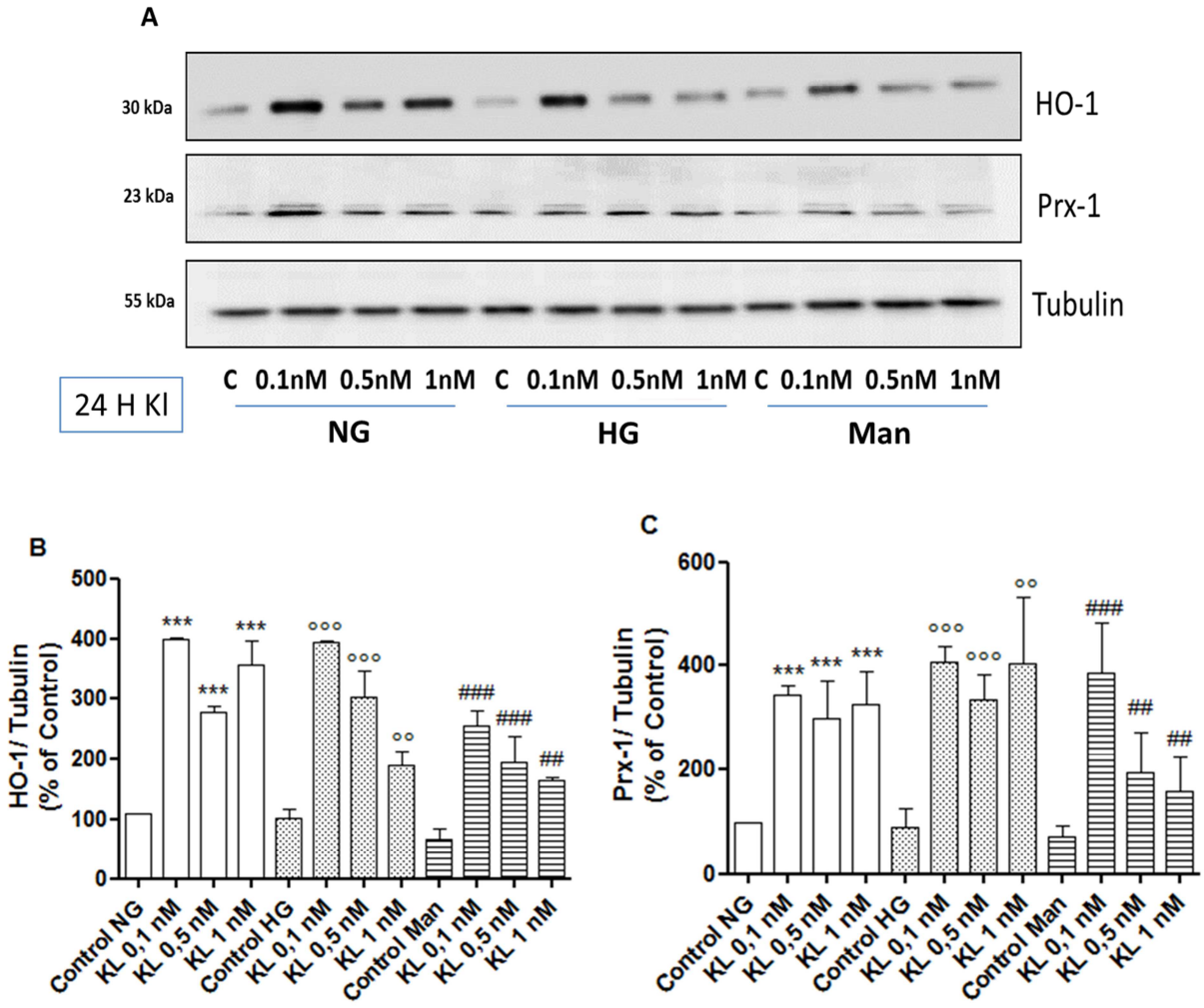


Fig. 25. Effect of 24 hours Klotho treatment on HO-1 and Prx-1 induction in HASMC maintained in Normal Glucose, High Glucose, or Mannitol conditions.

(A) Confluent HASMC were maintained in NG, HG and Man for 24 hours in DMEM, then treated with 0.1, 0.5, or 1 nM Kl for 24h. . Whole cell lysates were collected, electrophoresed, immunoblotted and revealed for anti-HO-1, anti-Prx-1 and anti-α-tubulin as control. A representative blot is shown. (B and C) Densitometric analysis of HO-1 and Prx-1 expression relative to α-tubulin in HASMC maintained in NG, HG and Man for 24 hours in DMEM, then treated with 0.1, 0.5, or 1 nM Kl for 24 hours. Data are expressed as means of three independent experiments and were analysed by one-way ANOVA followed by Bonferroni's test: ***p < 0.001 significantly different from control NG; ^{00}p < 0.01 significantly different from control HG, ^{000}p < 0.001 significantly different from control HG; ##p < 0.01 significantly different from control Man, ###p < 0.001 significantly different from control Man.

Data showed in Figs. 18B and 18C evidence that HASMC treated with exogenous Kl exhibit a strongly, significant increase in HO-1 and Prx-1 expression. Regarding to HO-1 (Fig. 18B), the highest increase is observed in NG and HG conditions, particularly corresponding to the lowest Kl concentration used. A similar trend is observed also in the case of Prx-1 expression (Fig. 18C). It has to be pointed out that the most active 0.1 nM Kl concentration is closer to the physiological

concentrations of soluble Klotho levels (Golembiewska, Safranow et al. 2013), confirming that Kl takes part on the natural antioxidant defences.

4.3.3 Effect of High Glucose condition on GSH intracellular levels in HASMC

Glutathione (GSH) is ubiquitously expressed in all cell types and it represents the major non-enzymatic regulator of intracellular redox homeostasis (Masella, Di Benedetto et al. 2005). Changes in endogenous antioxidants such as GSH and antioxidant enzymes may reflect a physiological consequence provoked by oxidative stress. It has been shown that long-term exposure of cultured mouse endothelial cells to high glucose (28 mM) resulted in impaired GSH synthesis and decreased GSH levels (Kline, Bassit et al. 2009).

Therefore, the GSH intracellular level of HASMC maintained in High Glucose condition was compared to that measured in the same cells maintained in Normal Glucose and Mannitol conditions. After 48 hours growth, the cells were maintained in High Glucose conditions for 6, 12, 24 or 48 hours and in NG or Mannitol conditions for 48 hours, then GSH intracellular level was determined by using OPA fluorescence as described in Materials and Methods section. The results reported in Fig. 19 show that High Glucose condition provoked a significant decrease in the intracellular GSH level after 48 hours.

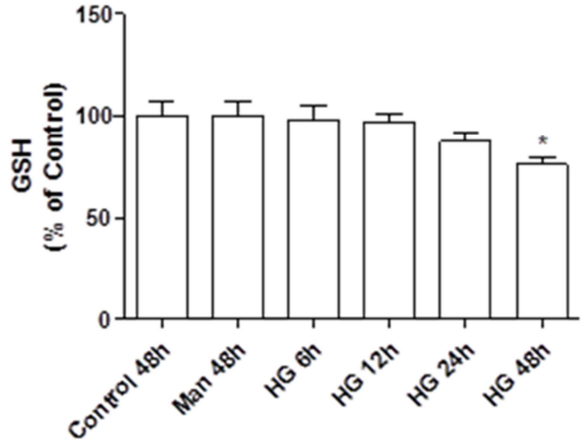


Fig. 26. Effect of different times of exposure to High Glucose condition on intracellular GSH levels in HASMC.
Confluent HASMC were maintained in HG for 6, 12, 24 or 48 hours, and in NG or Man for 48 hours. Intracellular GSH levels were determined employing *O*-phthaldialdehyde (OPA) fluorescence as described in Materials and Methods. Data are expressed as 100% Control, whose actual GSH concentration is 40-50 nmol/ mg of protein.. Data are expressed as means ± S.E.M of three independent experiments and were analysed by one-way ANOVA followed by Bonferroni's test: $*p < 0.05$ significantly different from control NG.

4.3.4 Effect of sulforaphane on GSH intracellular level in HASMC

Since it has been reported that SF enhances the total GSH levels in SH-SY5Y cells (Tarozzi, Morroni et al. 2009), it was investigated whether this molecule could be able to restore the depleted GSH level observed in HAMSC maintained in HG condition for 48 hours.

To this purpose, HASMC maintained in NG, HG or Man conditions for 24 hours in DMEM, were treated with 2.5 µM or 5 µM SF for 12 or 24 hours in the same three conditions, then the intracellular GSH levels were measured. As it can be seen in Figs. 20 A and 20 B, SF treatment provoked a significant increase in HASMC intracellular GSH content in all the condition tested, both at 12 and 24 hours. In particular, as reported in Fig. 20B, SF was able to completely restore the significant depletion of GSH levels observed after 48 hours exposition to HG condition.

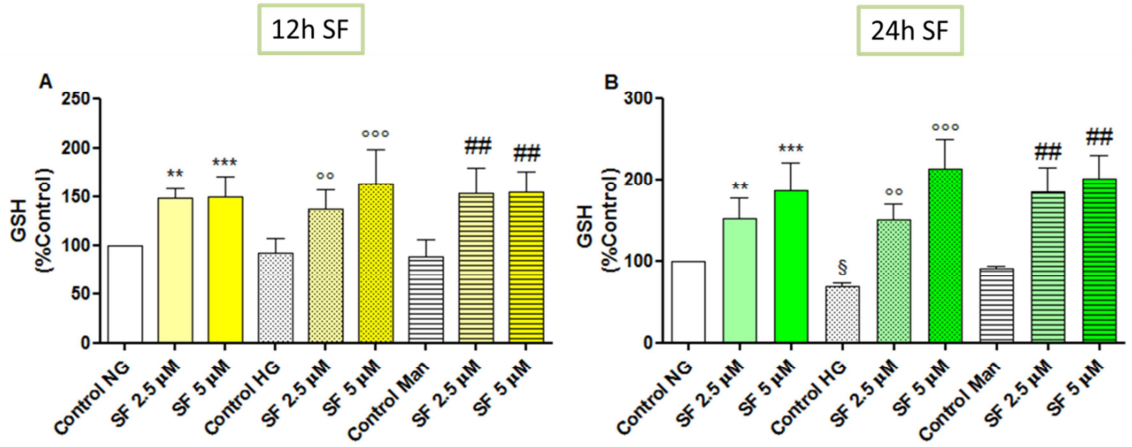


Fig. 27. Effect of different times of exposure to sulforaphane on intracellular GSH levels in HASMC.

Confluent HASMC were maintained in NG, HG or Man conditions for 24 hours in DMEM, then treated with 2.5 or 5µM SF for (A) 12 hours, (B) 24 hours. Intracellular GSH levels were determined employing *O*-phthaldialdehyde (OPA) fluorescence as described in Materials and Methods. Data are expressed as means ± S.E.M of three independent experiments and were analysed by one-way ANOVA followed by Bonferroni's test: ***p* < 0.01 significantly different from control NG, ****p* < 0.001 significantly different from control NG, °°*p* < 0.01 significantly different from control HG, °°°*p* < 0.001 significantly different from control HG, ##*p* < 0.01 significantly different from control Man, ###*p* < 0.001 significantly different from control Man; § *p* < 0.05 significantly different from control NG .

4.3.5 Effect of Klotho on GSH intracellular level in HASMC

GSH disorder plays a role in many pathological disease states such as ageing, diabetes and several chronic liver diseases. In aged mice, GSH levels are decreased (Wang, Liu et al. 2003). Since Klotho protein has been correlated to aging processes, it might be a relationship between the decrease of GSH levels and Klotho function.

To explore this possibility in the cellular model under investigation, HASM cells maintained in NG, HG or Man conditions for 24 hours in DMEM, were treated with 0.1, 0.5, or 1 nM Klotho for 8 or

24 hours in the same three conditions, then assayed for the intracellular GSH levels. Results in Fig. 21A and 21B show that, when HASMC are treated with Klotho, a significant increase in GSH intracellular levels occurs in all the tested conditions, with a little major extent corresponding to 24 hours Kl exposure (Fig. 21B).

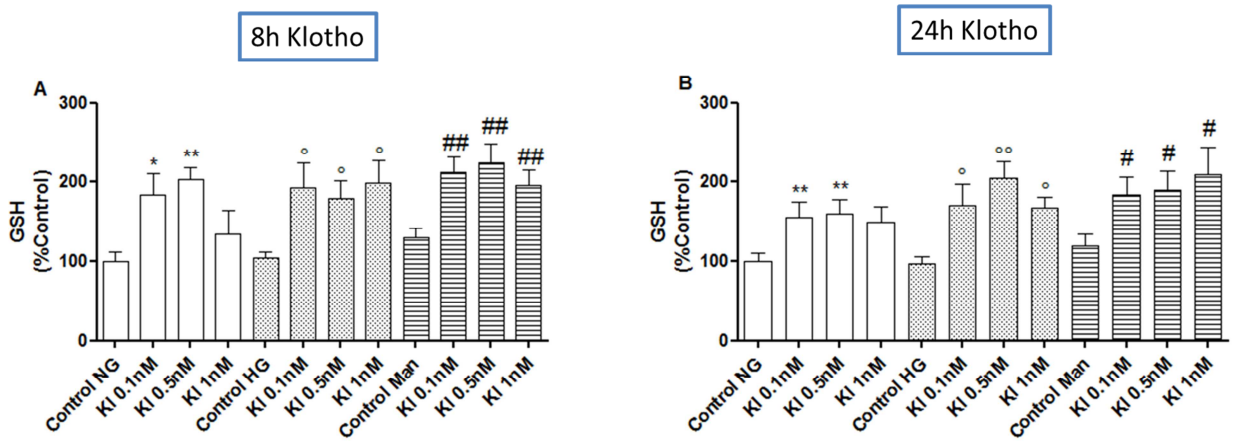


Fig. 28. Effect of different times of exposure to Klotho on intracellular GSH levels in HASMC.

Confluent HASMC were maintained in NG, HG or Man conditions for 24 hours in DMEM, then treated with 0.1, 0.5 or 1nM Kl for (A) 8 hours, (B) 24 hours. Intracellular GSH levels were determined employing *O*-phthaldialdehyde (OPA) fluorescence as described in Materials and Methods. Data are expressed as means ± S.E.M of three independent experiments and were analysed by one-way ANOVA followed by Bonferroni's test: *$p < 0.05$ significantly different from control NG, **$p < 0.01$ significantly different from control NG, °$p < 0.05$ significantly different from control HG, °°$p < 0.01$ significantly different from control HG, #$p < 0.05$ significantly different from control Man, ##$p < 0.01$ significantly different from control Man.

Also these results confirm that Klotho is involved in the physiological antioxidant defences, suggesting that a part of its antioxidant effect could proceed through the modulation of GSH intracellular levels.

4.4 Study of the antioxidant and anti-hyperglycaemic properties of a natural molecule

Topics that have been carried out at the King's College London and those in which the italian research group is interested, gave rise to the third problem studied, concerning the antioxidant and anti-hyperglycaemic role of steviol glycosides extracted from the perennial shrub *Stevia rebaudiana* Bertoni. Indeed, it was demonstrated that metabolic syndrome and obesity, which often include diabetes, are characterized by an altered oxidative/antioxidant status. In particular, hyperglycemia and inflammation, important components of these syndromes, increase the production of ROS, resulting in an increased oxidative stress with over-activation of NADPH oxidases (Hopps, Noto et al. 2010).

The plant *Stevia rebaudiana* Bertoni has been used by the native people of Paraguay and Brazil to treat diabetes, moreover, the plant's leaves give out a distinctly sweet taste, containing no calories, and are rich in metabolites such as β-carotene, vitamins, terpenes and flavonoids, which give the plant its therapeutic advantages (Mohd-Radzman, Ismail et al.). The zero-calorie properties are very beneficial to patients suffering from obesity and diabetes, as it will not elevate their blood glucose levels, furthermore, a number of animal studies have demonstrated that, in diabetic rats, steviosides exert anti-hyperglycemic and insulinotropic actions (Jeppesen, Gregersen et al. 2002).

Rebaudioside A and stevioside (the two main steviol glycosides found in this plant) are the two predominant derivatives used in high potency sweeteners alternative to sucrose, thus the antioxidant activity of Rebaudioside A and other commercial *Stevia* extracts was investigated. Furthermore, as above stated, the research group has been studied for a long time the role of glucose transport activity in leukaemia cell proliferation, thus, taking advantage from this expertise, the potential insulin-like properties of these steviosides were also studied.

The study was carried out in the neuroblastoma SH-SY5Y and the promyelocytic leukaemia HL-60 cell lines, since they both express the Glut1 isoform of glucose transporter, which is responsible for the basal glucose uptake, and Glut4, responsible for insulin-stimulated glucose uptake in peripheral tissues (Lord, Bunce et al. 1988; van der Heide, Ramakers et al. 2006). Furthermore, these cells express also the insulin-like growth factor-1 receptors (IGF-1) (Saltiel and Kahn 2001), thereby being susceptible to insulin action.

4.4.1 Effect of different Stevia extracts on cell viability and caspase-3 activity in SH-SY5Y and HL-60 cell lines

Four different *Stevia* extracts, differing by the relative content of Rebaudioside A and stevioside, were used for the following experiments:

R97: contains 97-98% Rebaudioside A

R60: contains about 60% Rebaudioside A and about 20% stevioside

SG: contains 50% Rebaudioside A and at least 25% stevioside

TRU: contains a mixture of steviol glycoside not analytically quantified.

First of all, the effect of the four *Stevia* extracts on cell viability was evaluated by MTT assay, as reported in Materials and Methods section. SH-SY5Y and HL-60 cells were treated with different concentrations of *Stevia* extracts (0.5-5 mg/mL, corresponding to 0.5-5 mM R97, which can be assumed as a pure compound) for 24 hours. Figs. 22A and 22B show that the extracts did not affect cell viability/proliferation, confirming that they are not cytotoxic within the concentration range tested.

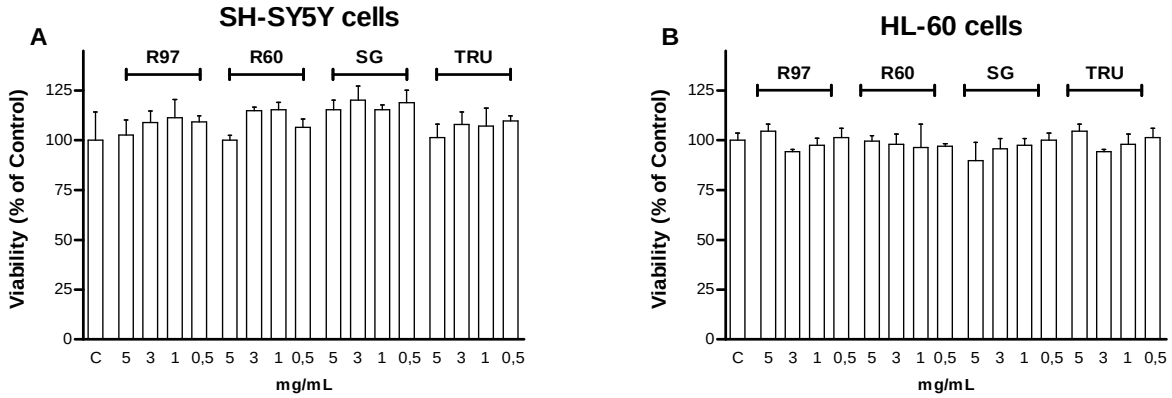


Fig. 29. Effect of different *Stevia* extracts on SH-SY5Y and HL-60 cell viability/proliferation.

SH-SY5Y (panel A) and HL-60 (panel B) cells were treated for 24 hours with different concentrations of the four extracts (0.1 mg/mL to 5 mg/mL). Viability/proliferation was evaluated by MTT assay as described in Materials and Methods, and compared to control (C). Results are expressed as means ± SD of three independent experiments (n=8). Statistical analysis was performed by Bonferroni's multiple comparison test following one-way ANOVA. Significant differences were not revealed.

Cytotoxicity was also evaluated by measuring the lactate dehydrogenase (LDH) activity, as reported in Materials and Methods section, which is an index of cell membrane integrity. As it can be seen in Figs. 23A and 23B, after 24 hours treatment with the different *Stevia* extracts, the LDH activity did not change in both cell lines. LDH activity measured in 100 µM H_2O_2 treated cells for 30 min was shown as positive control.

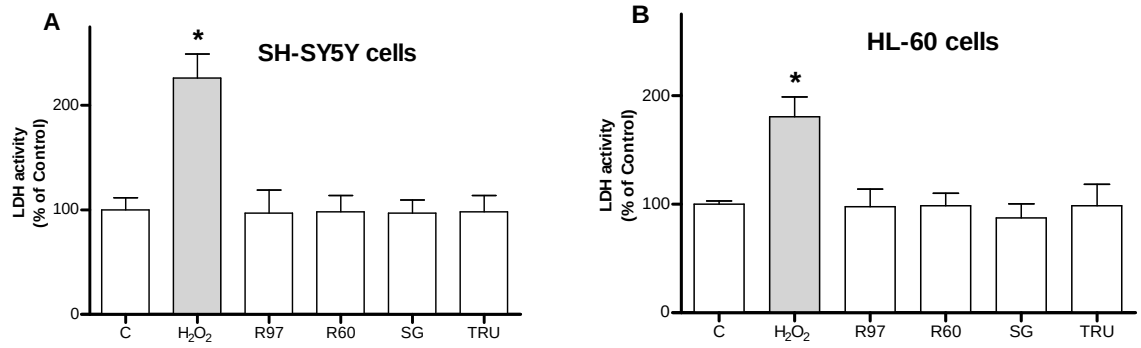


Fig. 30. Effect of different *Stevia* extracts treatment on lactate dehydrogenase (LDH) activity in SH-SY5Y and HL-60 cells.

LDH activity was measured as described in Materials and Methods. SH-SY5Y (panel A) and HL-60 (panel B) cells were treated with the four different extracts at 1 mg/mL final concentration for 24 hours, or cells were treated with 100 µM H_2O_2 for 30 min as control of LDH activity. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. Statistical analysis was performed by Bonferroni's multiple comparison test following one-way ANOVA. *$p < 0.05$, significantly different from control cells.

In order to evaluate a possible effect on apoptosis, the activity of caspase 3 was measured, as reported in Materials and Methods section. Caspases are a family of cysteine proteases with aspartate specificity, playing a central role in various apoptotic responses, where they are activated in a sequential cascade of cleavages (Alnemri, Livingston et al. 1996). To detect the enzymatic activity of caspase 3, the fluorogenic substrate Ac-DEVD-AMC was used, and staurosporine, a well known apoptosis inducer, was employed as positive control. Treatments of cells with *Stevia* extracts for 1 or 6 hours (data not shown) or for 24 hours (Figs. 24A and 24B) did not influence caspase 3 activity, indicating that the steviol glycosides tested did not induce programmed cell death.

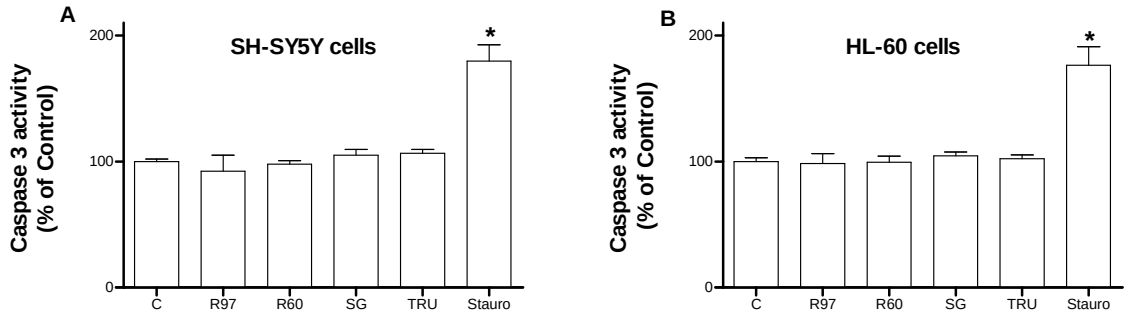


Fig. 31. Effect of different *Stevia* extracts treatment on Caspase 3 activity in SH-SY5Y and HL-60 cells.

Caspase 3 activity in SH-SY5Y (panel A) and HL-60 (panel B) cells was evaluated in the presence of 1 mg/mL steviol glycosides incubated for 24 hours. Staurosporine (Stauro, 1 µg/mL for 4 hours) was used as positive control. Caspase 3 activity was measured spectrofluorimetrically after 24 hours in cell lysates as reported in Materials and Methods. Each

column represents the mean ± SD of three independent experiments. Statistical analysis was performed by Bonferroni's multiple comparison test following one-way ANOVA. *$p < 0.05$, significantly different from control cells.

4.4.2 Effect of different Stevia extracts on ROS intracellular level in SH-SY5Y and HL-60 cell lines

The antioxidant activity of the four commercial *Stevia* extracts was investigated in SH-SY5Y and HL-60 cells by means of DCF fluorescence, as reported in Materials and Methods section. As shown in Fig. 25, the compounds did not exhibit any antioxidant activity, since they were neither able to decrease basal ROS level at the highest concentration used (Figs. 25A and 25B) nor to counteract intracellular ROS raise due to exogenous oxidative stress (100 μM H_2O_2 for 30 min) (Figs. 25C and 25D). This lack of antioxidant activity is in contrast with the data reported by other Authors (Shivanna, Naika et al. 2013; Tavarini and Angelini 2013), probably because the compounds used in the present study are commercial sweeteners containing 95-98% steviol glycosides with no appraisable amounts of polyphenols, naturally present in *Stevia* leaves, likely responsible for *Stevia* antioxidant activity.

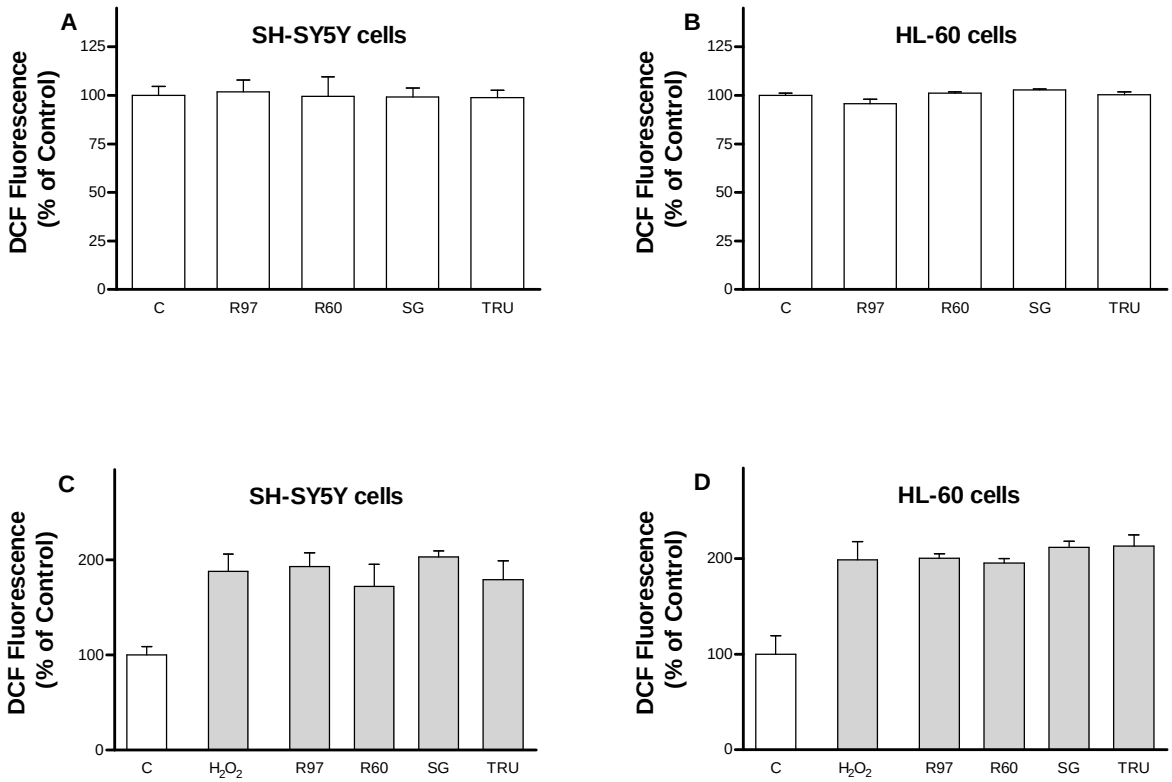


Fig. 32. Effect of different *Stevia* extracts treatment on intracellular ROS levels in SH-SY5Y and HL-60 cells.
SH-SY5Y (panel A) and HL-60 (panel B) cells were treated for 1 hour with different *Stevia* extracts (5 mg/mL), then basal ROS levels were measured by means of H_2DCFDA assay as described in Materials and Methods. Results are

expressed as means ± SD of four independent experiments (n=8). Statistical analysis was performed by Bonferroni's multiple comparison test following one-way ANOVA. Significant differences were not revealed. SH-SY5Y (panel C) and HL-60 (panel D) cells were pre-incubated for 1 hour with different *Stevia* extracts (5 mg/mL) then exposed to oxidative stress generated by 100 μM H_2O_2 for 30 min. ROS levels were measured by means of H_2DCFDA assay as described in Materials and Methods. Results are expressed as means ± SD of four independent experiments (n=8). Statistical analysis was performed by Bonferroni's multiple comparison test following one-way ANOVA. Significant differences were not revealed among H_2O_2 treated cells.

4.4.3 Effect of different Stevia extracts on glucose transport activity in SH-SY5Y and HL-60 cell lines

The effect of the four different *Stevia* extracts on glucose transport activity was evaluated in HL-60 and in SH-SY5Y cell lines, and compared to the effect of 100 nM insulin, a concentration value often reported in the literature (Udelhoven, Leeser et al. 2010). Figs. 26A and 26B show that all the extracts were able to enhance glucose uptake at similar extent after 1-hour incubation in both cellular lines.. More interestingly, results reveal that *Stevia* extracts and insulin behave similarly, being *Stevia* extracts as efficient as insulin in increasing glucose uptake. Moreover, the co-treatment with insulin and *Stevia* extracts causes a rise of glucose transport significantly higher than the increase due to insulin alone.

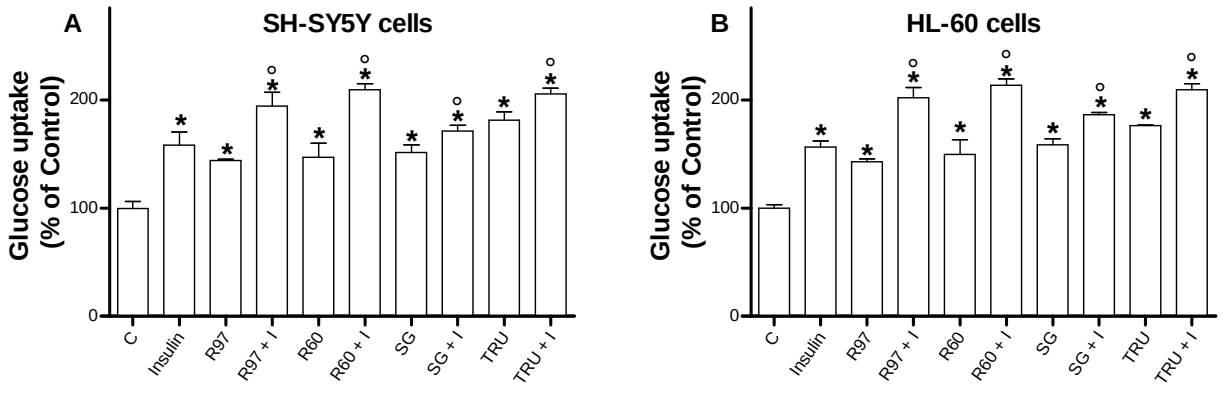


Fig. 33. Effects of different *Stevia* extracts and insulin treatment on glucose transport activity in SH-SY5Y and HL-60 cells.

SH-SY5Y (panel A) and HL-60 (panel B) cells were treated with steviol glycosides (1 mg/mL) or with 100 nM insulin (I) or with steviol glycosides and insulin simultaneously. Glucose uptake was assayed as described in the Materials and Methods. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. Statistical analysis was performed by Bonferroni multiple comparison test following one-way ANOVA. *$p<0.05$, significantly different from control cells; °$p<0.05$ significantly different from the corresponding cells not stimulated with insulin.

It is well known that insulin induces the translocation of Glut4 from cytosolic storage vesicles to the plasma membrane, thereby enhancing the glucose transport activity. The same behaviour has been described for Glut1 in acute leukaemia cell lines in response to different stimuli (Maraldi, Fiorentini et al. 2004; Maraldi, Prata et al. 2007). Interestingly, it has been recently shown for the first time in a neuronal system that in SH-SY5Y cells both Glut1 and Glut4 are translocated to the plasma membrane in response to insulin through a phosphatidylinositol 3-kinase (PI3K)–dependent mechanism similar to that described in muscle or adipose tissues (Benomar, Naour et al. 2006).

Therefore, the expression of Glut1 and Glut4 in neuroblastoma and leukaemia cells following treatment with insulin or *Stevia* extracts was assessed by Western blot analysis on cell lysates (as reported in Materials and Methods section). Figs. 27A and 27B report representative immunoblots for Glut isoforms and the densitometric analysis (Figs. 27C and 27D) in both cell lines. It can be seen that the increase in Glut1 and Glut4 content obtained following exposure to *Stevia* extracts is similar to that obtained by insulin stimulation. These results are in accordance with those observed in the evaluation of glucose transport activity.

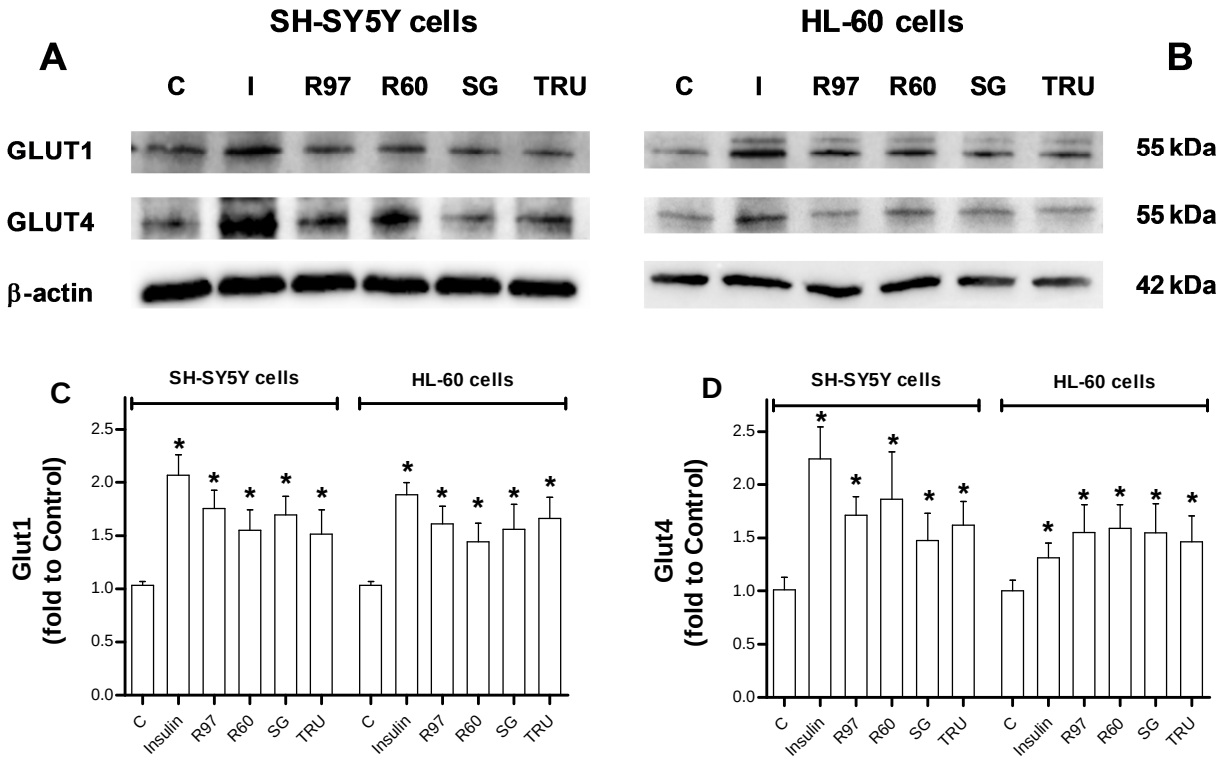


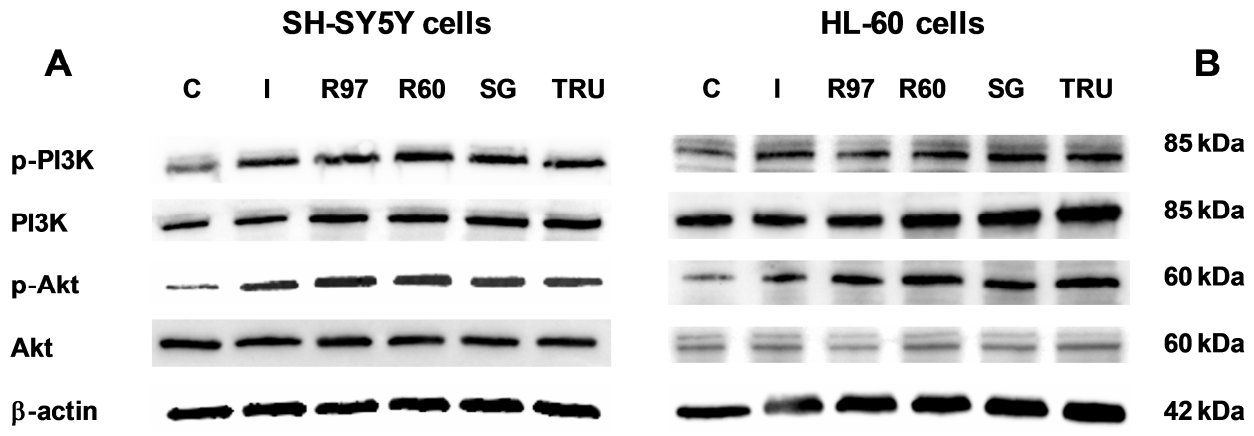
Fig. 34. Effects of different *Stevia* extracts and insulin treatment on Glut1 and Glut4 content in SH-SY5Y and HL-60 cells.

SH-SY5Y and HL-60 cells, treated with insulin or with steviol glycosides, were lysed with CelLytic M as described in Materials and Methods. Cell lysates were electrophoresed and immunoblotted with anti-Glut1 and anti-Glut4 antibodies, as described in Materials and Methods. β-Actin detection was used as control. Immunoblots representative of three independent experiments are reported for SH-SY5Y (A) and HL-60 (B) cell lines. Densitometric analysis

(normalized for β-actin content and expressed as fold of control) is shown for Glut1 (C) and Glut4 (D). Statistical analysis was performed by Bonferroni's multiple comparison test following one-way ANOVA. *$p < 0.05$, significantly different from control cells.

4.4.4 Effect of different Stevia extracts and insulin on PI3K pathway in SH-SY5Y and HL-60 cell lines

It is well known that insulin, through its receptor stimulation, provokes the phosphorylation of tyrosine receptor kinases and the activation of a signal transduction pathway involving PI3K and Akt (Hers 2007). Since the results thus far obtained show that the *Stevia* extracts mimic the insulin action, the possible involvement of the PI3K signaling pathway in the steviol glycoside action was investigated. Then the phosphorylation status of PI3K and Akt was evaluated following *Stevia* extract treatment and insulin stimulation of SH-SY5Y and HL-60 cell lines. Immunoblotting results reported in Figs. 28A and 28B and densitometric analysis reported in Figs. 28C and 28D show that, upon the treatment with insulin or *Stevia* extracts, the phosphorylated forms of both PI3K and Akt were significantly increased, indicating a possible similar mechanism of action or, at least, a common signalling pathway between these two stimuli.



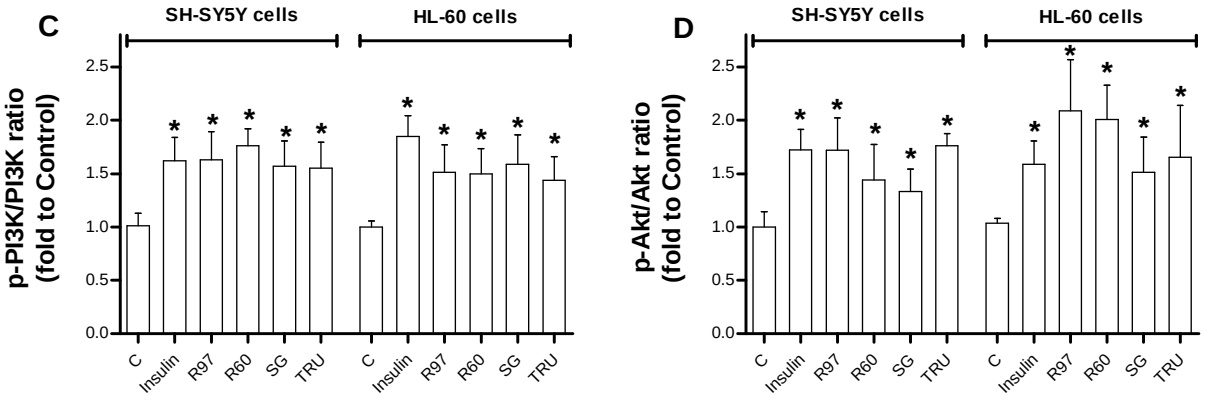


Fig. 35. Effects of different *Stevia* extracts and insulin treatment on PI3K/Akt pathway.

SH-SY5Y and HL-60 cells, treated with insulin or with steviol glycosides, were lysed with CelLytic M as described in Materials and Methods. Cell lysates were electrophoresed and immunoblotted with anti-PI3K, anti-Akt and anti-phospho-PI3k and anti-phospho-Akt, as described in Materials and Methods. β-Actin detection was used as control. Immunoblots representative of three independent experiments are reported for SH-SY5Y (A) and HL-60 (B) cell lines. Densitometric analysis of PI3K phosphorylation status is expressed as phospho-PI3K/total PI3K ratio (C) and expressed as fold of control. Densitometric analysis of Akt phosphorylation status is expressed as phospho-Akt/total Akt ratio (D) and expressed as fold of control. *$p < 0.05$, significantly different from control cells.

Chapter 5- Discussion

5 Discussion

The three major problems investigated and presented in this Thesis deal with the great universe of "ROS management". A fine balance between reactive oxidizing species generation and antioxidant levels allows either the cross talk between cells or the control of fundamental intracellular functions. Moreover, the generation of these double-edged molecules can lead to the activation of survival mechanisms or trigger the cell death. This scenario is complicated by the observation that different cell types seem to display different redox sensitivities, different signalling pathways and different defence mechanisms. Beside the role of ROS in normal physiological signalling, growing evidence implicates alterations in redox signalling or excessive ROS production as contributors to many disease processes. Cancer, insulin resistance and subsequent diabetes mellitus, aging and age-related regenerative capacity, neurodegenerative diseases and inflammation are correlated with intracellular redox state. In this context a critical issue is the important role played by antioxidants in human diet or as therapeutic agents, sometime considered "beneficial", sometime "deleterious". As stated in the "Aim of the Thesis", the first major problem investigated concerns the role of ROS in biosignalling.

After the discovery that NADPH oxidases are not only expressed in phagocytic cells, but also in a variety of cellular types, it has been demonstrated that these enzymes are the major source of intracellular ROS. NADPH oxidases are now recognized to have specific subcellular localization, required for localized ROS production and activation of specific redox signalling pathways (Ushio-Fukai 2009).

One of the surprises in redox biology was the relatively recent appreciation of hydrogen peroxide (H_2O_2) as a messenger molecule. H_2O_2 is considered a second messenger in different pathways, among them several growth factor-induced signalling cascades and insulin signalling (Sies 2014), which was probably the first transduction chain in which H_2O_2 resulted as a second messenger (May and de Haen 1979), inducing tyrosine phosphorylation and acting as a growth factor (Ushio-Fukai, Tang et al. 2002). To act as a signal molecule, H_2O_2 has to be spatially and temporally produced and this is the case of H_2O_2 originated by the regulated NADPH oxidase enzyme, which produces superoxide anion outside the cell. Owing to the spontaneous dismutation or to the action of SOD, superoxide anions give rise to H_2O_2. After its production, H_2O_2 is not free to move across the membrane, in agreement with its physicochemical properties: H_2O_2 has, in fact, a slightly larger polarity than water, making its non-facilitated diffusion through the hydrophobic lipid bilayer less rapid than that of water (Bienert and Chaumont 2014).

It was experimentally demonstrated that specific AQPs facilitate H_2O_2 diffusion, crossing this uncharged molecule with the same speed of water and more rapidly than by simple diffusion (Bienert and Chaumont 2014). This observation has been confirmed by using specific AQPs inhibitor, as silver or gold nitrate, which provoked a decrease of H_2O_2 movement into the cell (Niemietz and Tyerman 2002).

In the first set of experiments here reported, we demonstrated that, also in acute leukaemia-derived B1647 cell line, specific AQP isoforms constitute a possible way through which Nox-derived H_2O_2 can enter the cells to act as a signal molecule. This observation has been confirmed by the inhibition of AQP function obtained either by $AgNO_3$ or AQP silencing, which provoked similar results: the decrease of H_2O_2 transport when this peroxide is exogenously added or endogenously produced by Nox enzymes, as revealed by the evaluation of ROS intracellular levels.

Among the many AQP isoforms, AQP1, AQP3 and AQP8 were reported to channel H_2O_2 through the plasma membrane (Bienert and Chaumont 2014). B1647 cell line expresses, at least, AQP3 and AQP8. Silencing or overexpression of AQP3 and AQP8 demonstrated a pivotal role played by AQP8 in facilitating H_2O_2 diffusion across plasma membrane in B1647 cells.

The combined effect of an AQP inhibitor, $AgNO_3$, and of a Nox-inhibitor, DPI, allows to evaluate the correlation between Nox-generated ROS and AQP activity. The lack of an additive effect on intracellular ROS level exerted by these two inhibitors suggests that they act by preventing Nox-produced H_2O_2 to enter the cell in two different ways, *i.e.* either by blocking H_2O_2 production or transport.

It has been previously observed that H_2O_2 mimics the growth factor stimulation of glucose uptake in leukaemia cell lines, suggesting a key role for ROS in the signalling leading to Glut1 regulation (Maraldi, Fiorentini et al. 2007). Furthermore, in B1647 cells, constitutively expressing both VEGF and its receptor VEGFR-2, VEGF is supposed to utilize ROS as messenger intermediates downstream of the VEGFR-2, and glucose uptake is a cellular activity strictly bound to VEGF-induced cell proliferation. The measure of glucose transport activity obtained in AQP- and Nox-inhibition conditions allows to demonstrate that H_2O_2, produced by Nox and channelled *via* AQP through the plasma membrane, is indeed able to act as a signal molecule in B1647 cells, being responsible for the signal leading to Glut1 activity. Moreover, data here reported demonstrated that when AQP8 is overexpressed in B16478 cells, the ability of VEGF to trigger an increase of H_2O_2 intracellular level is enhanced; on the contrary, when AQP8 isoform was silenced, a decrease in intracellular ROS level triggered by VEGF was observed. These data confirm the importance of AQP8 basal expression in modulating VEGF-induced ROS generation.

There is now a consensus that VEGF family is crucial for vascular development and neovascularization in both physiological and pathologic processes. VEGF and VEGFR receptors, indeed, are not exclusively expressed by endothelial cells, but are also present in hematopoietic cell. This is not surprising, since during embryonic development hematopoietic and early endothelial cells originate from a common precursor known as hemangioblast, thus several pathways are shared by hematopoietic and vascular cells (Choi 1998). In recent studies, the expression of VEGF/VEGFR in acute myeloid leukaemia patients has been detected and the increased levels of plasma VEGF have been correlated with reduced survival and low remission rates (Aguayo, Kantarjian et al. 2002).

The role of caveolae and lipid rafts in the regulation of signal transduction and protein trafficking is now well established. Rafts may contain incomplete signalling pathways that are activated when a receptor or other required molecules are recruited into them, or, on the contrary, they can limit signalling by physical sequestration of signalling components, which become in their active form when are released from rafts (Pike 2003; Parton and del Pozo 2013). As above mentioned, this concept is peculiar for redox signalling: in order to act as signal molecules, ROS must be generated in discrete compartments and in a controlled manner.

Regarding this point, recent experimental evidence shows that, in endothelial cells, VEGF promotes the release of VEGFR-2 from caveolae/lipid rafts and its consequent activation, possibly stimulating NADPH oxidases (Ushio-Fukai 2007). Moreover, in endothelial cells, VEGFR-2 is present in caveolae through association with Caveolin-1 (Cav-1), which negatively regulates the receptor activity in basal state. Dissociation of VEGFR-2 from caveolae/Cav-1 seems to be essential for VEGFR-2 autophosphorylation and activation of downstream signalling events (Labrecque, Royal et al. 2003).

To investigate the potential involvement of plasma membrane caveolae/lipid rafts in VEGF-mediated redox signalling in B1647 cells, the experimental model employed cells cultured either in the presence or absence of serum. In fact, this cell type is able to auto-produce VEGF, and serum starved conditions allow to evaluate the cellular basal state, ruling out other growth factor effects.

The results obtained in the second set of experiments here reported, show that when B1647 cells are serum starved, VEGFR-2 is present in fractions corresponding to raft and non-raft regions, whereas when cells are cultured in the presence of serum, VEGFR-2 content partially shifts towards non-raft fractions. This behaviour is consistent with higher or lower VEGF concentrations: serum starved cells have a lower VEGF concentration, thus VEGFR-2 is partially sequestered into caveolae/lipid raft regions, where it is probably linked to Cav-1; on the contrary, in serum added cells, the VEGF

concentration is higher, thus causing the breakage of the link Cav-1/VEGFR-2 and the consequent displacement of a major portion of VEGFR-2 from caveolae/lipid raft to non-raft regions.

The methyl-β-cyclodextrin-induced disruption of caveolae/lipid rafts allows more interaction between VEGF and its receptor VEGFR-2, causing the shift towards non-raft regions in both serum-starved and serum added conditions. This facilitated formation of the VEGF/VEGFR-2 complex causes VEGFR-2 phosphorylation and the consequent activation of downstream signalling pathway, leading to the increase of intracellular ROS level and glucose transport activity.

Moreover, CD treatment increases glucose uptake through Glut1 recruitment into the plasma membrane from intracellular pool, in accordance with evidence reporting that Glut1 transporters are localized in part in caveolae/lipid rafts in many cell types (Sakyo and Kitagawa 2002) and that cholesterol depletion triggers its translocation from cellular stores toward the cell surface in leukaemia cells (Caliceti, Zambonin et al. 2012).

In agreement with data obtained in endothelial cells (Labrecque, Royal et al. 2003), it has been demonstrated that, also in B1647 cells, VEGFR-2, in the absence of serum, is more linked to Cav-1; instead, in serum added cells, VEGFR-2 binding to Cav-1 significantly decreases promoting its activation, as demonstrated by the higher level of phosphorylation observed.

It is interesting to consider the role of the protein Cav-1, which has been reported to influence both positively and negatively various aspects of tumour progression and to act as tumour suppressor or poor prognostic factor in many human cancers (Goetz, Lajoie et al. 2008). The Cav-1 scaffolding domain (CSD) mimics the action of Cav-1 protein, representing the portion of the protein essential for both Cav-1 oligomerization and the interaction with other proteins (Couet, Li et al. 1997). CSD binds many signalling molecules, and in many cases it negatively regulates their functions (Drab, Verkade et al. 2001). Results obtained in the evaluation of intracellular ROS level and glucose uptake activity in the presence of CSD suggest that Cav-1 may act as a negative regulator of the VEGFR-2 function also in B1647 cells.

The results obtained with Bevacizumab (Ferrara, Hillan et al. 2004) and Semaxinib (Fong, Shawver et al. 1999), able to inhibit the VEGF/VEGFR-2 interaction with different mechanism of action, confirm the importance of this molecular complex in maintaining the redox loop leading to high intracellular ROS level and glucose uptake activity in B1647 cells. The importance of inhibit tumour angiogenesis is now recognized as an helpful tool in therapeutic strategy, nevertheless, the wide adverse effects induced by anti-VEGF agents demonstrated that these drugs have a broad impact on vasculatures in multiple healthy tissues and organs (Folkman 1971).

The second major problem investigated concerns the role of natural or physiological molecules in maintaining or altering the ROS balance inside the cells.

The issue of protective *versus* deleterious roles for both endogenous and diet-derived antioxidants in health and diseases is highly debated. From the many reviews published on this topic, G.E. Mann and co-workers observed that, although epidemiological evidence suggests that diets containing high amounts of natural antioxidants afford protection against coronary heart disease, antioxidant supplementation trials have largely reported only marginal health benefits (Mann, Rowlands et al. 2007). A convincing point of view comes from B. Halliwell, who claims that, provided one is not deficient in vitamins, the antioxidant defences that we synthesize ourselves are far more important than any antioxidant benefit deriving from large supplementation of vitamins E, C, carotenoids or flavonoids. Thus, agents that stimulate our adaptation systems and elevate our endogenous antioxidant defences and other protective systems may be more protective against tissue damages due to oxidative stress than the consumption of large amounts of vitamins or antioxidants (Halliwell 2012).

One of the most important of the adaptation system is the nuclear factor erythroid 2-related factor 2, Nrf2, a transcription factor that increases levels of several protective agents, such as reduced glutathione, and the expression of endogenous antioxidants, such as heme oxygenase 1 (HO-1) or peroxiredoxin-1, (Prx-1), in response to toxic agents (Kasper et al, 2009).

Many nutraceuticals compounds, extracted from a variety of plants, are able to activate Nrf2 pathways, such as resveratrol, pterostilbene, sulforaphane, curcumin, cafestol, quercetin, and epigallocatechin-3-gallate (Balstad, Carlsen et al. 2011). To this regard, it has been shown that vascular cells respond to an environmental oxidative stress by inducing endogenous antioxidant defence mechanisms and that dietary antioxidants may counteract oxidative stress in vascular and inflammatory diseases by modulating key redox sensitive gene transcription *via* NF-κB and Nrf2/ARE signalling pathways (Mann, Rowlands et al. 2007). Among the endogenous antioxidant defences, the recent discovery of the Klotho protein raised a great appeal, since the Klotho gene was identified as an “aging suppressor” in mice (Kuro-o 2011).

In the experiments performed at the King's College London, human aortic smooth muscle cells (HASMC) were subjected to three different conditions, in order to mimic an hyperglycaemic status, and treated with sulforaphane or exogenous Klotho. Results show that both these treatments induced significant increases in HO-1 and Prx-1 expression, confirming that sulforaphane is able to stimulate Nrf2/ARE signaling pathways and that the Klotho protein takes part on the natural antioxidant defenses. Moreover, both sulforaphane and Klotho treatments were able to strongly elevate GSH intracellular levels and to completely restore the significant depletion of GSH levels observed after 48 hours HASMC exposition to HG condition. These data contribute to partially

clarify the antioxidant effect of Klotho, which could proceed through the modulation of GSH intracellular levels.

Nevertheless, the similar results observed in the three experimental conditions tested, suggest that these conditions are not stressing enough to appreciate significant differences between a normal glucose concentration and a simulated pathological status. Alternative experimental strategies should be adopted to carry on this study in the future.

The third faced problem, *i.e.* the study of the antioxidant and antihyperglycemic properties of natural molecules (*Stevia* extracts) used for their anti-diabetes effect, deals once again with the ROS balance inside the cells, providing a more applied approach.

At present, researchers worldwide agree on the antidiabetic *Stevia* effect, but they differ on how this effect proceeds through. Some assert that *Stevia*'s utility is due to the antioxidant properties of its leaves, which have been reported both *in vitro* and *in vivo* (Shivanna, Naika et al. 2013; Tavarini and Angelini 2013). Some other state that steviosides are able to down-regulate the NF-kB pathway and TNFα expression, modulate insulin secretion and sensitivity and inhibit hepatic gluconeogenesis (Mohd-Radzman, Ismail et al.). Therefore, many points concerning the mechanism of action of these compounds need to be clarified.

In order to study the potential insulin-like properties of the most used *Stevia* extracts, the human neuronal cell line SH-SY5Y is of particular interest, since it has been recently reported that in these cells Glut1 translocation in response to insulin-like growth factor occurs and, for the first time in a neuronal cell system, also Glut4 translocated to the plasma membrane in response to insulin (Choeiri, Staines et al. 2002). The evaluation of the intracellular ROS level demonstrated that the *Stevia* extracts tested failed to exert any antioxidant effect in both SH-SY5Y and HL-60 cells, in contrast with data reported in the literature. On this regard, it has to be pointed out that *Stevia* leaves were properly reported to have antioxidant activity, probably due to their naturally elevated amount of polyphenols (Shivanna, Naika et al. 2013), whereas the compounds used in the present study are commercial sweeteners, containing 95-98% steviol glycosides with non appraisable amount of polyphenols.

Interesting results were obtained in the evaluation of the glucose transport activity, since the different *Stevia* extracts tested were as efficient as insulin in increasing the glucose uptake of both cell lines. Moreover, the co-treatment with insulin and *Stevia* extracts causes a rise in glucose transport significantly higher than the increase due to insulin alone, revealing an additive effect between these two stimuli. The expression of glucose transporter isoforms observed in neuroblastoma and leukaemia cells following treatment with insulin or *Stevia* extracts demonstrated that both these stimuli provoke a Glut1 and Glut4 recruitment from intracellular pools to the plasma

membrane of HL-60 and SH-SY5Y cells, thereby inducing the increase in the glucose transport activity. These data provide important evidence supporting the insulin-like effect of steviol glycosides in human cells and clarify one of their possible mechanism of action. On this regard, the investigation of the signal transduction pathway involved in the effect of these two stimuli demonstrated that the phosphorylated forms of both PI3K and Akt were significantly increased upon the treatment with insulin or *Stevia* extracts, indicating a possible similar mechanism of action or, at least, a common signalling pathway shared by these two stimuli, which can explain their observed additive effect.

In conclusion, these data suggest that steviol glycosides act by modulating Gluts translocation through PI3K/Akt pathway, giving evidence about their recommended use as natural sweeteners for diabetic patients.

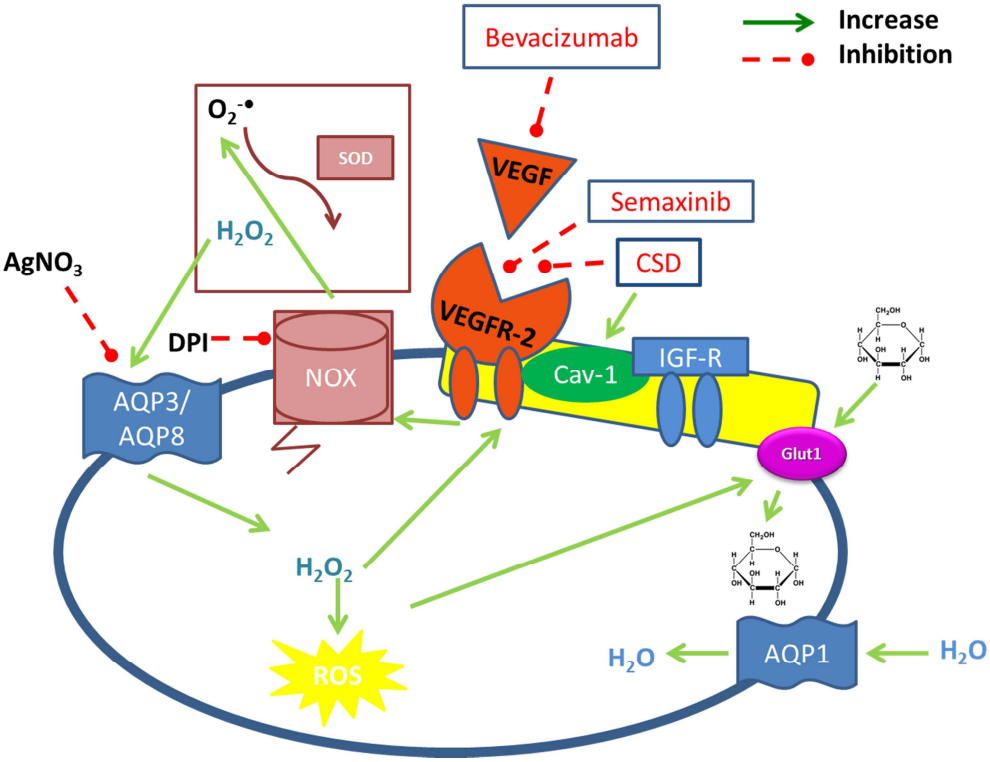


Fig. 36. H_2O_2 signalling in B1647 human acute myeloid leukaemia cell line.

The major source of intracellular ROS is the NAD(P)H oxidase family (Nox), which play an important role in biological processes, producing extracellular superoxide ($O_2^{-\bullet}$). Two molecules of $O_2^{-\bullet}$ can dismutate and produce hydrogen peroxide (H_2O_2) in the presence of the enzyme superoxide dismutase (SOD) (Lambeth 2004). AQPs is a channel through which H_2O_2 or H_2O can enter into the cell; in particular AQP1 isoforms facilitate the passive diffusion of H_2O across biological membranes, whereas AQP3 and AQP8 are more specific for H_2O_2 diffusion. $AgNO_3$ inhibits AQPs, while DPI blocks Nox. Caveolae/lipid rafts are membrane regions rich of cholesterol and sphingolipids and act as platforms for compartmentalization of proteins involved in signal pathway (Patel and Insel 2009). Vascular endothelial growth factor receptor (VEGFR)

colocalizes with Caveolin-1 (Cav-1) into caveolae, and also insulin growth factor receptor (IGFR) and the glucose transporter 1 (Glut1) are partially localized in these domains.

In B1647 cells, constitutively expressing both VEGF and its receptor VEGFR-2, VEGF is supposed to utilize ROS as messenger intermediates downstream of the VEGFR-2. Upon the formation of VEGF/VEGFR-2 complex, Nox is activated and H_2O_2 produced extracellularly is channelled *via* AQP3/AQP8 through the plasma membrane. Inside the cell, H_2O_2, is able to act as a signal molecule, being responsible for the signal leading to Glut1 activity.

Bevacizumab inhibits VEGF; Semaxinib inhibits VEGFR-2, Caveolin scaffolding domain peptide (CSD) mimics Cav-1, acting as a negative regulator of the VEGFR-2 function. All these compounds confirm the importance of VEGF/VEGFR-2 complex in maintaining the redox loop leading to high intracellular ROS level and glucose uptake activity in B1647 cells.

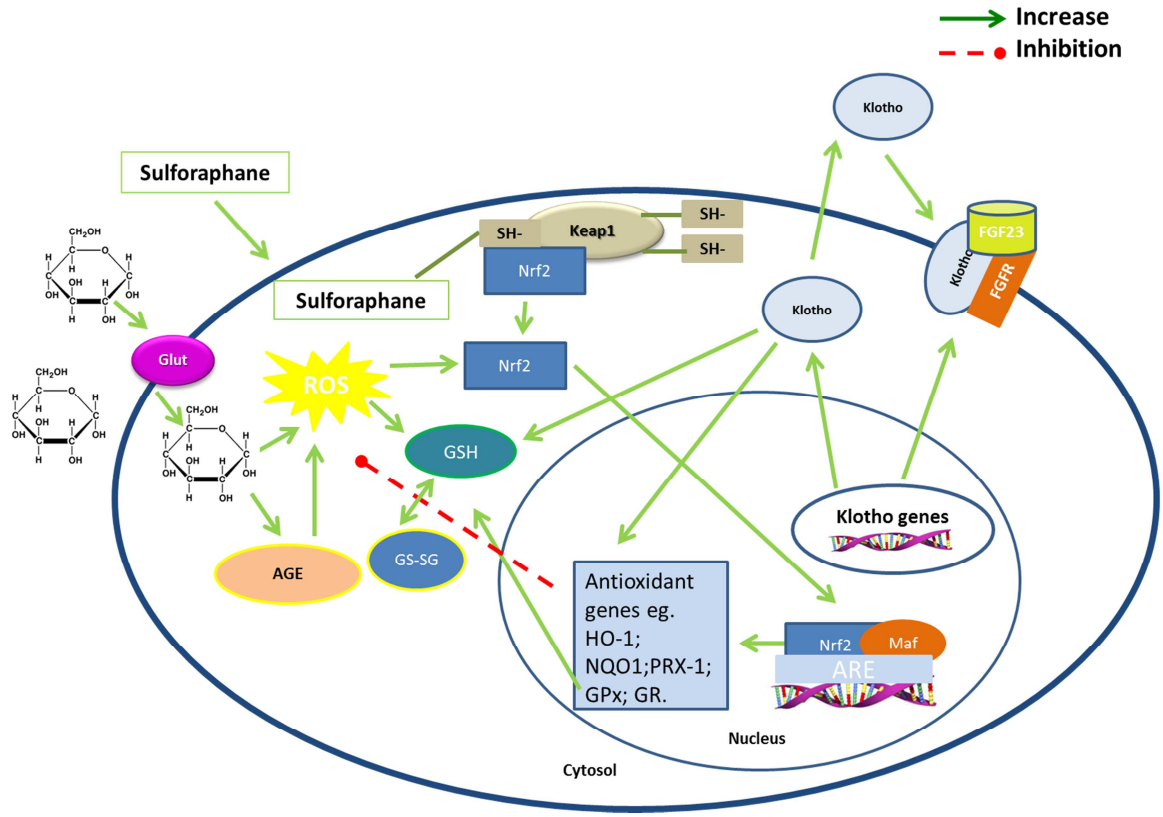


Fig. 37. Proposed mechanism of effects provided by SF and Klotho in HASMC.

The representative figure of sulforaphane (SF) and Klotho mechanisms is an adaptation of (Tarozzi, Morroni et al. 2009). Human aortic smooth muscle cells (HASMC) were subjected to different conditions, in order to mimic an hyperglycaemic status, and treated with SF or exogenous soluble Klotho. High glucose and AGEs increase ROS production (Chen and Kong 2004).

SF is an isothiocyanate present in cruciferous vegetables that induces antioxidant defence enzymes through Keap1/Nrf2 transcriptional activation (Angeloni, Leoncini et al. 2009; Alfieri, Srivastava et al. 2011). Under basal conditions, Keap1, that contains cysteine residues, binds Nrf2. When SF, or other compounds or oxidative stress change the eletrofilic balance, the cysteine residues are oxidized and Nrf2 is released (Baird

and Dinkova-Kostova 2011). Nrf2 translocates to the nucleus where binds ARE (Antioxidant Responsive Element) in the presence of Maf proteins (Baird and Dinkova-Kostova 2011). ARE increase the synthesis of many ARE-dependent antioxidant enzymes in different cell systems (Angeloni, Leoncini et al. 2009), such as glutathione reductase (GR), glutathione peroxidase (GPx), heme oxygenase (HO-1), NAD(P)H-Quinone:Oxidoreductase (NQO1), peroxiredoxin-1 (Prx-1), and glutaredoxin, thioredoxin, thioredoxin reductase. It has been shown that SF directly interacts with Keap1 by covalent binding to its thiol groups (Dinkova-Kostova, Holtzclaw et al. 2002).

Klotho, a protein with multiple pleiotropic effects, exists in membrane and secreted forms with distinct functions. The trans-membrane protein form is a co-receptor for FGF23, although the mechanisms involved in its action remain to be fully elucidated.

Klotho increases HO-1 and Prx-1 expression. Both SF and Klotho treatments were able to elevate GSH intracellular levels and to completely restore the significant depletion of GSH cell contents observed after 48-h exposition to high glucose conditions.

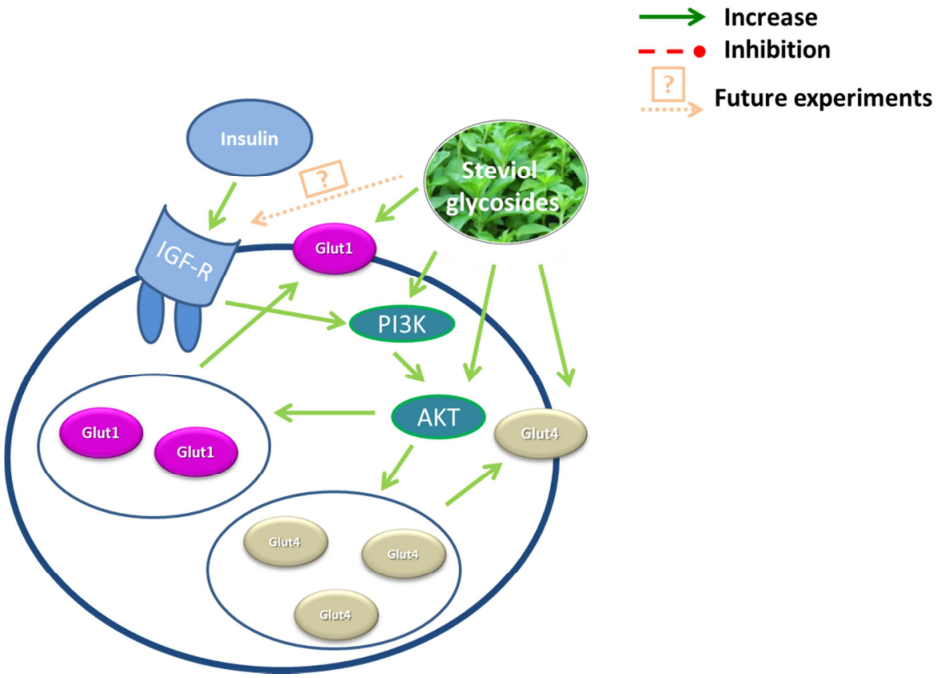


Fig. 38. Proposed mechanism of Steviol Glycosides effects on SH-SY5Y and HL60.

Steviol glycosides, primarily stevioside and rebaudioside A, are natural constituents of the plant *Stevia rebaudiana* Bertoni. Insulin increases glucose uptake in cells by stimulating the translocation of the glucose transporters (Glut1 and Glut4) from intracellular stores to the cell surface.

The PI3K/AKT pathway is a key component of the insulin signalling cascade and is essential for glucose transport, inducing the translocation of Glut4. Rebaudioside A and three different mixtures of steviol glycosides activate the same PI3K/Akt pathways, showing an insulin-like effect.

Chapter 6- Conclusions

6 Conclusions

Results reported in this Thesis contribute to the comprehension of the complicated world of “redox biology”.

Having a clear understanding of how ROS regulate signalling pathways both in physiological responses and in pathogenesis and progression of diseases will allow to develop strategies in order to improve well-being or to uncover new therapeutic targets.

In cancer cells, the increase in ROS generation from metabolic abnormalities and oncogenic signalling may trigger a redox adaptation response, leading to an up-regulation of antioxidant capacity in order to maintain the ROS level below the toxic threshold. Thus, cancer cells would be more dependent on the antioxidant system and more vulnerable to further oxidative stress induced by exogenous ROS-generating agents or compounds that inhibit the antioxidant system. The modulation of ROS content above or below the threshold level could lead to cell death, and this might constitute a biochemical basis to design therapeutic strategies to selectively kill cancer cells.

Results here reported indicate that the development of new drugs targeting specific Nox isoforms, responsible for intracellular ROS generation, or AQP isoforms, involved in the transport of extracellular H_2O_2 toward intracellular targets, might be an interesting novel anti-leukaemia strategy.

Furthermore, also the use of CSD peptide, which simulate the VEGFR-2 segregation into caveolae in the inactive form, might be a strategy to stop the cellular response to VEGF signalling.

As above stated, in the understanding of the redox biology, it is also important to identify and distinguish the molecular effectors that maintain normal biological and physiological responses, such as agents that stimulate our adaptation systems and elevate our endogenous antioxidant defences or other protective systems. Data here reported indicate that the nutraceutical compound sulforaphane and the Klotho protein are able to stimulate the HO-1 and Prx-1 expression, as well as the GSH levels, confirming their antioxidant and protective role.

Finally, results here reported demonstrated that *Stevia* extracts are involved in insulin regulated glucose metabolism, suggesting that the use of these compounds goes beyond their sweetening power and may also offer therapeutic benefits hence improving the quality of life.

List of abbreviations

•OH Hydroxyl Radical

Ac-DEVD-AMC acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin

AGE Advanced glycation end products

AML Acute myeloid leukemia

AngII Angiotensin II

ANOVA Analysis of variance

AQP Aquaporin

ARE Antioxidant response element

BSA Bovine serum albumin

B1647 Human erythromegakaryocytic leukemia cell line

bZip Basic leucine zipper

Cav-1 Caveolin-1

CD Methyl-β-cyclodextrin

CD71 Transferrin receptor

CSD Cav-1 Scaffolding Domain

CVD Cardiovascular diseases

Cyp Cytochrome

DAG Diacylglycerol

DCF 2',7'-dichlorofluorescein

DCFH-DA Dichlorofluorescin diacetate

DMSO Dimethyl sulfoxide

DMEM Dulbecco's modified Eagle's medium

DNA Deoxyribonucleic acid

DOG 2-deoxy-D-glucose

DPI Diphenyleneiodonium chloride

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme-linked immunosorbent assay

eNOS Endothelial nitric oxide synthase

ERK Extracellular signal-regulated kinases

FADH Flavin adenine dinucleotide

FBS Foetal bovine serum

FGF Fibroblast growth factor

FOXO Forkhead box O

GF growth factor

GLUT Glucose transporter

GPx Glutathione peroxidase

GRed Glutathione reductase

GSH Glutathione

GSK3 Glycogen synthase kinase 3

GSSG Oxidised glutathione

GST Glutathione S-transferase

H_2DCFDA 2',7'-dichlorodihydrofluorescein diacetate

H_2O_2 Hydrogen peroxide

HAD highly efficient and unaltered antioxidant defences

HBSS Hanks' Balanced Salt Solution

HG High Glucose

HIF-1 Hypoxia inducible factor-1

HO-1 Heme oxygenase-1

HRP Horseradish peroxidase

HS Human serum

HUVEC Human umbilical vein endothelial cell

I Insulin

IGF Insulin-like growth factor

IL Interleukin

iNOS Inducible nitric oxide synthase

IR Insulin receptor

IRE1 Inositol-requiring kinase 1

IRS Insulin receptor substrate

LAD low antioxidant defences

LDH Lactate dehydrogenase

JNK c-jun N-terminal kinase

Keap1 Kelch-like ECH-associating protein 1

Kl Klotho

MAPK Mitogen-activated protein kinase

MnSOD Manganese superoxide dismutase

mRNA Messenger ribonucleic acid

MTT (3-(4,5 – dimethylthiazol – 2 – yl) – 2,5 – diphenyltetrazolium bromide

Man Mannitol

NAD Nicotinamide-adenine dinucleotide

NADH Reduced nicotinamide-adenine dinucleotide

NADP+ Oxidised nicotinamide-adenine dinucleotide phosphate

NADPH Nicotinamide adenine dinucleotide phosphate

NG Normal Glucose

NO^+ Nitrosonium Cation

NO^- Nitroxyl Anion

NF-κB Nuclear factor kappa B

NO Nitric oxide

NOS Nitric oxide synthase

Nox NAD(P)H oxidase

NQO1 NAD(P)H quinone oxidoreductase 1

Nrf2 Nuclear factor erythroid 2-related factor 2

O_2^- Superoxide

$ONOO^-$ Peroxynitrite

OPA O-phthaldialdehyde

oxLDL Oxidised low-density lipoproteins

PBS Phosphate-buffered saline

PH pleckstrin homology

PI3K Phosphatidylinositol 3-kinase

PIP3 Phosphatidylinositol-(3, 4, 5)-trisphosphate

PKB (or Akt) Protein Kinases Protein Kinase B

Prx Peroxiredoxin

PTP Protein Phosphatases

PVDF Polyvinylidene difluoride

RNS Reactive nitrogen species

ROS Reactive Oxidizing Species

SD Standard Deviation

SEM Standard Error Mean

SDS-PAGE Sodium dodecyl sulfate- poly acrylamide gel electrophoresis

SF Sulforaphane

siRNA Small interfering ribonucleic acid

SMC Smooth muscle cell

SOD Superoxide dismutase

STZ Streptozotocin

TCA Trichloroacetic acid

TCA cycle Tricarboxylic acid cycle

TEMED Tetramethylethylenediamine

TGF-β Transforming growth factor beta

TNF-α Tumour necrosis factor α

Trx Thioredoxin

TrxR Thioredoxin reductase

VCAM-1 Vascular cell adhesion molecule-1

VSMC Vascular smooth muscle cell

VEGF Vascular endothelial growth factor

VEGFR-1 Vascular endothelial growth factor receptor

WHO World Health Organisation

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