

## **Exercise, Calorie Restriction and Insulin Sensitivity in Aging**

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Mammalian evolution occurred in the context of vigorous exercise, and humans are genetically adapted for a physically active lifestyle. Regularly performed exercise prevents obesity and, thus, protects against insulin resistance, while acute exercise induces short term increases in insulin sensitivity and responsiveness. Regular exercise is normal, and people who exercise regularly age normally, meaning that exercise has no effect on primary aging. Like exercise, calorie restriction (CR) to prevent weight gain can prevent development of insulin resistance, and CR to induce loss of abdominal fat can cure type 2 diabetes and lower the risk for diseases that cause secondary aging. In contrast very severe, long term CR with optimal nutrition sufficient to stunt growth in young animals, or cause emaciation in adults, slows aging and increases maximal longevity in rodents. It is not known whether severe long-term CR increases maximal longevity in humans. However, members of the CR Society who have been practicing severe CR for years, show the same adaptations as CR rodents. Although the CR Society members have low fasting glucose and insulin levels, a subgroup of them have impaired glucose tolerance that appears to be due to down regulation of the insulin/IGF-1 signaling pathway.

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## **Nutrition, aging and longevity: the strategy of the new EU large Project NU-AGE (2011-2016)**

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A major characteristic of the aging process is the progressive development of a systemic, low grade inflammation which I proposed to call INFLAMM-AGING and which constitutes the common pathogenetic background shared by all major age-associated diseases, including cardiovascular diseases, diabetes, cancer, dementia, sarcopenia, depression and frailty, among others. Inflamm-aging is much more complex than we previously thought and the underpinning biological basis are still largely unknown. Nutrition is one of the most important strategy to be used in order to counteract the age-related decline of all major functions in humans, owing to its pervasive and profound biological effect on molecular and cellular mechanisms and pathways. This is a major aim of the 5-year new EU Large Project “New dietary strategies addressing the specific needs of the elderly population for healthy ageing in Europe (acronym: NU-AGE; FP7 KBBE-2010-2-2-02: Diet and prevention of functional decline of the elderly). NU-AGE is a multidisciplinary consortium consisting of 31 partners from 17 EU countries. Involved are research institutes across Europe, large food industries, traditional food companies, one biotech SME and associations of the European food and drink industry. The basic hypothesis and rationale of NU-AGE is that an appropriate WHOLE DIET (an *ad hoc* fortified “mediterranean diet”) will decrease the level of inflamm-aging by an *ad hoc* whole diet approach tailored for the elderly. To this aim the NU-AGE project will start by designing a new food pyramid for those over 65 years old. This will be developed from food based dietary guidelines used in Europe, illustrating the proportions of different foods that should be included in a balanced diet. The NU-AGE 65+ food pyramid will be designed to meet the nutritional needs of the elderly by emphasising nutrient-density, water, dietary fibre, vitamin D, vitamin B12 and folate, among others. To study the effects of the NU-AGE food pyramid on health and ageing factors, seniors across Europe will receive advice, fortified foods and other support to adjust their diets to match the pyramid. A total of 1250 individuals (65-79 years of age of both sexes) categorized as not frail or prefrail will be recruited in 5 European countries (Italy, France, UK, The Netherlands and Poland), and divided into 2 groups: the first will follow for 1 year a whole “mediterranean” *ad hoc* fortified/modified diet while the second will continue its own diet (control group). At time zero and after 12 months all 1250 subjects will be fully characterized for their physical and cognitive ability, nutritional status. Blood, urine and feces will be collected to measure a variety of parameters according to a comprehensive protocol capable of exploring a variety of body functional activities (hematological profiles, immunology and inflammation, cognition, genetics, body composition). Alongside the dietary intervention, socio-economic determinants for food choice in the elderly will be investigated. In a subcohort of 120 elderly subjects in depth immunology (in plasma/serum), transcriptomics (PBMC), oxido-lipidomics (in plasma), metabolomics (in urine), gut microbiota composition by HITChip microarray and metagenomics (in feces), epigenetics (whole genome methylation on DNA from PBMC and on candidate genes involved in inflamm-aging) will be performed. Based on the knowledge gained about influences of diet on ageing and its potential to prevent age-related disease, foods designed especially for elderly consumers will be developed and the best ways to communicate dietary recommendations to those over 65 will be explored. The results will be valuable to a wide range of stakeholders - from the scientific community and health professionals to industry and policy makers. - and contribute to the work of the European Commission's recently launched Pilot European Innovation Partnership on Active and Healthy Ageing.

## Understanding the mechanisms of calorie restriction

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Calorie Restriction (CR) induces complex metabolic changes and stress responses in organisms. It is currently unclear whether and which of these factors play a major role in mediating CR-induced beneficial effects including life span extension. Recent studies have suggested that mitochondrial metabolism and NAD<sup>+</sup> (nicotinamide adenine dinucleotide) homeostasis play important roles in CR. To further understand the molecular basis of CR, we utilize the genetically tractable model system, the budding yeast *Saccharomyces cerevisiae*, to unravel potential novel metabolic/longevity factors that affect mitochondrial activity and NAD<sup>+</sup> metabolism. Our studies will help elucidate the regulation and interplay of CR-related metabolic and stress response pathways and may provide insights into the mechanisms of CR.

## **Transcriptional biomarkers of calorie restriction in adipose tissue and modulation by dietary interventions**

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Calorie restriction (CR) is the only dietary intervention shown to increase maximum lifespan by slowing the aging process in diverse species. Studies using whole-genome transcriptional profiling have identified thousands of genes that are changed in expression in response to a CR diet, but many of these changes are specific to the genetic background of the organism being studied. Thus, there is great interest in identifying robust biomarkers of CR that are conserved across species and are applicable to humans.

We used gene expression profiling to identify transcripts that were consistently changed in expression in response to short-term CR in white adipose tissue from seven strains of mice. Of the 20,789 genes represented on the microarray, 414 genes were changed in 6/7 strains ( $p < 0.01$ ). The expression of 20 genes was confirmed by RT-qPCR and a subset of 11 genes was selected as the final panel of CR biomarkers. We then used this panel of genes to assess the ability of selected dietary interventions to mimic the effect of CR in mice. Pioglitazone (a known insulin sensitizing drug) had the greatest ability to mimic the effect of CR (significant mimicry for 8 of 11 biomarkers). Interestingly, consumption of a low dose of either resveratrol or quercetin alone only modestly mimicked the effect of CR, whereas a diet containing both compounds had a greater effect than either compound alone.

In summary, we have identified robust transcriptional biomarkers of CR in white adipose tissue and have successfully used these biomarkers to screen for compounds that have the ability to mimic CR at the transcriptional level. This study was supported by NIH/NIA Grant Number R43 AG034833 to JLB.

## **Flavonoids and brain health: multiple effects underpinned by common mechanisms**

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Evidence suggests that dietary phytochemicals, in particular flavonoids, may exert beneficial effects on the central nervous system by protecting neurons against stress-induced injury, by suppressing neuroinflammation and by improving cognitive function. Historically, they were believed to do this via an ability to express classical antioxidant activity in the brain. However, their poor brain bioavailability and extensive metabolism means that this is unlikely. Instead, their actions on the brain appear to be mediated by two separate mechanisms. Firstly, they interact with critical protein and lipid kinase signalling cascades in the brain, leading to an inhibition of neurotoxin-induced apoptosis, neuroinflammation and the promotion of synaptic plasticity. For example, their ability to activate the extracellular signal-regulated kinase (ERK1/2) and the protein kinase B (PKB/Akt) signalling pathways leads to the activation of the cAMP response element-binding protein (CREB), a transcription factor responsible for increasing the expression of a number of neurotrophins critical in memory processing. Secondly, they induce effects on both the peripheral and cerebrovascular system that lead to changes in improve blood flow to the brain capable of causing angiogenesis, neurogenesis and changes in neuronal morphology. Through these mechanisms, the consumption of flavonoid-rich foods throughout life holds the potential to limit neurodegeneration and to prevent or reverse age-dependent losses in cognitive performance. In addition, flavonoids may represent important precursor molecules in the quest to develop a new generation of brain enhancing drugs.

## A diet for health and longevity. How do we get there?

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1) An inexpensive intervention could delay the degenerative diseases accompanying aging, such as cancer, cardiovascular disease, cognitive decline, and immune dysfunction. Most of the world's population, even in developed countries, has inadequate intake of one or more micronutrients (~40 essential vitamins, minerals, fatty acids and amino acids) that a varied and balanced diet should provide. My *triage theory* (PNAS 103, 17589, 2006; AJCN 90, 889, 2009; M.A.D. 2010) posits that, as a result of recurrent shortages of micronutrients during evolution, natural selection developed a metabolic rebalancing response to shortage. The rebalancing favors micronutrient-dependent proteins needed for short-term survival while starving those only required for long-term health. Triage theory predicts that the consequence of moderate shortages of even a single micronutrient, though insufficient to cause overt clinical symptoms, will impair functions essential for long-term health. This impairment will result in insidious damage (e.g. increased DNA damage) that, over time, leads to the acceleration of age-associated diseases (e.g. increased cancer). As people with modest deficiencies have no overt clinical symptoms, there has been little incentive to correct these deficiencies, though this could change if it can be shown that they are resulting in biochemical changes, e.g. chromosome breaks, that are markers of increased risk of age-related diseases, e.g. cancer. The considerable experimental and theoretical support for the triage idea will be discussed as will a strategy for determining the optimum level of each micronutrient in humans. A perfect balanced diet (and adequate sunshine) would optimize levels of all micronutrients, but few reach this standard; fortunately inexpensive supplements and fortification can help. The triage theory should help to put micronutrient nutrition on a firm foundation and lead to preventive medicine for age-related diseases. 2) Too much refined food causes a shortage of micronutrients and fiber and an excess of calories (sugar, fat, and alcohol) which contributes to chronic inflammation, obesity, and associated diseases, such as diabetes. 3) How do we translate this new knowledge into improved health and lower costs?

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## Cellular Stress Response, Mitostress and Carnitine Insufficiency as critical determinants in Aging and Neurodegenerative Disorders: Role of Hormesis and Vitagenes

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Protein quality control is a critical feature of intracellular homeostasis<sup>1</sup>. In addition, modulation of endogenous cellular defense mechanisms via the stress response signaling represents an innovative approach to therapeutic intervention in diseases causing chronic tissue damage, such as neurodegeneration and cancer<sup>1</sup>. Protein thiols play a key role in redox sensing, and regulation of cellular redox state is crucial mediator of multiple metabolic, signalling and transcriptional processes. Under optimal conditions long-term health protection is accomplished by protein homeostasis, a highly complex network of molecular interactions that balances protein biosynthesis, folding, translocation, assembly/disassembly, and clearance<sup>2,3</sup>. Efficient functioning of maintenance and repair processes is crucial for both survival and physical quality of life. This is accomplished by a complex network of the so-called longevity assurance processes, which are composed of several genes termed vitagenes<sup>4-8</sup>. The term vitagenes refers to a group of genes which are strictly involved in preserving cellular homeostasis during stressful conditions. The vitagene family is actually composed of the heat shock proteins (Hsp) Hsp32, Hsp70, the thioredoxin system and the sirtuin system<sup>9</sup>. Dietary antioxidants, such as polyphenols and L-carnitine/acetyl-L-carnitine, have recently been demonstrated in vitro to be neuroprotective through the activation of hormetic pathways, including vitagenes<sup>10-12</sup>. Over the past decade there has been a remarkable increase of interest in hormesis as a result of more significance being given to low dose effects and the use of more powerful study designs which have enabled to identify rational approaches to detect hormetic biphasic dose responses in the low dose zone. The hormetic dose–response, challenging long-standing beliefs about the nature of the dose–response in a lowdose zone, has the potential to affect significantly the design of pre-clinical studies and clinical trials as well as strategies for optimal patient dosing in the treatment of numerous diseases, including oxidant disorders. Given the broad cytoprotective properties of the heat shock response there is now strong interest in discovering and developing pharmacological agents capable of inducing stress responses. We have recently focused our research on the role of acetylcarnitine in the defense mechanisms against cellular stress and neurodegeneration. In addition, with a redox proteomics approach, we identified mitochondrial oxidatively modified proteins as a function of brain aging, specifically in those brain regions, such as cortex and hippocampus, that are commonly affected by the aging process. In all brain regions examined, many of the identified proteins were energy-related, such as pyruvate kinase, ATP synthase, aldolase, creatine kinase, and a-enolase. These alterations were associated with increased expression of Hsps, as well as carnosinase and thioredoxin reductase and with significant changes in both cytosolic and mitochondrial redox status in all brain regions analyzed. This findings are relevant to potential pharmacological interventions in healthy medicine strategy, pointing to maximize cellular stress resistance of the brain thus providing neuroprotection<sup>9-14</sup>, and will be extended to other systemic oxidant disorders such as diabetic nephropathy or cancer.

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## Promotion of metabolic health and lifespan by transiently increasing oxidative stress

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Recent evidence suggests that calorie restriction and specifically reduced glucose metabolism induces mitochondrial metabolism to extend life span in various model organisms, including *S. cerevisiae*, *D. melanogaster*, *C. elegans* and possibly mice. In conflict with Harman's free radical theory of aging (FRTA), these effects may be due to *increased* formation of reactive oxygen species (ROS) within the mitochondria causing an adaptive response that culminates in subsequently increased stress resistance assumed to ultimately cause a long-term reduction of oxidative stress. This type of retrograde response has been named mitochondrial hormesis or mitohormesis, and may in addition be applicable to the health-promoting effects of physical exercise in humans and, hypothetically, impaired insulin/IGF1-signaling in model organisms. Consistently, abrogation of this mitochondrial ROS signal by antioxidant supplements impairs the lifespan-extending and health-promoting capabilities of glucose restriction and physical exercise, respectively. In summary, the findings discussed in this review indicate that ROS are essential signaling molecules which are required to promote health and longevity. Hence, the concept of mitohormesis provides a common mechanistic denominator for the physiological effects of physical exercise, reduced calorie uptake, glucose restriction, and possibly beyond.

For details see: Exp Gerontol, 45, 410-8 [2010], PubMedID 20350594



## **Redox systems biology: Mapping the metabolic and proteomic networks**

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Oxidative stress and redox signaling research has led to improved understanding of radical and non-radical mechanisms of diet and nutrition. Importantly, a large number of redox processes occur concurrently within biological systems so that imbalances can occur selectively among biochemical systems and subcellular compartments. This presentation will summarize research applying metabolomic and proteomic approaches to understand function and control of thiol redox systems. These redox systems depend upon the amino acid cysteine (Cys), the Cys-containing antioxidant glutathione (GSH) and about 214,000 specific Cys residues in proteins. A relatively small subset of these protein Cys residues function in cell signaling, while a larger number coordinate cell functions in response to redox state. The Cys residues that function in signaling are termed redox-signaling thiols while the latter are defined as redox-sensing thiols. Bulk measurements of protein thiols are not very informative for systems biology because reactivity of thiols in proteins differs by over six orders of magnitude. Analysis of percentage oxidation of specific Cys residues in proteins by mass spectrometry shows that the average peptidyl Cys is about 15% oxidized under steady-state conditions in cell culture. Analyses of percent oxidation of Cys residues show that functional redox networks can be identified that differ in terms of steady-state levels of oxidation. Application of new high-resolution mass spectrometry methods are beginning to provide capability to link metabolic changes induced by diet to redox proteomic effects, with selective effects in different subcellular compartments. The results suggest that systems biology models can be used to connect metabolic pathways to central GSH and thioredoxin control hubs and functional redox networks. Development of such models will be useful to support customized diet and nutrition for personalized health and medicine.

## **Redox regulation of muscle-nerve interactions in age-related loss of muscle function**

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Correlative data indicate that the substantial loss of skeletal muscle fibres in peripheral muscles that occurs with ageing is accompanied by loss of whole motor units including disruption of motor neurons and neuromuscular junctions. Reactive oxygen and nitrogen species have been implicated in fundamental processes of ageing for a considerable time but although all tissues from aged organisms contain increased amounts of markers of oxidative damage, the precise role of these species in loss of neuromuscular function remains unclear. Our recent studies have focussed on a model of accelerated age-related loss of skeletal muscle fibres that is seen in mice lacking Cu,Zn superoxide dismutase (SOD1). These mice show premature loss of axons, disruption of neuromuscular junctions, loss of muscle fibres and weakness of the remaining fibres. Recent data indicate that the lack of SOD1 in these mice does not cause any detectable increase in muscle superoxide availability, but leads to a substantial increase in peroxynitrite formation with upregulation of peroxiredoxin V (a peroxynitrite reductase). This increase is dependent upon the availability of nitric oxide. Understanding the nature of how reactive oxygen and nitrogen species can cause premature loss of relevant components of the neuromuscular system holds considerable promise for development of interventions to reduce or prevent age-related loss of skeletal muscle mass and function.

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## **An ectopic source of reactive oxygen species in skin aging and atherosclerosis**

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Deleterious modifications of macromolecules resulting from the production of reactive oxygen species (ROS) have long been associated with progressive age-associated disorders of skin and increased cardiovascular risk from coronary artery disease. However, the source of these ROS, which may appear quite distant from sources usually regarded as ROS generators, and the manner by which they are directed to specific targets, has been inadequately addressed. For example, mitochondria appear not to be a major source of ROS contributing either to oxidation of skin matrix proteins or to the oxidation of low density lipoproteins (LDLs). Our work has identified a potentially important source of extracellular ROS, a special class of age-related cell surface proteins measurable in man after about age 30 as well as in late passage tissue culture cells that has been designated as age-related NADH oxidases or arNOX proteins. The arNOX proteins are unique in that they generate superoxide at the cell surface and, as shed proteins, in the circulation and other body fluids (Morré et al., 2003). Generation of superoxide anion is estimated from superoxide dismutase-inhibited reduction of ferricytochrome c. Recently cloned, the arNOX proteins have been identified as members of the TM-9 superfamily, initially membrane anchored, all functionally similar, with their N-termini exposed at the cell's exterior. A ca. 30 kDa N-terminal fragment is proteolytically cleaved and accumulates in body fluids (serum, saliva, urine, perspiration). Reduced quinones, i.e., reduced coenzyme Q, of the plasma membrane are its natural substrates at the cell surface. NADH is oxidized as an artificial substrate. Substrates for the shed forms of arNOX appear to be proteins contacted by arNOX-containing body fluids. Circulating lipoproteins and skin matrix proteins emerge as potentially important health-related targets. arNOX in the blood is structured as an integral component of the LDL particle through site-specific binding. As such, arNOX is implicated as a major risk factor for cardiovascular disease due to specific oxidation of LDLs. Through oxidation of collagen and other proteins of the skin matrix, arNOX is a major contributor to skin aging through tyrosine and thiol oxidation and subsequent cross linking (Morré et al., 2008). In addition to the transfer of electrons and proteins from NAD(P)H or quinols to oxygen to form water, arNOX proteins generate superoxide during one phase of its activity cycle. The main destructive action of arNOX may be to directly oxidize proteins such that the superoxide produced and its dismutation to hydrogen peroxide would be only one part of the potentially destructive properties of the arNOX proteins. However, the amounts of hydrogen peroxide produced are substantial and contribute to lipid oxidation. Thus, inhibition of arNOX provides a rational basis for anti-aging interventions. Included would be dietary or oral interventions to retard formation of aging-related arterial lesions through prevention of LDL oxidation and a reduction in the formation of foam cells and the use of topical arNOX inhibitors to prevent, reduce or even reverse external manifestations of skin aging.

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## Ageing, Proteolysis, and Stress Adaptation

### Kelvin J. A. Davies

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Proteins are major targets for oxidative damage, and both the intracellular and extracellular accumulation of oxidized proteins has been reported in many ageing and disease models. In young and healthy individuals, moderately oxidized soluble cell proteins are selectively and rapidly degraded by the Proteasome in the cell cytoplasm, nucleus, and endoplasmic reticulum. Inside mitochondria, the matrix proteinase called 'Lon' selectively degrades oxidized soluble proteins. Severely oxidized, aggregated, and cross-linked proteins, however, are poor substrates for degradation and actually inhibit the Proteasome and the Lon protease. Some mildly aggregated and cross-linked proteins are degraded by autophagy, but more severely aggregated and cross-linked proteins seem to accumulate as inclusion bodies or lipofuscin inside lysosomes. It is, therefore, vitally important that cells rapidly and selectively degrade mildly oxidized proteins, before they undergo more severe oxidation, aggregation, and cross-linking. Young mammalian cells can readily adapt to mild oxidative stress such that they become (temporarily) much more resistant to oxidative damage. Such adaptive responses include the immediate disassembly of the 26S proteasome (catalyzed by HSP70) to form free 20S proteasome and 19S regulator complexes, at which point and ATP/Ubiquitin-dependent proteolysis is temporarily lost. The additional free 20S Proteasomes are of immediate help in degrading oxidized proteins. Next, over a 3 – 20 hour period, new 20S Proteasomes, Immunoproteasomes, and both 11S (PA28) and PA200 proteasome regulators are synthesized, partially under the control of the Nrf2 signal transduction pathway. The original 26S Proteasomes are re-assembled, and ATP/Ubiquitin-dependent proteolysis is restored. In the mitochondria, as much as a seven-fold increase in the synthesis of Lon protease is seen, permitting dramatic increases in the capacity to degrade oxidized intramitochondrial proteins. Indeed, our recent work suggests that Lon, and certain proteasome subunits and regulators should be classified as true stress proteins. Induction of Proteasome/Lon expression provides significantly greater capacity to remove oxidized proteins, and such adaptive responses contribute to overall stress resistance. In older cells, however, both Lon and Proteasome activities decline, and adaptational responses become sluggish or even ineffectual. Studies in animals and humans suggest that declining Proteasome/Lon activities and, perhaps, declining responsiveness to stress, may contribute to the ageing process, and to various age-associated diseases.

## **Proteasome activation delays aging in human cells**

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Aging and longevity are two multifactorial biological phenomena whose knowledge at molecular level is still limited. Specifically, we have studied proteasome function in replicative senescence and cell survival. We have observed reduced levels of proteasome content and activities in senescent cells due to the down-regulation of the catalytic subunits of the 20S complex (J Biol Chem 278, 28026-28037, 2003). In support, partial inhibition of proteasomes in young cells by specific inhibitors induces premature senescence which is p53 dependent (Aging Cell 7, 717-732, 2008). Stable over-expression of catalytic subunits or POMP resulted in enhanced proteasome assembly and activities and increased cell survival following treatments with various oxidants. Importantly, the developed “proteasome activated” human fibroblasts cell lines exhibit a delay of senescence by approximately 15% (J Biol Chem 280, 11840-11850, 2005). Our recent findings indicate that the recorded proteasome activation by many inducers is Nrf2-dependent (J Biol Chem 285, 8171-8184, 2010). Finally, we have studied the proteolysis processes of various age-related proteins and we have identified that CHIP is a major p53 E3 ligase in senescent fibroblasts (Free Rad Biol Med 50, 157-165, 2011).

## Role of protein repair in aging and protection against oxidative damage

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The accumulation of oxidatively modified protein is a hallmark of aging. This accumulation results, at least in part, from the increase of reactive oxygen species coming from both cellular metabolism and external factors including environment, but the efficacy of protein maintenance systems is also involved. Most organisms, from bacteria to humans, have developed a specific reductase system, the Methionine Sulfoxide Reductase (Msr), which allows the repair of oxidized methionines in proteins. The Msr system, composed of the two stereo specific enzymes: MsrA and MsrB, plays a major role in the maintenance of protein homeostasis during aging and has also been involved in cellular defenses against oxidative stress, by scavenging ROS (Ugarte et al., 2010). To analyse more precisely the relationships between the Msr system, oxidative stress and oxidative protein damage, we stably overexpressed MsrA and MsrB2, a mitochondrial member of the MsrB family, in cellular models. We showed that Msr overexpressing cells are more resistant to H<sub>2</sub>O<sub>2</sub> cytotoxicity by delaying apoptosis and protecting against necrosis. Moreover, we demonstrated that the mechanisms by which MsrB2 protects against oxidative stress include: maintenance of a lower level of intracellular ROS, prevention of oxidized protein accumulation and protection of the proteasome against oxidative stress-induced inactivation (Cabreiro et al., 2008). In addition, MsrB proteins being Zinc binding proteins, we showed that this micronutrient can potentiate the protective effect of the Msr system (Cabreiro et al., 2009).

Finally, we generated stable human embryonic kidney cell lines (HEK) where MsrA, MsrB1 or MsrB2 gene expressions are silenced by using RNAi technology. These knocked down clones have been used in 2D-DIGE experiments (2D fluorescence difference gel electrophoresis). Most of the proteins found to be differentially expressed in Msr depleted cells were identified by mass spectrometry as proteins related to redox homeostasis.

Altogether, our results suggest that the Msr proteins, in addition to be oxidized protein repair enzymes, may play an important role in protein homeostasis and cellular anti-oxidant defenses

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## **The role of mitochondrial oxidative stress and Bak-mediated apoptosis in age-related hearing loss**

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Caloric restriction (CR) extends the life span and health span of a variety of species and slows the progression of age-related hearing loss (AHL), a common age-related disorder associated with oxidative stress. We have previously reported that caloric restriction suppresses the expression of pro-apoptotic genes in the cochlea, including the mitochondrial pro-apoptotic gene Bak. Mice deficient for Bak display prevention of AHL, and Bak deficient cochlear cells are resistant to oxidative stress. More recently, we have reported that CR reduces oxidative DNA damage in multiple tissues and prevents AHL in wild-type mice but fails to modify these phenotypes in mice lacking the mitochondrial deacetylase Sirt3, a member of the sirtuin family. In response to CR, Sirt3 directly deacetylates and activates mitochondrial isocitrate dehydrogenase 2 (Idh2), leading to increased NADPH levels and an increased ratio of reduced-to-oxidized glutathione in mitochondria. In cultured cells, overexpression of Sirt3 and/or Idh2 increases NADPH levels and protects from oxidative stress-induced cell death. Therefore, our findings identify Sirt3 as an essential player in enhancing the mitochondrial glutathione antioxidant defense system during CR and suggest that Sirt3-dependent mitochondrial adaptations may be a central mechanism of aging retardation in mammals. Thus, our studies show that age-related mitochondrial dysfunction and its prevention under CR play a central role in AHL. We postulate that mitochondrial adaptations under CR may also delay multiple aging phenotypes in mammals.

## Regulation of mitochondrial function, ROS and yeast chronological life span by the TORC1 signaling pathway

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Inhibition of the nutrient-sensing, target of rapamycin 1 (TORC1) signaling pathway extends life span in organisms ranging from yeast to mice, yet the precise mechanisms involved remain to be elucidated fully. Likewise, mitochondria have been implicated in aging and age-related pathology, with their production of reactive oxygen species (ROS) often implicated as major effectors via their damaging effects. However, the precise roles of ROS and mitochondria in aging and longevity are still debated. I will show new results demonstrating that inhibition of the TORC1 signaling pathway causes an adaptive mitochondrial ROS signal that is important for extension of yeast chronological life span (CLS). Specifically, *tor1Δ* strains have efficiently coupled respiration that results in greater mitochondrial membrane potential and superoxide production during growth, that, in turn, supplies an adaptive ROS signal promoting enhanced stationary phase survival. This conclusion is bolstered by experiments showing that uncoupling respiration during growth or over-expressing mitochondrial MnSOD (SOD2) significantly curtails CLS extension in *tor1Δ* strains, and treatment of wild-type strains with either rapamycin or menadione (to generate mitochondrial ROS) just during growth extends CLS. Finally, extension of CLS by reduced TORC1/Sch9p-mitochondrial signaling occurs independently of the stress-related Rim15p kinase and is not a function of media composition. These results strongly implicate signaling functions of mitochondrial ROS as a key component of their effects on aging and life span.



## **Decline in mitochondrial bioenergetics and shift to ketogenic profile in brain during reproductive senescence**

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We previously demonstrated that mitochondrial bioenergetic deficits precede Alzheimer's pathology in the female triple transgenic Alzheimer's (3xTgAD) mouse model. Herein, we sought to determine the impact of reproductive senescence on mitochondrial function in the normal non-transgenic (nonTg) and 3xTgAD female mouse model of AD. *Methods:* Both nonTg and 3xTgAD female mice at 3,6,9, and 12 months of age were sacrificed and mitochondrial bioenergetic profile as well as oxidative stress markers were analyzed. *Results:* In both nonTg and 3xTgAD mice, reproductive senescence paralleled a significant decline in PDH, and Complex IV cytochrome c oxidase activity and mitochondrial respiration. During the reproductive senescence transition, both nonTg and 3xTgAD mice exhibited greater individual variability in bioenergetic parameters suggestive of divergent bioenergetic phenotypes. Following transition through reproductive senescence, enzymes required for long-chain fatty acid (HADHA) and ketone body (SCOT) metabolism were significantly increased and variability in cytochrome c oxidase (Complex IV) collapsed to cluster at a ~40% decline in both the nonTg and 3xTgAD brain which was indicative of alternative fuel generation with concomitant decline in ATP generation. *Conclusions:* These data indicate that reproductive senescence in the normal nonTg female brain parallels the shift to ketogenic / fatty acid substrate phenotype with concomitant decline in mitochondrial function and exacerbation of bioenergetic deficits in the 3xTgAD brain. *General Significance:* These findings provide a plausible mechanism for increased life-time risk of AD in postmenopausal women and suggest an optimal window of opportunity to prevent or delay decline in bioenergetics during reproductive senescence.

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## **ApoE genotype, diet, oxidative stress, inflammation, and disease risk**

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In Westernised societies the apoE4 genotype it is associated with increased morbidity and mortality, and represents a significant risk factor for cardiovascular disease (CVD), late-onset Alzheimer's disease and other chronic age-related disorders.

ApoE is an important modulator of many stages of lipoprotein metabolism and traditionally the increased risk was attributed to higher lipid levels in E4 carriers. However, more recent evidence in cultured cells, targeted gene replacement mice, and humans demonstrates the multifunctional nature of the apoE protein. The impact of the apoE genotype on disease risk may be due to an impact on the oxidant/antioxidant status (e.g. paraoxonase-1 activity), the immunomodulatory/anti-inflammatory as well as the gene-regulatory properties (e.g. Nrf2 signalling) of apoE.

Information regarding the impact of apoE genotype on disease risk is often derived from observational studies or small intervention trials in which retrospective genotyping of the cohort results in small group sizes in the rarer E2 and E4 subgroups. Either larger well-standardised intervention trials or smaller trials with prospective recruitment according to apoE genotype are needed to fully establish the impact of diet on genotype-CVD associations and to establish the potential of dietary strategies to counteract the increased CVD burden in apoE4 carriers.

Flavonoids may counteract inflammatory processes in vitro as well as in vivo. Similarly, flavonoids are inducers of hepatic paraoxonase-1, an enzyme that prevents LDL oxidation. Interestingly, the apoE3 genotype is more responsive than the apoE4 genotype in terms of potential health promoting effects of dietary flavonoids as far as anti-inflammatory properties, inhibition of LDL oxidation (Boesch-Saadatmandi et al. 2010), and blood pressure lowering effects (Egert et al. 2010) are concerned.

Boesch-Saadatmandi C, Niering J, Minihane AM, Wiswedel I, Gardeman A, Wolffram S, Rimbach G. (2010): Impact of apolipoprotein E genotype and dietary quercetin on paraoxonase 1 status in apoE3 and apoE4 transgenic mice. *Atherosclerosis* 211(1):110-113

Egert S, Boesch-Saadatmandi C, Wolffram S, Rimbach G, Müller MJ. (2010): Serum lipid and blood pressure responses to quercetin vary in overweight patients by apolipoprotein E genotype. *Journal of Nutrition* 140(2):278-84.

## Neurotrophic interactions with diet and oxidative stress in the aging brain

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Various neurotrophic factors, including insulin-like growth factor I (IGF-I), a prototype neuroprotective signal, decrease during aging. While we still have an incomplete understanding of the impact of this decrease on the aging brain, it appears likely that reduced neurotrophic input is detrimental rather than adaptive. An important mechanism purportedly associated to aging is oxidative stress, which in turn may contribute to reduced neurotrophic input by interfering with intracellular neurotrophic pathways. This is exemplified by cell type-specific resistance to neuroprotection by IGF-I induced by oxidative stress. In response to acute oxidative stress neurons become insensitive to the rescuing actions of IGF-I while astrocytes show an enhanced anti-oxidant response. While oxidative stress is a by-product of normal cell metabolism, additional disturbances of cell metabolism may arise from imbalanced diets, a common situation in industrialized societies. We recently reported that high blood triglycerides as a result of inadequate diet disturb trophic IGF-I input to the brain. Therefore, a compound effect on brain aging of these 3 factors: reduced neurotrophic input, cumulative oxidative stress and impaired cell metabolism may be envisaged. Effective measures for healthy brain aging should include regular physical activity to maintain neurotrophic input and prime against oxidative stress together with balanced diets. As these are common recommendations for healthy aging, implementation of public health policies will greatly contribute towards this end.

## **Up-regulation of longevity associated genes by moderate wine consumption is conserved from drosophila to human beings**

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For more than two decades, it has been known that those populations who take a moderate intake of red wine suffer fewer cardiovascular diseases than those who have a similar diet but without red wine consumption. This was termed “the French paradox” because it was first observed in the wine producing areas in France. However, this epidemiological study required a clear-cut test in controlled populations in which external factors, particularly those involved in the lifestyle, could be ruled out.

Moreover, to our knowledge, the number of studies in which red wine had been given as part of the normal diet of experimental animals to measure longevity had been scarce.

The discovery of sirtuins and their up-regulation by resveratrol was another important hallmark in the modulation of life span by wine and its components. However, the importance of resveratrol in the control of life span has been debated recently.

We have shown recently that sirtuin expression is subjected to tight redox control by the NADH/NAD ratio. It is well known from pioneer work by Krebs and his co-workers that ethanol shifts this ratio to a more reduced state. We showed that ethanol, which indeed reduces the NADH/NAD ratio causes a clear up-regulation of sirtuins.

With this framework in mind, we decided to test the effects of wine consumption on longevity and on longevity-associated genes in experimental animals: invertebrates (*Drosophila*), in vertebrates (mice) and in human beings (Catholic nuns).

We tested longevity in *drosophila* and both with and without red wine added to their food. We found that moderate wine consumption increases the average life span of *drosophila* by 11%, i.e., a significant increase. Maximal life span was not affected. We measured two longevity-associated genes, sirtuins and catalase and both of them were up-regulated in *drosophila* which were fed with red wine.

We also tested the effect of longevity-associated genes in rats which were fed red wine for three months. The ingestion of red wine did not affect the weight of these animals. Importantly, the expression of longevity-associated genes was markedly increased particularly for catalase and for sirtuin.

Finally, we tested the effect of red wine consumption in a population of human beings who are under strict control of lifestyle. Catholic nuns are an excellent model for this because they live in convents and they have exactly the same daily activity and food intake. Blood samples were taken before and after three weeks in which the nuns were given two glasses of (150 ml) of Spanish red wine. A battery of longevity-associated genes was measured in mononuclear cells of the nuns. It is noteworthy that in all cases each of the three longevity-associated genes that were tested (sirtuin, p53, and catalase) did indeed increase their activity by percentage ranging the 30-40%. The significance of these results in terms of a possibility of providing biological bases for the French paradox will be discussed.

## Environmental stress, Sirt1, and lung inflammaging

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Oxidative stress as a result of cigarette smoking and inflammation are major hallmarks of various chronic inflammatory diseases, cancer, and premature aging. The yeast Sir2 or mammalian ortholog Sirtuin1 (Sirt1) has been identified as a key regulator of lifespan in several model organisms. Sirt1, a NAD<sup>+</sup>-dependent protein/histone deacetylase, which regulates inflammation, aging, energetics, mitochondria biogenesis, stress resistance, senescence/aging, endothelial functions, apoptosis/autophagy, and circadian rhythms through deacetylation of transcription factors and histones. Sirt1 deacetylase level and activity are decreased in chronic inflammatory conditions and aging where oxidative stress occur. It is oxidatively down-regulated by cigarette smoke/oxidants/aldehydes, leading to post-translational modifications, inactivation and protein loss via the proteasome. Furthermore, Sirt1 is shown to be carbonylated and glutathionylated *in vivo* in mice exposed to CS, that these modifications could be attenuated by increasing intracellular thiols by NAC *in vitro* and *in vivo* in mice over-expressing Glrx1, an enzyme that reverses glutathionylated proteins. It has been shown that oxidant/carbonyl stress-mediated reduction of Sirt1 leads to loss of its control of acetylation of its target proteins p53, RelA/p65 and FOXO3, thereby enhances the inflammatory, senescence and apoptosis responses (hallmarks of various chronic inflammatory diseases and premature aging). Furthermore, Sirt1 regulates cigarette smoke-mediated pro-inflammatory mediators releases via NF- $\kappa$ B, implicating a role of Sirt1 in sustained inflammation and aging of the lungs. In addition, Sirt1 protects mice against pulmonary emphysema through a novel mechanism involving negative regulation of MMP-9 by deacetylating TIMP-1. Dietary polyphenols, such as resveratrol activates Sirt1 and protects against cigarette smoke-induced lung inflammation. Hence, activation of Sirt1 by polyphenols may have beneficial effects in regulation of inflammation, cellular senescence, autophagy/apoptosis, mitochondria biogenesis, circadian rhythm, and endothelial dysfunction. Current knowledge on environmental stress/cigarette smoke/oxidant-mediated post-translational modifications of Sirt1 deacetylase, NF- $\kappa$ B activation, FOXO3 regulation, and chromatin remodeling leading to pro-inflammatory gene transcription, and their regulation by dietary polyphenolic compounds in inflammation-aging (inflammaging) will be presented.

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## Micronutrients – a global perspective



Dr. Eggersdorfer Manfred

Sufficient intake of micronutrients is essential for optimal health. It is well established that in developing countries hundreds of million people suffer from micronutrient and mineral deficiencies. Less obvious and accepted is that this is also an issue in developed countries. However there is growing evidence from food and intake surveys in countries like USA, Canada, Germany, France, Great Britain and many others that a sufficient intake of micronutrients is not reached according recommendations. As the insufficient intake does not result in immediate consequences like deficiency symptoms the impact and long term effects on health, wellness and healthcare costs are often neglected. Assessments by different research groups indicate that the financial burden on direct and indirect health care costs is for single countries in the range of tens of billion Euros. The presentation will focus on the analysis of data available for US and Germany.

For instance, taking the data from the German Nutritional Survey (NVSII), several micronutrients were below recommended intake levels: The mean intakes for vitamin D and folic acid were markedly below the D-A-CH reference values in both, men (83% and 79%, respectively) and women (91% and 86%, respectively), for all age groups and 48% men and 49% women did not meet recommended vitamin E intakes. Similar results were seen for vitamin C. Although overall vitamin A intake looks good, it is important to mention that pre-formed vitamin A was not sufficient to cover vitamin A needs. Beta-carotene contributed (especially in women) highly to achieve recommended overall vitamin A intake. Similar results on insufficient vitamin intakes were reported in other studies (e.g. VELS, Dietary Habits in Denmark Survey, NHANES).

It is known that a number of factors have impact on vitamin status such as age, gender and genetic makeup. The crucial role of vitamins for a number of important biological processes suggests a more in depth analysis of inter-individual differences in candidate genes of vitamin pathways as contributing factor to health related outcomes. Besides the well known SNPs in 5,10-Methylenetetrahydrofolate reductase (NADPH) (*MTHFR*), Vitamin D-binding protein (*GC*) and the beta-carotene 15,15'-monooxygenase 1 (*BCMO1*), a number of other variants in vitamin metabolism have been reported to impact on various metabolic pathways.

In this context of inadequate vitamin supply in the developed world Ames et al. suggested that long-term (underlying) inadequate vitamin intake is potentially linked to several diseases (triage theory). Based on these existing nutrient intake surveys it is warranted to pay more attention to nutrient intake levels in the general population and in particular with a focus on vulnerable groups at high risk.

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## **Skin aging: role of UV and infrared radiation**

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UV, visible light and infrared are all parts of the solar spectrum reaching the earth surface. Although, the energy of photons in the infrared spectrum is rather low, a substantial part of the total solar energy output reaching the earth surface is due to this part of the solar spectrum. Therefore, exposure of humans to infrared is common. However, whether and how infrared or infrared A is acting on human skin cells is still under debate. In contrast the effects of UVB and UVA are often demonstrated and substantial mechanistic evidence exists for molecular targets of this irradiation. On the other side molecular targets for infrared are missing and the potential role of this part of the solar irradiation is not clear.

Interestingly, the generation of reactive oxygen species by water-filtered-infrared-A (wIRA)-irradiation was postulated recently. wIRA shows a similar spectral distribution as solar irradiation at Earth's surface and is emitted by an artificial infrared lamp and filtered through a thin layer of water. We were able to demonstrate that in human dermal fibroblasts the reactive oxygen species generation by wIRA is dependent on heat formation and can be reproduced by thermal exposure. wIRA-irradiation had no detectable effect besides those of heat formation: if the temperature in the cells was maintained constant no effect was observed.

We concluded that the major skin damaging and, therefore skin aging, effects are due to UV irradiation if the skin temperature is balanced by the physiological blood flow.

## Connexin 43 and metabolic effect of fatty acids in stressed endothelial cells.

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Cellular stressors cause changes in the inner mitochondrial membrane potential ( $\Delta\psi$ ) leading to mitochondrial swelling and cell apoptosis, as well as induction of protective for cell survival autophagy. Connexin 43 (Cx43) is a main gap junction protein in endothelial cells. The redistribution of Cx43 in the cellular membranes during the ischemia-induced cellular stress has been recently reported. Free fatty acids (FFA) as well as TNF $\alpha$ , especially in the postprandial period, are inducers of oxidative stress that lead to development of metabolic syndrome vascular complications,

**Aim of the study** is to follow effects of selected dietary FFA on the Cx43 expression and mitochondrial function in the endothelial cells challenged with TNF $\alpha$ .

**Methods:** HUVECs were incubated with non-toxic, physiological (10-30 $\mu$ M) concentrations of albumin-bound palmitic (PA), oleic (OA), eicosapentaenoic (EPA) or arachidonic (AA) acids for 24 hours. 5ng/mL TNF $\alpha$  was added for the last 4 hours of incubation.. The expression of *Cx43* gene was analyzed by the quantitative real-time PCR (qRT-PCR) method. The Cx43 protein concentration in whole cells, as well as in isolated mitochondria was measured by western blot. Changes in the mitochondrial membrane potential ( $\Delta\psi$ ) were measured by flow cytometry, while  $\Delta\psi$  and the localization of Cx43 protein were analyzed by BD Pathway 855 Bioimager. The generation of ATP was measured by ATPlite™ Luminescence ATP Detection Assay.

**Results:** The significant decrease of  $\Delta\psi$  following incubation with TNF $\alpha$  (p=0.003) as well as with PA (p=0.042) and OA (p=0.002) was observed. On the contrary, AA (p=0.047) as well as EPA (p= 0.004) led to increase of  $\Delta\psi$ . Initial incubation with EPA or AA also partially prevented the TNF $\alpha$ -induced decrease of  $\Delta\psi$ . Incubation with AA resulted in the up-regulation of *Cx43* gene expression. Addition of AA as well as PA significantly increased Cx43 protein cellular content.

**Conclusions:** The up-regulation of *Cx43* expression and Cx43 protein concentration along with normalization of the mitochondrial function ( $\Delta\psi$ ) and increased ATP generation seems to be one of the mechanisms of EPA-mediated protective effect in the endothelial cells.

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**Age-dependent accumulation of proteins modified with oxidation products of polyunsaturated fatty acids in the brain: Possible involvement in the pathogenesis of neurodegenerative disorders.**

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“Free radical theory” is generally proposed as molecular mechanism behind aging-associated deterioration of brain function, but it remains enigmatic how the “free radicals” induce neuronal loss. In the brain, neurons are most vulnerable, because of the continuous exposure to oxidative stress caused by high oxygen consumption and limited radical scavenging capacity. In addition, neuronal membrane is rich in polyunsaturated fatty acids (PUFA) including  $\omega$ -3 docosahexaenoic acid (DHA) and  $\omega$ -6 arachidonic acid. These PUFAs are required for the membrane fluidity, which is essentially associated with synaptogenesis and signal transduction.

However, PUFAs are highly sensitive to oxidative stress and produce reactive lipid peroxide. In this paper, we present our recent results on the accumulation of abnormal proteins produced by oxidative modification with PUFA-derived lipid peroxide. To this study, we prepared anti-body specific for detection of lipid peroxide produced from  $\omega$ -3 or  $\omega$ -6 PUFAs, respectively. In old (24 month) rat brain, propanoylated lysine residues of proteins as an indicator of  $\omega$ -3 peroxidation were detected in CA1 area of the hippocampus. Immunoprecipitation and confocal microscopy studies identified propanoylated protein as cytoskeleton protein, such as tau.

In the postmortem brains of Alzheimer disease, high contents of propanoylated proteins were detected, whereas they were not found in the normal human brain. Propanoylated protein was localized in the neuronal cells and accumulated in the senile plaques. The cytotoxicity of amyloid  $\beta$  protein (A $\beta$ ) was increased by propanoylation, as shown by injection of synthesized propanoylated A $\beta$  into the rat brain.

These results indicate that protein modified with lipid peroxide from PUFAs accumulates in neurons, mostly in membrane, and might be involved in ageing-dependent brain dysfunction and neurodegenerative disorders, such as Alzheimer disease.

## **Proteome alteration in human myoblasts upon oxidative stress and replicative senescence**

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Age-related decline in skeletal muscle mass during ageing has been attributed at least in part to a reduction in regenerative potential of resident stem cells, also known as satellite cells. Although increased oxidative stress is associated with the impairment of the proliferation and function of these cells, the proteins either involved in the response to oxidative stress or those damaged by oxidation have not yet been identified. In this work changes in the proteome of human myoblasts during replicative senescence and in response to acute oxidative stress have been addressed. A parallel proteomic analysis aimed at identifying differentially expressed proteins as well as those targeted by carbonylation has been performed. The carbonylated proteins identified either during replicative senescence or upon acute oxidative stress are mainly cytosolic and involved in key cellular functions, such as carbohydrate metabolism, cellular motility, cellular homeostasis, protein synthesis and protein degradation. Interestingly, almost half of the proteins identified as increasingly carbonylated were the same under the two different experimental conditions, suggesting a particular susceptibility of some proteins for oxidation. In addition, data mining indicates that these proteins can be grouped in a pathway analysis pointing to skeletal and muscle disorders, cell death and cancer as the main molecular networks altered. We next evidenced the proteins which expression level has changed. A different set of proteins were found to be upregulated or down regulated in both approaches suggesting different effects on the proteome upon the two experimental conditions used. As expected, most of the proteins found to be increased upon oxidative stress were those involved in the antioxidant stress response, however, proteins involved in cell morphology, cell motility and energetic metabolism were found to be differentially expressed during replicative senescence. Taking together, our results indicate that proteins involved in key cellular pathways are affected upon oxidative stress and replicative senescence and the impairment of them may be implicated in cellular dysfunction. In addition, this study underscores the importance of performing proteomic analyses looking at different aspects, such as the expression level and specific post-translational modifications, in order to have a broader view of changes affecting the cellular proteome.

## Lipid peroxidation and protein tyrosine oxidation are mechanistically-associated events in hydrophobic biocompartments: consequences in biomembranes and lipoproteins

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Oxidative stress encompasses a number of oxidative modifications in biomolecules, many of which are triggered by free-radical dependent processes. Prime examples of these are lipid peroxidation and protein oxidation, which can lead to direct alteration of biological function and/or formation of secondary reactive intermediates/products. In biologically-relevant hydrophobic biocompartments such as biomembranes and lipoproteins, lipid peroxidation and protein oxidation may be intertwined due to the organization of these structures resulting in close proximity among the different participating biomolecules. In the past years<sup>1-4</sup>, we have explored how lipid peroxidation and tyrosine oxidation/nitration are interrelated, using model membrane systems, red blood cell membranes and isolated LDL. Mechanistic studies have been performed by incorporation of a series of hydrophobic tyrosine analogs to the tested lipidic systems and exposing them to a variety of oxidizing systems including peroxytrite, peroxy radical donors and hemin. Overall, we have found that lipid peroxidation and tyrosine oxidation processes in hydrophobic biocompartments are connected *via* the reaction of lipid peroxy radicals (LOO<sup>•</sup>) with tyrosine residues which results in the one-electron oxidation of tyrosine to tyrosyl radicals (Tyr<sup>•</sup>):



The overall proposed mechanism connecting both processes predicts that 1) tyrosine oxidation yields will be influenced by oxygen levels, as molecular oxygen is a critical reagent in the propagation phase of lipid peroxidation<sup>1</sup> and 2) that a fraction of the tyrosyl radicals could combine with lipid peroxy radicals to yield termination adduction products. Indeed, in the different tested systems the levels of oxidized and/or nitrated tyrosine (to 3,3'-dityrosine and/or 3-nitrotyrosine, respectively) were significantly decreased under low oxygen tensions as happened with lipid peroxidation products (lipid hydroperoxides and malondialdehyde). Second, peroxy radical formation from PLPC (13S)-OOH by MeOAMVN (2, 2'-azobis (4-methoxy-2,4-dimethylvaleronitrile) in the presence of BTBE or hydrophobic tyrosine-containing peptides resulted in the formation of novel compounds which were HPLC purified and analyzed by MS and NMR-based techniques and identified as of Diels-Alder type tyrosyl-lipid tricyclic adducts. The data showed herein further support the connection between lipid peroxidation and protein oxidation processes *in vitro* and *in vivo*<sup>5</sup>, revealing a previously unrecognized influence of oxygen levels on tyrosine oxidation and nitration yields and identifying mixed oxidation products which may represent novel biomarkers and mediators of membrane and lipoprotein oxidative damage.

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## Pepsinogen is nitrated in the stomach in vivo: modulation by NSAIDs, oral bacteria and salivary nitrite

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Dietary nitrate, long believed to be an inert product of nitric oxide (<sup>•</sup>NO) oxidation, is now recognized as a critical mediator of physiological functions in the gastrointestinal tract (GIT) due to its stepwise reduction to nitrite and <sup>•</sup>NO. In the stomach, <sup>•</sup>NO is engaged in gastroprotection but nitrite can also generate other reactive nitrogen oxides with nitrating capacity, which may have an impact on gastric pathophysiology. Here we studied the impact of dietary nitrite supplementation on the nitration *status* of the healthy and ulcerated rat gastric mucosa. Additionally, the *in vivo* nitration of specific gastric mediators, namely pepsinogen, was screened in connection with the impact of such post-translational modification on pepsin function. Finally, germ-free mice were used to elucidate the role of bacteria in gastric nitration reactions in the stomach.

Wistar rats were divided in two groups: a healthy untreated group and another one experiencing acute gastric inflammation induced by diclofenac (30 mg.Kg<sup>-1</sup>). Animals were further divided in two subgroups, one fed with nitrite 1.38 mg.Kg<sup>-1</sup> and the other with water. All compounds were given by oral gavage. Protein tyrosine nitration was evaluated by immunohistochemistry and immunoprecipitation. Nitrotyrosine (NT) labeling was detected in the deep mucosa of untreated rats, suggesting that nitration is a physiological event in the stomach. NT yields increased in the stomach of rats with acute gastric ulceration ( $p < 0.01$ ) and were further enhanced in the subgroup fed with nitrite ( $p < 0.01$ ). NT staining was located within the *lamina propria* and blood vessels but also in cells of the oxyntic glands, where an intense cytoplasmic staining suggests nitration of specific gastric mediators stored in cytoplasmic vesicles, such as pepsinogen. Indeed, pepsinogen nitration occurs under basal conditions but increases under gastric ulceration. Unexpectedly, healthy nitrite-fed rats showed reduced levels of both, overall and pepsinogen nitration in respect to untreated rats. Pepsinogen nitration had no effect on activation to form pepsin, but it strongly inhibited the proteolytic activity of pepsin ( $p < 0.001$ ).

Mechanistically, both <sup>•</sup>NO-dependent (e.g. iNOS and nitrite-derived <sup>•</sup>NO) and independent (e.g., via myeloperoxidase activity) nitration pathways can be envisaged. In order to address the origin of the nitrating species *in vivo* we used germ-free (GF) mice because these animals lack nitrate-reducing bacteria and, therefore, do not reduce nitrate to nitrite, thereby preventing <sup>•</sup>NO formation in the gastric lumen. GF mice showed lower levels of NT under basal conditions than wild-type. When nitrite (but not nitrate) was added to the drinking water (1 mM for 7 days) NT yields decreased ( $p < 0.01$ ) whereas in wild-type mice both, nitrate and nitrite, induced a decrease in NT. No differences in gastric iNOS expression were found between GF and conventional animals. The results support that protein nitration is in part regulated by salivary-derived nitrite, gastrointestinal bacteria and intragastric generation of reactive nitrogen oxides.

We demonstrate that pepsinogen is nitrated under physiological conditions in the stomach and that nitration increases under acute ulceration. Dietary nitrite seems to have a dual role in modulating gastric nitration as it enhances nitration under inflammatory conditions but has the opposite effect in healthy animals. The results also suggest that pepsinogen nitration impairs the proteolytic function of the derived pepsin, thereby impacting on protein function. Expectedly, an inefficient protease would impair the digestion of dietary proteins but, on the other hand would also prevent the breakdown of endogenous proteins (mucins, collagen) vital for gastric integrity, thus preventing peptic ulcer disease.

Overall, these results unravel a role of dietary nitrite in the gastro physiopathology via nitrative modification of local proteins.

## Involvement of mitochondrial and oxidative mechanisms in glyphosate-induced epidermal cell death

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A deregulation of programmed cell death mechanisms in human epidermis accelerates skin aging. We previously showed that glyphosate provoked cytotoxic effects on cultured human keratinocytes, affecting their antioxidant capacities (1-2) and impairing morphological and functional cell characteristics (3). This glyphosate-induced oxidative stress investigation can provide us a possible way to an interesting *in vitro* skin aging model. The aim of the present study, led on the human epidermal cell line, HaCaT, was to examine the part of apoptosis in the cytotoxic effects of glyphosate and the intracellular mechanisms involved in the apoptotic events. We have conducted different incubation periods to reveal events implied in glyphosate-induced cell death. We observed an increase in the number of early apoptotic cells for a low cytotoxicity level (15%), and then, a decrease, in favor of late apoptotic and necrotic cell rates for stronger cytotoxicity conditions. At the same time, we showed that the mitochondrial membrane potential disruption could be a cause of apoptosis in keratinocyte cultures. It is now apparent that the oxidative imbalance is not only glyphosate dose-dependent, but also directly related to the exposure time; this conceptual advance could concern many other environmental toxic agents. Moreover, an original and complementary study by Atomic Force Microscopy (AFM) allows us to combine imaging and mechanical properties measurements on HaCaT cells for different oxidative stress conditions. Finally, a better understanding of the glyphosate-induced deleterious mechanisms will help us in a relevant choice of antioxidant molecules able to reverse these cellular impairments.

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## **Calorie restriction improves diabetic complications via reduction of mitochondrial reactive oxygen species in Type II diabetic rats**

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We determined the effects of calorie restriction (CR) on the dynamic aspects of mitochondrial reactive oxygen species (ROS) production, uncoupling protein 2 (UCP2), and the nitric oxide (NO)-cGMP pathway in the cardiovascular tissues of type II diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Some rats were on restricted diets (30% reduction from free intake) from age 29 to 42 weeks. Blood glucose, hemoglobin A1c, plasma levels of free fatty acid, triacylglycerol, etc. in OLETF rats were significantly higher than those in nondiabetic control rats at 29 weeks. Mitochondrial ROS production and UCP2 expression significantly increased in the heart and aorta of OLETF rats compared with those in LETO rats. A fibrogenic growth factor, TGF- $\beta$ 1 in the coronary vessels, endothelial nitric-oxide synthase, and aortic nitrotyrosine were increased in OLETF rats at 42 weeks. In contrast, an index of the NO-cGMP pathway, phosphorylated vasodilator-stimulated phosphoprotein, and superoxide dismutase activity in the aorta were significantly diminished. The relationship between UCP2 and ROS production in the cardiovascular function of diabetic rats being fed a calorie-restricted diet is unknown. These abnormalities in OLETF rats were reversed to normal levels by CR. CR significantly improved the NO-cGMP pathway via normalizing ROS generation in OLETF rats. A decrease in UCP2 expression by CR may be a compensatory mechanism to counteract decreased intracellular oxidative stress. The data suggest that CR may prevent cardiovascular tissues. [This work was supported by Grant-in-Aid for Scientific Research (C)]

## The impact of flavonoid-rich blueberries on memory and learning

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Phytochemical-rich foods have been shown to be effective at reversing age-related deficits in memory and learning in both animals and humans. Evidence suggests that these effects are underpinned by the ability of flavonoids to modulate neuronal signalling and to inhibit neuroinflammation. We have investigated the effects of a chronic blueberry (BB) intervention on spatial working memory in both young and aged animals, and have determined the molecular changes in the brain underlying these behavioural changes. In two independent experiments, young (1 month) and aged (18 month) rats were fed a placebo control or a control + BB (2% w/w) diet for a total of 6 weeks. During feeding, spatial memory was assessed in an 8-Radial Arm Radial Maze (young) or T-maze (old) model. The results indicated that blueberry intervention significantly increased spatial working memory task performance in both young ( $p=0.023$ ) and aged animals ( $p=0.001$ ), although the effect observed in the young animals was less pronounced. Furthermore, chronic supplementation of aged animals with pure flavanols (catechin and (-)-epicatechin) or a purified BB anthocyanin extract, revealed that both flavanol and anthocyanin intervention resulted in a pattern of cognitive improvement very similar to that observed following blueberry intervention ( $p=0.001$ ;  $p=0.004$  respectively). The changes at the behavioural level were accompanied by changes in total protein levels of BDNF along with region-specific BDNF gene expression (DG and CA1) in the hippocampus. Our data provide further weight to the growing body of evidence linking flavonoid-rich foods with improvements in both memory and cognition.

*Blueberry; Spatial; Memory; Learning; Flavonoids*

## Alternative mechanisms of quercetin neuroprotection involving Nrf2-dependent modulation of antioxidant defenses

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There is a growing interest in the neuroprotective potential of flavonoids although the exact mechanisms by which these compounds exert their benefits are not fully understood. Though these favorable effects have been largely related to their classical hydrogen-donating antioxidant activity, in the past 10 years, evidence from cell studies suggests that flavonoids can influence cellular fate by other mechanisms of action involving protein and lipid interactions leading to enzymatic modulation, interaction with several receptors, modulation of intracellular signaling cascades, and modulation of gene expression.

In the present work we describe the protective effects of the flavonoid quercetin against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in 24h-pretreated neuronal cultures. In particular we explored quercetin availability and subcellular fate through the use of HPLC-Diode Array Detection (DAD), epifluorescence, and confocal microscopy. Furthermore, we focused on the analysis of ROS and lipoperoxidation products formation (DCFH-DA, and MDA assays, respectively). Besides, we focused on quercetin activation of the protective NF-E2-related factor 2 (Nrf2)-dependent signaling pathway, on the modulation of total glutathione (GSH) levels, and on the expression of the  $\gamma$ -glutamate–cysteine ligase catalytic subunit (GCLC), the rate-limiting enzyme of GSH synthesis (by immunocytochemistry and Western blot, HPLC, and real-time PCR, respectively).

Our results showed rapid quercetin internalization into neurons, reaching the nucleus after its addition to the culture. At the moment of H<sub>2</sub>O<sub>2</sub> insult, intracellular quercetin or related metabolites were undetectable in the cultures. Nevertheless, quercetin pretreatment prevented the formation of ROS and lipoperoxidation products, and finally prevented neuronal death from the oxidant exposure as well. Furthermore, quercetin treatment caused Nrf2 nuclear translocation, and it increased GCLC gene expression, and total GSH levels.

Our findings suggest alternative mechanisms of quercetin neuroprotection beyond its long-established direct antioxidant (hydrogen-donating) properties, involving Nrf2-dependent modulation of endogenous antioxidant defense systems. Further gene-silencing studies should clarify the relevance of Nrf2 pathway as a novel target involved in quercetin neuroprotective effects.



## Evidence of health benefits of polyphenols enriched foods : from *in vitro* studies to clinical trials performed at University – CHU of Liège, Belgium

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Oxidative stress results from a decrease of endogenous antioxidant capacities or an increase of reactive oxygen species (ROS) concentration in organisms. It can cause deleterious damages on cell constituents like DNA, proteins or lipids and finally, could induce several pathologies. Phytochemicals of fruits and vegetables such as polyphenols have been considered of crucial nutritional importance in the prevention of chronic diseases such as cancer, cardiovascular and neurodegenerative diseases. This may be related to their antioxidant activity as well their ability to regulate cellular activities of inflammation-related cells.

A large number of methods and variations have been developed and applied for the measurement of *in vitro* antioxidant capacity and efficacy of food matrix. The most popular assay is the ORAC assay (Oxygen Radical Antioxidant Capacity) initially developed by the US Agriculture Minister and using 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH) as free radical generator. However, our expertise shows that results are strongly dependent of experimental conditions so that comparison between laboratories is impossible (Food Chem 113, 1226-1233, 2009). It is therefore required to better standardize the method as we recently proposed it (ORAC®). Another problem is that ORAC values do not necessarily correlate with the concentration in polyphenols present in the food matrix but also with data obtained with other tests for evaluating the antioxidant capacity such as DPPH, ABTS or FRAP assays. Moreover, a weakness of all these methodologies is that they do not use physiological free radical generator. So, we proposed to integrate all these results as a global antioxidant scoring® using the model of Chernoff's figures. An example will be given with 18 commercial orange juices.

The great challenge of the future will be the demonstration of nutritional and health assertions of polyphenols enriched foods via conclusive clinical trials. Recently, Afssaps has, however, rejected the "antioxidant" assertion since no convincing data are available on the regulation of the *in vivo* oxidative stress due to a lack of sensitivity and specificity of used methodologies. This will be discussed in our presentation. Nevertheless polyphenols are well – known to contribute to largely increase the release of nitric oxide or NO (a free radical produced by endothelial cells) that is implicated in the regulation of the blood flow and, consequently, of the arterial blood pressure. As ROS conditions strongly react with NO, it could be speculated that polyphenols may also exert NO protection provided that their blood concentration at least reaches 10 µM. We are convinced that clinical trials on polyphenols must be therefore focused on the regulation of *in vivo* endothelial function (and thus blood pressure) in order to get a chance to evidence a health assertion linked to antioxidant properties as now required by the European Food Safety Authority (EFSA). Interesting data both on aorta segments of rats or in humans have already been obtained with different polyphenols enriched foods (red wine, blackcurrant, ginkgo biloba extract, black chocolate). Nevertheless, this beforehand requires to evidence the *in vivo* biodisponibility of the active ingredient(s).

## **Taurine and Beauty: Alleviation of ageing; Cosmeceuticals to prevention of hair loss**

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Ageing is love by none; even one does not like but can not escape. Ageing is a complex process and is composite in nature. Ageing can be marked by variety of symptoms, external and internal. Some of the external components includes; wrinkles, loss of hair, hydration of skin (aged skin) etc. Wrinkles now have a great social impact because people live longer. Same is true for hair loss and loose skin. All these happening definitely affect the totality of beauty subsequently the personality.

It is believed that wrinkles, hair loss, look skin etc are generally due to prominence of reactive oxygen species (ROS), changing osmotic conditions, as well as ion concentration mainly calcium ions in T cells. An ideal therapeutic agent must have properties to prevent such happening and also to some extent reverse the effects. In short cosmetic approach to wrinkles follow these steps; cleaning, photo-protection and application of active ingredients. First two are routine in nature but active ingredients play important role. There are several such functional ingredients like AHAS to anti-oxidants. Anti oxidants provides trap to ROS. Taurine is well recognized anti oxidants and currently is part of several anti ageing formulas, anti wrinkle agents and behaves as cosmeceuticals. Similarly taurine is a systemic anti-fibrotic agent and hopefully a definite boon for hair growth. In case of loose skin, skin keratinocytes are subjected to changing osmotic conditions. In principle osmolyte transporters serves maintenance of cell volume in hyper-toxic environment. Hyper osmotic stress significantly decreases the proliferation of HaCaT keratinocytes. Supplementation of taurine and some others are able to reverse the trends. Thus taurine gets unique "Status" to be classifying as such agent. However, amino acids are highly polar molecules there fore unable to penetrate into deeper epidermal layers after topical applications. Even higher doses due to its zwitterionic nature takes considerable longer duration for clinical efficiency, hence lipophilic analogues will be more suitable. As of now taurine is regarded as components of functional foods and drugs; after careful evaluation taurine and analogues may constitute a new class of cosmeceuticals and hair growth promoters.

## Modification of alpha-synuclein by lipidperoxide derived from PUFA – The relevance of cell death in PD

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Protein fibril formation is generally considered to be associated with neuronal death in neurodegenerative disorders, such as Parkinson disease (PD) and Alzheimer disease. Lewy body (LB), intracellular inclusions in dying dopaminergic neurons of substantia nigra, is the pathological hallmark of PD.  $\alpha$ -Synuclein ( $\alpha$ -SYN) protein is a major component of LB and play an important role in maintaining function of synaptic vesicle and mitochondria, producing dopamine and regulating cellular redox state. Docosahexaenoic acid (22:6n-3, DHA) is the one of the most common long chain polyunsaturated fatty acids (PUFA), that is essential fatty acids for human and critical component of membrane lipids involved in the signal transduction systems. DHA is highly enriched in the brain and retina as a component of phosphatidylethanolamine and phosphatidylserine in neural membrane and it is essential for neural functions (membrane-fluidity, development, synaptic plasticity, signal transduction). DHA has six double bonds and functions as a potent antioxidants. However, DHA itself is easily oxidized by reactive oxygen species (ROS) to produce lipid peroxides highly reactive to proteins.

In this paper, we present our recent results on the modification of  $\alpha$ -SYN with DHA-derived lipid peroxide, in relation to the generation of inclusion body in PD.  $\alpha$ -SYN oligomerization and fibrillization was enhanced by DHA in the dose-dependent way *in vitro*. This oligomer of  $\alpha$ -SYN was found to be modification by lipid peroxides derived from DHA, propanoyl (PRL)-lysine and succinyl (SUL)-lysine as proved by specific antibodies. In addition, to determine the effect of DHA modification of  $\alpha$ -SYN on the viability of neural cells, we established SH-SY5Y cells, in which human  $\alpha$ -SYN were transfected (Syn-SH cells). Syn-SH cells transiently expressed  $\alpha$ -SYN (20k) and truncated  $\alpha$ -SYN (16k) as proved by Western blotting analysis. After incubation with DHA for 3 days, death of Syn-SH cells was induced and intracellular ROS production was increased in a dose dependency way. PRL-positive protein was increased in mitochondria and nuclei/membrane fraction as quantified with ELISA system. In Syn-SH cells treated with DHA, Lewy body-like protein aggregate was observed in the cytoplasm and they are simultaneously stained with anti- $\alpha$ -SYN and anti-PRL antibody. These results suggest that age-dependent accumulation of oxidatively modified  $\alpha$ -SYN may induce neuronal death, though the changes in the higher structure, including oligomerization, fibrillization and aggregation, in PD.

## Connexin 43 and metabolic effect of fatty acids in stressed endothelial cells.

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Cellular stressors cause changes in the inner mitochondrial membrane potential ( $\Delta\psi$ ) leading to mitochondrial swelling and cell apoptosis, as well as induction of protective for cell survival autophagy. Connexin 43 (Cx43) is a main gap junction protein in endothelial cells. The redistribution of Cx43 in the cellular membranes during the ischemia-induced cellular stress has been recently reported. Free fatty acids (FFA) as well as TNF $\alpha$ , especially in the postprandial period, are inducers of oxidative stress that lead to development of metabolic syndrome vascular complications,

**Aim of the study** is to follow effects of selected dietary FFA on the Cx43 expression and mitochondrial function in the endothelial cells challenged with TNF $\alpha$ .

**Methods:** HUVECs were incubated with non-toxic, physiological (10-30 $\mu$ M) concentrations of albumin-bound palmitic (PA), oleic (OA), eicosapentaenoic (EPA) or arachidonic (AA) acids for 24 hours. 5ng/mL TNF $\alpha$  was added for the last 4 hours of incubation.. The expression of *Cx43* gene was analyzed by the quantitative real-time PCR (qRT-PCR) method. The Cx43 protein concentration in whole cells, as well as in isolated mitochondria was measured by western blot. Changes in the mitochondrial membrane potential ( $\Delta\psi$ ) were measured by flow cytometry, while  $\Delta\psi$  and the localization of Cx43 protein were analyzed by BD Pathway 855 Bioimager. The generation of ATP was measured by ATPlite TM Luminescence ATP Detection Assay.

**Results:** The significant decrease of  $\Delta\psi$  following incubation with TNF $\alpha$  (p=0.003) as well as with PA (p=0.042) and OA (p=0.002) was observed. On the contrary, AA (p=0.047) as well as EPA (p= 0.004) led to increase of  $\Delta\psi$ . Initial incubation with EPA or AA also partially prevented the TNF $\alpha$ -induced decrease of  $\Delta\psi$ . Incubation with AA resulted in the up-regulation of *Cx43* gene expression. Addition of AA as well as PA significantly increased Cx43 protein cellular content.

**Conclusions:** The up-regulation of *Cx43* expression and Cx43 protein concentration along with normalization of the mitochondrial function ( $\Delta\psi$ ) and increased ATP generation seems to be one of the mechanisms of EPA-mediated protective effect in the endothelial cells.

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## Olive oil and nuts in the Mediterranean diet enhance antioxidant enzyme activities and spare NO in metabolic syndrome patients

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Oxidative stress is involved in metabolic syndrome pathologies and its attenuation could ameliorate their symptoms. Mediterranean diet is especially rich in olive oil and is associated with a higher degree of plasma antioxidant capabilities. The aim of this work was to study the effects of the introduction of olive oil or nuts in the diet on the plasma activities and levels of some antioxidant and prooxidant enzymes, on the levels of nitric oxide metabolites and on the levels of oxidative and nitrosative damage markers in patients with metabolic syndrome. The subjects were included in the PREDIMED study. Three groups of twenty paired matched subjects were analyzed. All subjects presented almost two indicators of metabolic syndrome and their diet was controlled for 5 years. The control group consumed a low fat diet, the olive oil group consumed a high fat diet with olive oil as the main source of fat, and the nuts group also consumed a high fat diet but with nuts as the main responsible for the high fat intake. Ten millilitres of blood were extracted in basal conditions after overnight fasting. Plasma was obtained after blood centrifugation at 900g, frozen immediately and stored at -20°C until analysis in the two next days. The plasmatic activities of extracellular superoxide dismutase (ec-SOD), catalase (CAT), and myeloperoxidase (MPO) were measured using standardized methods. The protein levels of CAT, xanthine oxidase (XOD) and ec-SOD were also determined in plasma by western blot techniques. Nitrite and nitrate plasma levels were determined by detection of the liberated NO by gas-phase chemiluminescence reaction with ozone using a Nitric Oxide Analyzer. Nitrotyrosine and protein carbonyls were determined by immunoblot techniques using primary antibodies against nitrotyrosine residues and derivatized carbonyl groups, respectively. The adherence to the Mediterranean Diet was determined from a frequency questionnaire of food intake in accordance with the PREDIMED criteria (Martínez-González et al 2004) and it is validate for a rapid estimation to the adherence to the Mediterranean diet (Schröder et al 2011).

The diet supplementation with nuts or olive oil significantly increased the plasma activity and levels of ec-SOD, the plasma activity of CAT and the plasma levels of nitrate but unaffected the plasma levels and activity of MPO, XOD and the levels of nitrite, nitrotyrosine and protein carbonyls respect the control group. The adherence to the Mediterranean Diet significantly positively correlates with plasma ec-SOD and CAT activities.

The supplementation of the Mediterranean diet with nuts or olive oil enhances plasma antioxidant enzyme activities and spares NO without a major degree of oxidative and nitrosative damage in patients with Metabolic Syndrome.

Martínez-González et al, Eur. J. Clin. Nutr. 58:1550-1552, 2004

Schröder et al, J. Nutr. Apr 20. [Epub ahead of print], 2011.

## Formulation Curcumin loaded nanoparticles improves brain targeting to treat Alzheimer's disease.

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A rapid increase in the incidence of neurodegenerative disorders has been observed with the aging of the population. Among them, Alzheimer's disease (AD) is one of the most common neurodegenerative disorders, characterized by progressive memory loss, loss of lexical access, spatial and temporal disorientation. The accumulation of beta-amyloid peptides and the hyperphosphorylation of tau neurofilament protein are major characteristics of this disease. Oxidative stress plays an important role in the physiology of the onset and the progression of AD.

Many medicinal plants have been traditionally used in Asian countries to enhance cognition and memory processes. Here we show the neuroprotective effects of Curcumin on SK-N-SH cells (human neuroblastoma cells). Curcumin is a yellow colored polyphenol present in turmeric (*Curcuma longa*) that has been reported to have antioxidant and anti-inflammatory actions. However, curcumin is poorly soluble in water, which is one of the reasons for its poor absorption and low bioavailability, thus limiting its clinical application. In the present study, we have formulated curcumin-loaded nanoparticles (Nps Cur), which are environmentally safe, biocompatible and resorbability through natural pathways.

The objective of the research was whether Nps Cur could protect neurons against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) toxicity, widely used as *in vitro* model of oxidative insults. The SK-N-SH cells were co-treated with curcumin, nanoparticles (Nps) and Nps Cur with H<sub>2</sub>O<sub>2</sub> (500 µM) for 24 hrs. The cell survival was measured by LDH and Resazurin assays. The effect of these compounds on intracellular production of reactive oxygen species (ROS) was measured by DCFDA assay. The cellular uptake was observed by fluorescence microscopy.

However, due to their special physicochemical properties, the Nps may cause neurotoxicity after entering into the brain. Therefore, the evaluation of the potential neurotoxic effect of these Nps is required, as specific mechanisms and pathways through which these Nps may exert their toxic effects remain largely unknown.

Like most new technologies, there is a rising debate concerning the possible side effects derived from the use of NPs. The risk associated with exposure to NPs, the routes of entry and the molecular level mechanisms of any cytotoxicity need to be well understood. The toxicity of NPs depend on whether they are persistent or whether the brain can raise an effective response to dispose them. Therefore, the risk/benefits ratio for the use of NPs has to be evaluated. Thus, the development of novel engineered nanocarriers for neuropharmacology, therapeutics and diagnostics must proceed in tandem with assessment of any toxicological and side effects of these particles. Finally, the effects of NPs in the environment after clearance from biological human fluids should also be analyzed.

**Age-dependent accumulation of proteins modified with oxidation products of polyunsaturated fatty acids in the brain: Possible involvement in the pathogenesis of neurodegenerative disorders.**

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“Free radical theory” is generally proposed as molecular mechanism behind aging-associated deterioration of brain function, but it remains enigmatic how the “free radicals” induce neuronal loss. In the brain, neurons are most vulnerable, because of the continuous exposure to oxidative stress caused by high oxygen consumption and limited radical scavenging capacity. In addition, neuronal membrane is rich in polyunsaturated fatty acids (PUFA) including  $\omega$ -3 docosahexaenoic acid (DHA) and  $\omega$ -6 arachidonic acid. These PUFAs are required for the membrane fluidity, which is essentially associated with synaptogenesis and signal transduction.

However, PUFAs are highly sensitive to oxidative stress and produce reactive lipid peroxide. In this paper, we present our recent results on the accumulation of abnormal proteins produced by oxidative modification with PUFA-derived lipid peroxide. To this study, we prepared anti-body specific for detection of lipid peroxide produced from  $\omega$ -3 or  $\omega$ -6 PUFAs, respectively. In old (24 month) rat brain, propanoylated lysine residues of proteins as an indicator of  $\omega$ -3 peroxidation were detected in CA1 area of the hippocampus. Immunoprecipitation and confocal microscopy studies identified propanoylated protein as cytoskeleton protein, such as tau.

In the postmortem brains of Alzheimer disease, high contents of propanoylated proteins were detected, whereas they were not found in the normal human brain. Propanoylated protein was localized in the neuronal cells and accumulated in the senile plaques. The cytotoxicity of amyloid  $\beta$  protein (A $\beta$ ) was increased by propanoylation, as shown by injection of synthesized propanoylated A $\beta$  into the rat brain.

These results indicate that protein modified with lipid peroxide from PUFAs accumulates in neurons, mostly in membrane, and might be involved in ageing-dependent brain dysfunction and neurodegenerative disorders, such as Alzheimer disease.

## Effect of amyloid-beta peptide upon lipid peroxidation and 4-hydroxy-2-nonenal formation by copper ion

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Alzheimer's disease is characterized by deposition of aggregated amyloid- $\beta$  peptide ( $A\beta$ ) and oxidative stress marked by protein and RNA oxidation and lipid peroxidation. The primary peroxidation products are phospholipid hydroperoxides having a hydroperoxy unsaturated fatty acyl residue. The phospholipid hydroperoxides are degraded to reactive aldehydes, such as 4-hydroxy-2-nonenal (HNE), malondialdehyde and alkenals, as secondary peroxidation products, and the HNE has been demonstrated to cause neuron death. We investigated the role of amyloid- $\beta$  peptide ( $A\beta$ ) in the formation of phospholipid hydroperoxides and HNE by copper ion bound to  $A\beta$ . The  $A\beta_{1-42}$ - $Cu^{2+}$  (1:1 molar ratio) complex showed an activity to form phospholipid hydroperoxide, 1-palmitoyl-2-(13-hydroperoxy-cis-9, trans-11-octadecadienoyl) phosphatidylcholine (PLPC-OOH), from phospholipid, 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC), through  $Cu^{2+}$  reduction in the presence of ascorbic acid. When  $Cu^{2+}$  was bound to two molar equivalents of  $A\beta_{1-42}$  ( $2 A\beta_{1-42}$ - $Cu^{2+}$ ), the lipid peroxidation was inhibited. HNE was generated from the PLPC-OOH by free  $Cu^{2+}$  in the presence of ascorbic acid through  $Cu^{2+}$  reduction and degradation of PLPC-OOH. HNE generation was markedly inhibited by equimolar concentrations of  $A\beta_{1-40}$  (92%) and  $A\beta_{1-42}$  (92%). However,  $A\beta_{1-42}$  binding two or three molar equivalents of  $Cu^{2+}$  ( $A\beta_{1-42}$ - $2Cu^{2+}$ ,  $A\beta_{1-42}$ - $3Cu^{2+}$ ) act as pro-oxidant to form HNE from PLPC-OOH. These findings suggest that  $A\beta$  acts primarily as an antioxidant to prevent  $Cu^{2+}$ -catalyzed oxidation of biomolecules at moderate concentrations of copper, but that, in the presence of excess copper, pro-oxidant complexes of  $A\beta$  with  $Cu^{2+}$  are formed.



## **Preclinical evaluation of tocotrienols for preventing prostate cancer,**

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Prostate cancer is the most frequently diagnosed cancer in American men and the second leading cause of cancer. Prostate cancer is primarily a disease of aging and is unique in having a long latency period during which cancer growth is slow and can cause few symptoms. Prostate cancer is often preceded by prostatic intraepithelial neoplasia (PIN) characterized by an increased proliferative ability of prostate gland cells and morphological alterations. Chemopreventive agents are well suited for decreasing prostate cancer mortality by extending the time during which PIN progresses to prostate cancer. Our long-term objective is to explore the use of tocotrienols (unique forms of vitamin E) as chemopreventive agents for prostate cancer. Vitamin E is at least four tocopherols (alpha-, beta-, gamma- and delta-) and four corresponding tocotrienols. Most research linking vitamin E with cancer does not distinguish between these various isoforms and primarily focuses on alpha-tocopherol ( $\alpha$ -T), the primary vitamin E isoform found in plasma and in most dietary supplements. In this study, we compared the abilities of different vitamin E isoforms to inhibit the growth of both androgen-dependent (LNCaP) and androgen-independent cell (PC-3) lines compared to a nontumorigenic prostate epithelial cell line (RWPE-1).

$\alpha$ -T was not effective at inhibiting prostate cancer cell growth whereas tocotrienols (T3s), particularly  $\gamma$ -T3 and  $\delta$ -T3 were effective at physiological levels (1  $\mu$ M) in inhibiting the growth of both PC-3 and LNCaP cells while having little impact on nontumorigenic prostate epithelial cells (RWPE-1). PC-3 prostate cancer cell line cells were found to take up T3s (particularly  $\delta$ -T3) to a greater extent than tocopherols (Ts). The T3s were found to inhibit prostate cell growth by induction of apoptosis. Our recent mechanistic studies show that the inhibition of prostate cancer cell growth by T3s occurs through though a partial PPAR dependent mechanism and results in the inactivation of the NF $\kappa$ B pathway. Moreover, we have found that  $\gamma$ -T3 also induces growth arrest in PC-3 cells though a novel mechanism involving the down regulation of TGF $\beta$ 2.

Our research suggests that T3s, particularly  $\delta$ -T3, may be a cost effective chemopreventive agent with low toxicity, no known side-effects and suitable for long-term intake. The use of chemopreventive agents to decrease the incidence of prostate cancer mortality is generally recognized as an optimal approach. The preclinical work presented here suggests that T3s are effective at selectively inhibiting the growth of prostate cancer cells at levels achievable with an inexpensive dietary supplement. We believe that sufficient data now exists to warrant a large-scale primary clinical intervention study.

## Substituent Effect on the Radical-Scavenging Reactivity of Vitamin E Analogues

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The molecular design for novel phenolic antioxidants with high antioxidative and low pro-oxidant activities is of considerable importance with regard to the development of effective chemopreventive agents against oxidative stress and associated diseases. However, very little is known about the detailed structure–activity relationship of phenolic antioxidants. In this study, we examined the radical-scavenging rates of  $\alpha$ -tocopherol and its analogues with various substituents on the 6-chromanol ring.

$\alpha$ -Tocopherol efficiently scavenged galvinoxyl radical (GO $\cdot$ ) with the second-order rate constant ( $k$ ) of  $3.3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  in deaerated acetonitrile at 25 °C. When the phytyl group at the C2 position in  $\alpha$ -tocopherol was replaced by methyl group, no significant change in the  $k$  value was observed ( $3.2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ ). On the other hand, a carboxyl group at the C2 position significantly retarded the GO $\cdot$ -scavenging rate ( $k = 4.9 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ ). Among vitamin E analogues used in this study, 8-amino-2,2-dimethylchroman-6-ol showed the highest GO $\cdot$ -scavenging activity ( $k = 1.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ). The logarithm of the  $k$  values for  $\alpha$ -tocopherol and its analogues were linearly correlated with the corresponding ionization potentials (IP) determined by the density functional theory calculations. The smaller the IP value is, the stronger the radical-scavenging activity becomes.

## Activity of pinosylvin administered in monotherapy and in combination with methotrexate on the development of rat adjuvant arthritis

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Oxidative stress (OS) has been implicated in various pathological conditions involving several diseases and aging. Rheumatoid arthritis (RA) is a common severe joint disease that involves all age groups. The pathogenesis of RA is associated predominantly with the formation of free radicals at the site of inflammation. However, knowledge on the role of OS in the progression of RA is scarce and the link between OS and inflammation status in arthritis should be more precisely investigated. Pinosylvin (PIN), 3,5-dihydroxy-trans-stilbene, is mainly found in the heartwood of *Pinus sylvestris*. PIN used in this study was synthesized in the Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague, Czech Republic by Ing. Juraj Harmatha, PhD. The aim of the present study was to examine the effect of PIN on the progression of adjuvant-induced arthritis (AA) in rats in monotherapy and in combination with methotrexate (MTX), which is a classical immunosuppressant drug.

AA was induced by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* in incomplete Freund's adjuvant. The experiments included healthy animals, arthritic animals not treated, arthritic animals treated with MTX, with PIN, and with a combination of PIN and MTX. The two latter groups received a daily oral dose of 50 mg/kg b.w. of PIN, either alone or with MTX in an oral dose of 0.4 mg/kg b.w. twice a week during 28 experimental days.

We found that PIN potentiated both the antiarthritic (decrease of hind paw volume) and the antioxidant effect of MTX (reduction of plasmatic levels of TBARS). Activity of GGT in spleen, level of MCP-1 and CRP in plasma were not improved by addition of PIN to MTX due to the prominent effect of MTX alone on these parameters. Arthritic animals showed increased OS, evaluated as plasma levels of isoprostanes. PIN alone or in combination with MTX strongly reduced isoprostane levels (about 50%). On the contrary, a significant decline in Nrf2-regulated antioxidant defences, such as hemeoxygenase-1 (HO-1), was observed in the lung (about 40%) but not in the liver from AA rats. In the AA lung, PIN alone increased the levels of HO-1 by about 30% more than MTX. Moreover, the combination therapy was the most effective in increasing the levels of HO-1 (3-fold in respect to AA values). OS can also activate NF- $\kappa$ B, which plays a critical role in the transcription of proinflammatory genes. Our data showed a marked increase in NF- $\kappa$ B in the lung and liver from AA animals. This increase was strongly reduced by PIN alone as well as in combination with MTX. Our results suggest that the anti-inflammatory activity of PIN is mediated by suppression of NF- $\kappa$ B activation in the liver and lung of arthritic animals.

In summary, combined administration of PIN and MTX suppressed arthritic progression in rats more effectively than did MTX alone. This natural compound is able to reduce OS *in vivo* and may help improve the treatment of rheumatoid arthritis.

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## Antioxidant and anti-inflammatory activities in vivo of thymoquinone isolated from *Nigella sativa* essential oil extracted by microwaves

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Accelerated steam-distillation assisted by microwave (ASDAM) is a combination of microwave heating and steam distillation, performed at atmospheric pressure at very short extraction time. Isolation and concentration of volatile compounds are performed by a single stage. (ASDAM) has been realized with cryogrinding of seeds (CG) in order to eliminate the thermal and hydrolysis reaction.

The ASDAM essential oil of black cumin seed (*Nigella sativa* L.) was investigated for its composition and antioxidant and anti-inflammatory properties in vivo. After analysis by GC, GC-MS and GC-GC-MS, 40 compounds were identified in the oil, obtained in 1.3% (v/w) yield. Among them, *p*-cymene (23.15%) and thymoquinone TQ (57.05%) were the major components. TQ was isolated from essential oil at -8°C during 72 hours at 96 % purity.

The in vivo study of antioxidant and anti-inflammatory activities of thymoquinone was investigated after rectal administration to animals (Swiss, *Mus musculus*) at 1mg/Kg on a experimental model of DNBS-induced colitis the results showed that TQ reduced significantly inflammation and the lipid peroxidation induced by DNBS reducing the neutrophil infiltration into the colon.

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**Antioxidant, antiinflammatory and vasodilatation properties of a commercial extract of *Punica granatum*. Comparison with *Ginkgo biloba* standardized extract.**

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Pomegranate which contains antioxidant polyphenols (ellagic acid, punicalagin), is a [fruit-bearing deciduous shrub](#) or small [tree](#) growing between five and eight meters tall. Introduced into [Latin America](#) and [California](#) by [Spanish](#) settlers in 1769, pomegranate is now cultivated in parts of California and [Arizona](#) for juice production. In the present work, we tested both antioxidant and inflammatory activities of a commercial extract of pomegranate (Oxylent GR<sup>®</sup>, Stiernon, Belgium) and compared them to a *Ginkgo biloba* extract as a polyphenolic reference (Pharma Nord, Denmark). Its concentration in total polyphenols was 31.78 mg acid gallic equivalent/100 mg against 19.50 for *Ginkgo biloba* extract. Using the ORAC (Oxygen Radical Antioxidant Capacity) assay, we found a global antioxidant capacity of 221  $\mu\text{M}/100$  mg (Trolox equivalent) against 343  $\mu\text{M}$  for *Ginkgo biloba*. Opposite data were, however, getted by using the DPPH test : 382  $\mu\text{M}/100$  g (Trolox equivalent) for pomegranate vs only 33  $\mu\text{M}/100$  g for *Ginkgo biloba*. Using electron spin resonance spectrometry, we found that the extracts were also able to similarly inhibit the production of superoxide anion produced by xanthine/xanthine oxidase system :  $\text{IC}_{50}$  : 5  $\mu\text{g}/\text{mL}$  pomegranate; 7.5  $\mu\text{g}/\text{mL}$  *Ginkgo biloba*. Besides the results, our data reveal that the choice of the test is of primordial importance for evaluating the global antioxidant capacity. The effect of the extracts was studied on the oxidant response on isolated human neutrophils and myeloperoxidase (MPO) both playing key roles in inflammation. The production of reactive oxygen species (ROS) by phorbol myristate acetate stimulated neutrophils was evaluated by lucigenin dependent chemiluminescence while MPO released by neutrophils was measured by ELISA (kit MPO ELIZEN, Zentech SA, Liège, Belgium). Pomegranate extract inhibits ROS production by 50% at a concentration of 5  $\mu\text{g}/\text{mL}$  ( $\text{IC}_{50}$ ) against 21  $\mu\text{g}/\text{mL}$  for *Ginkgo biloba*. With respect to the release of MPO, we found an  $\text{IC}_{50}$  of 7.5  $\mu\text{g}/\text{mL}$  for pomegranate vs 42  $\mu\text{g}/\text{mL}$  for *Ginkgo biloba*. At least, experimentations on isolated rat aorta mounted in standard organ baths and precontracted with noradrenalin (0.1  $\mu\text{M}$ ) have shown that pomegranate and *Ginkgo biloba* at a concentration of 3.5 mg/mL were respectively able to induce 60% and 50% of vasorelaxation. Taken together, our data confirm the potential interest of pomegranate and *Ginkgo biloba* for nutritional and health purposes.

## **Xanthine Oxidase Inhibition and Antioxidant Activities of Ten Crude Drugs Extracts, Usually Used in Indonesian Traditional Medicine for Hyperuricemia**

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Xanthine oxidase inhibition and antioxidant activities of ten crude drugs extracts, usually used in Indonesian traditional medicine for hyperuricemia had been done. The ethanol extract of the leaf of *Anredera cordifolia* (Ten.) Steenis, concentration 0,1 %, inhibited the activity of xanthine oxidase *in vitro* better than the other extracts (76,3%). This inhibition of enzyme activity was found to be dose dependent, with IC 50 value approximately 0,066 % while IC 50 allopurinol is 0,0002 %.

Antioxidant activity of extracts was evaluated by DPPH free radical scavenging, with ascorbic acid is used as comparison solution. The ethanol extract of rhizome of *Curcuma mangga*, concentration 0,1 %, showed better activity than the other extract, with value 85,3 % while ascorbic acid (concentration 0,1 %) is 94 %.

## **DPPH Free Radical Scavenging Activity and Catechins Content of Fifteen Grades of Indonesian Black Teas.**

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The antioxidant activities of fifteen grades of Indonesian Black Teas was determined by DPPH free-radical scavenging. The first grade of Indonesian Black Teas (PF) show a better ability to scavenging of DPPH free radical, with lowest IC<sub>50</sub> 218 µg/mL followed BOPF, Dust, BOP and BT each 227, 240, 252 and 260 µg/mL respectively.

The catechin content was determined by High Performance Liquid Chromatography (HPLC). The total catechins concentration was found to vary from 6.27 to 10.18 %. The antioxidant activities well correlated with catechin content.

## **Basil (*Ocimum basilicum* L.) as a valuable source of antioxidants**

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As one of the reasons, human aging is attributed to the increasing concentration of high-reactive free radicals (superoxide anion radical, hydroxyl radical and others) in the organism. These active species of oxygen are formed in the process of normal metabolism, but their amount considerably increases under stress conditions (air pollution, low-grade food, irradiation, exposure to heavy metals, treatment with some forms of medicines and others). For compensating the negative effect of free radicals it is recommended to consume more natural antioxidants. The antioxidants include antioxidant enzymes and the low-molecular antioxidants, such as, ascorbic acid, reduced glutathione,  $\alpha$ -tocopherol, flavonoids, carotenoids etc.

The preservative effect of many plant spices and herbs suggests the presence of antioxidative constituents in their tissues. Many medicinal plants contain large amounts of antioxidants other than vitamin C, vitamin E, and carotinoids.

Among the plants known for medicinal value, the plants of genus *Ocimum* belonging to family *Lamiaceae* are rich in phenolic compounds and are very useful for their therapeutic potentials. They are widely used in traditional systems of medicine. *Ocimum basilicum* L. is examples of species of genus *Ocimum*, which grow in different parts of the world and are known to have medicinal properties. *Ocimum basilicum* usually named common basil or sweet basil is an annual plant, with extraordinary medicinal properties and contains several antioxidant compounds. In traditional medicine, *Ocimum basilicum* has been used as an antiseptic, preservative, sedative, digestive regulator and diuretic.

There are many varieties of *Ocimum basilicum*, as well as several related species or species hybrids also called basil. The type used in Italian food is typically called sweet basil, as opposed to Thai basil (*O. basilicum* var. *thyrsoflora*), lemon basil (*O. × citriodorum*) and [holy basil](#) (*Ocimum tenuiflorum*), which are used in Asia.

The purpose of present study was to estimate the antioxidant properties of leaves of six varieties of basil (4 varieties – plants with green leaves, 2 varieties - plants with the violet leaves). In basil leaves were determined ascorbic acid, riboflavin, carotinoids, chlorophylls a and b, some of phenolic compounds (rutin, anthocyanins, catechins, and tannins), total phenolic content, and antioxidant activity.

It was established that all investigated varieties were characterized by the high summary content of phenol compounds (variation between the t varieties - 26- 45%), by antioxidant activity (variation - 15- 23%), ascorbic acid (variation - 34-46%). Significant difference in the content of carotinoids and riboflavin in basil leaves of different varieties was not revealed. The especially significant difference between the varieties was determined in the content of bioflavonoids in leaves. Plants with violet leaves were characterized by the considerably higher content of anthocyanins and rutin.

Thus, it was shown that the basil plants can be used as valuable source of antioxidants. In this case the level of biologically active materials in leaves of plants, especially phenolic nature, strongly depends on the variety of plant.



## Redoxmodulation and dopamine induced cell death in Parkinson disease

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The main pathological feature of Parkinson disease (PD) is the loss of dopamine producing neurons in substantia nigra, as a result of dopamine induced apoptosis. Oxidative stress has a central role here, by contributing to the release of dopamine into the cytosol from the vesicle membrane resulting in formation of toxic metabolites. Another hallmark for PD is the malfunction of Complex I in the electron transport chain, which consequently leads to an increased amount of oxygen radicals. Due to the involvement of oxidative stress in PD we aimed to elucidate the involvement of redox proteins belonging to the thioredoxin and glutaredoxin systems. Immunohistochemistry performed on paraffin embedded sections from substantia nigra in Parkinson patients revealed that thioredoxin reductase 1 (TrxR1) and thioredoxin 1 (Trx1) were significantly decreased compared to age matching controls. On the contrary there was not a significant change in the levels of Grx1, Grx2 and Trx2 in PD compared to controls. The involvement of redox proteins was further investigated during complex I inhibition and under dopamine induced cell death. *In vitro* cell viability experiments showed a protective effect of Q10 and Se in dopamine induced cytotoxicity. The protective effects were associated with an upregulation of the mRNA level of Trx1, Grx1 and Grx2. In conclusion, our results indicate a clear connection of redoxproteins in PD.

## **Expression pattern of redox proteins in Alzheimer's disease**

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Neurodegenerative disorders are characterized by neuronal impairment that eventually leads to neuronal death. Oxidative stress plays an important role in the pathological process of these disorders, among them Alzheimer's disease (AD). A shift in the redox balance may lead to accumulation of reactive oxygen species in the cell and cause oxidative stress. Both the glutaredoxin (Grx) and the thioredoxin (Trx) systems play a central role in maintaining a reduced environment in the cell, thus protecting the cells from oxidative stress. Trx1, Trx2, Thioredoxin reductase 1, Grx1 and Grx2 expression pattern were characterized immunohistochemically in human brain tissue from Alzheimer patients. In the CA1- region of hippocampus from patients with AD the expression of Grx1 and Grx2 decreased while the level of Trx1 increased compared to control. In addition, liquor samples from Alzheimer patients, were analyzed with sandwich ELISA, showing release of both Trx1 and Grx1 in liquor. The levels of Trx1 and Grx1 were further found to be increased in early stages of AD. A correlation was seen between these proteins and S-tau and P-tau, validated biomarkers for clinical use to diagnose AD. In conclusion, we introduce members of the thioredoxin superfamily of proteins as potential early markers in the diagnostics, and a possible role of these enzymes in the pathogenesis of AD.

## **Oxidative stress in Parkinson's disease: Regulation of mitochondrial redox state by neuromelanin**

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Parkinson's disease (PD) is characterized by selective loss of dopaminergic neurons in the substantia nigra and accumulation of modified protein called Lewy bodies. Specified vulnerability of neuromelanin (NM)-containing dopaminergic neurons suggests the association of NM and dopamine (DA) in the pathogenesis of PD. NM is synthesized in human brain from DA-quinone produced by DA autoxidation, but it contains significant amounts of protein, lipid and reactive metals as its components. In this paper, we present our recent finding that NM induces apoptosis through perturbation of the redox state in mitochondria.

The effect of NM isolated from human brain was investigated using human DA neuroblastoma SH-SY5Y cells. NM induced apoptosis in the cells, whereas the NM sample, whose protein component was removed by protease-K treatment, and synthetic DA melanin did not. Only NM increased free reduced glutathione (GSH) by release of GSH from *S*-glutathionylated proteins in mitochondria. NM dissociated the higher structure of complex I and III in the oxidative phosphorylation chain, and impaired mitochondrial function. This reaction was prevented by superoxide dismutase, deferoxamine and EGCG. Superoxide radicals generated by iron from NM was associated with the *S*-glutathionylation and the SH residues in the protein components of NM functioned catalytically. On the other hand, synthetic DA melanin and DA itself caused cell death through increase in reactive oxygen species, especially superoxide radicals.

These results indicate that redox state in mitochondria is regulated by *S*-glutathionylation independently from cellular redox state and associated with neurodegenerative disorders, such as PD.

## **The red-ox imbalance in cells treated with phosphatidylcholine chlorohydrins**

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In the term of oxidative stress, the modified lipids and by-products of lipid peroxidation process are usually regarded as markers or indicators of pathologic conditions represented by the excessive production of oxidants or deficiency of antioxidants. But some of them have biological activity inducing ROS generation and induction of cell death. Therefore, we decided to check the effects of phosphatidylcholine chlorohydrins, arising during the reaction of chloric acid (I) with unsaturated bonds of fatty acid chains, on the red-ox state of selected cell lines (A549, MCF-7, HUVEC-ST). The red-ox balanced was estimated on the basis of dihydrorhodamine oxidation, generation of superoxide anion by mitochondria with MitoSOX Red. Fluorescence was read with flow cytometry. The concentration of GSH and GSSG was determined with o-phtalaldehyde, the antioxidant capacity of cell extracts on the basis of: ABTS cation radical scavenging ability and protection of pyrogallol red against oxidation induced by HOCl. We found the increased release of superoxide anion from mitochondria accompanied by increased ROS level as indicated by rhodamine 123 fluorescence after 1 h cell incubation with chlorohydrins in all cell lines employed in our study. Simultaneously, we observed the reduction of GSH/GSSG ratio and the decrease of antioxidant ability of cell lysate extracts. The intensity of the effects found was strongly dependent on the number of HOCl molecules attached to the phosphatidylcholine molecule.

## Drug delivery nanoparticles are autophagic inducers in macrophages

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Recently, we reported that empty Eudragit® RS (ERS<sup>-</sup>) nanoparticles prepared by nanoprecipitation techniques (NP/ERS<sup>-</sup>) or double emulsion (DE/ERS<sup>-</sup>) impair cellular membrane integrity and mitochondrial functions of a NR8383 macrophage cell line (Eidi et al, 2010). Moreover, NP/ERS<sup>-</sup> particles were more toxic than DE/ERS<sup>-</sup>. To fill a gap in toxicology assessment, complementary preclinical study of ERS nanoparticles was needed. Therefore, we investigated both biochemical and morphological effects of NP/ERS<sup>-</sup> nanoparticles on NR8383 cells. Electron microscopic results showed that NP/ERS<sup>-</sup> nanoparticles were internalised by macrophages. The particles reached the mitochondria that lost their physiological shape and integrity. Nanoparticle effects on mitochondria were confirmed by a transcriptomic analysis that revealed over-expression of *opal*, a dynamin-related GTPase located in the inner membrane of mitochondria. They also induced a dose-dependent down-regulation of the anti-apoptotic genes: *bcl-2*, *pdc4* and *nfkb*, as well as a dose-dependent down-regulation of the pro-apoptotic genes encoding for *caspase 8* and *tnf-α* proteins. A dose-dependent up-regulation of the anti-apoptotic *ras* homolog gene family, namely *rhov*, *rho* GTPase activating protein 22 (*arhgap22*) and Rho effector Rhotekin (*rtnk*) as well as of the gene related to the G-proteins apelin (*apln*) was obtained. It was also shown that the proautophagic gene - *atg16l1* - was up-regulated. Autophagy involved genes were reported to play a role in innate, and adaptive immune response against viruses, quantum dots and water soluble fullerenes. In this study, we reported for the first time that such polymeric drug nanocarriers (NP/ERS<sup>-</sup>) could be considered as a novel class of autophagy activators as are viruses. Thus, attention should be made in using Eudragit® RS as drug nanovectors, since they might alter functions of immune system with potential consequence on human health...

Keywords: Nanoparticle, toxicity, apoptosis, autophagy.

Eidi H, Joubert O, Attik G, Duval RE, Bottin MC, Hamouia A, Maincent P, Rihn BH. Cytotoxicity assessment of heparin nanoparticles in NR8383 macrophages. Int J Pharm. 2010 Aug 30;396(1-2):156-65. Epub 2010 Jun 11. PubMed PMID: 20542101.

## **Human atherosclerotic plaque lipid moieties enhance ROS production and inflammation while modifying antioxidant enzyme expression and activity**

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During ongoing atherosclerosis development, cells in the vasculature are widely exposed to oxidative stress and inflammation. Previous studies have suggested that arterial plaques, apart from being the consequence of atherosclerotic disease development, are also a cause of its progression. In this study, we challenged this idea by exposing monocytes to human carotid plaque lipid extract (LE). An exposure of up to 72 h to LE caused 70% elevation in the cellular oxidative state. Subsequent determination of the antioxidant protein expression and activity revealed a significant reduction of  $0.73 \pm 0.051$  and  $0.78 \pm 0.071$  in catalase activity and expression, respectively. In contrast, superoxide dismutase activity and expression were augmented by  $1.768 \pm 0.07$  and by  $1.25 \pm 0.07$ , after 72 h exposure to LE, respectively. GSH levels did not change, while the relative oxidized GSH form (GSSG) increased significantly following LE exposure. Glutathione peroxidase and thioredoxin reductase enzyme activities were significantly reduced by  $0.77 \pm 0.02$  and  $0.57 \pm 0.05$ , respectively. Furthermore, the transcription level of the proinflammatory cytokines, interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$ , were upregulated by 2.9 and 100.2 fold, respectively. These findings imply that established lesions may promote the development and progression of atherosclerosis in adjacent regions by elevating the oxidative and inflammation state, while modifying antioxidant enzyme activity and expression.

## **An Age-Related Circulating Apolipoprotein B Oxidase as a Coronary Artery Disease Biomarker**

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Age and oxidative stress are major risk factors for heart disease. The causal link is the generation in the circulation of oxidized low density lipoproteins (LDLs) and their subsequent clearance by macrophages and delivery to the arterial wall. Internalizing the oxidized particles transforms the macrophages into foam cells. Activity of a family of age-related hydroquinone oxidases (arNOX) corresponding to the TM9 Super Family of transmembrane proteins capable of generating superoxide, appears at about age 30 and increases steadily thereafter. The ectodomain (N terminus) of the protein is shed into blood and other body fluids including saliva where at least one TM9 family member, SF2, is associated specifically with the apoB protein of low density lipoprotein as a major source of oxidative damage potentially leading to coronary artery disease. A second source of damage is via oxidation of protein amino acids by arNOX as a source of electrons to generate superoxide and disruption of protein structure attributable to arNOX. arNOX family member TM9SF2 contains a 25 amino acid sequence with 50% similarity to the cell recognition sequence of apoB. A 3 kDa peptide having that sequence binds to apoB of human LDL and the 30 kDa shed form of TM9SF2 appear to co-isolate as a 130 kDa complex. The apoB-bound TM9SF2 abstracts electrons from the apoB resulting in oxidation of protein thiols and tyrosines to generate  $O_2^-$  which dismutates to form  $H_2O_2$ . The latter results in lipid peroxidation and formation of thiobarbituric acid-reactive lipid adducts. Reducing the production of superoxide and other reactive oxygen species through inhibition of arNOX has the potential to represent a significant advance in the prevention of atherosclerosis.

## **Inhibition and molecular modeling of ATR protein kinase by Schisandrin B during DNA damage response.**

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ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad-3-related) protein kinases play a crucial role in cellular DNA damage responses. The inhibition of ATM and/or ATR leads to an abolishment of one such signal pathway termed as checkpoints. It is expected that the development of checkpoint inhibitor will be an effective assistance of anti-cancer therapy. While several ATM inhibitors have been reported, ATR-specific inhibitor is currently unavailable. Here, we report inhibitory effects and the mechanism of Schisandrin B (SchB), an active ingredient of *Fructus Schisandrae*, on DNA damage response. Addition, a molecular modeling was performed (MOE, CCG Inc.) to understand the mechanism of interaction between ATR and SchB. The treatment of SchB significantly decreased the A549 adenocarcinoma cell viability after UV exposure. Of importance, SchB treatment also disrupts G2/M checkpoint in the UV-exposed cells. *In vitro* immunoprecipitation protein kinase activity of ATR was clearly decreased by increased concentration of SchB. A molecular modeling speculated that the formation of hydrogen bonds with oxygen atoms at methylenedioxy group in SchB and Val2380 and/or Lys2327 in ATR which conducting the methylenedioxy ring of SchB may play a similar role to an adenine ring of ATP in the ATP-binding site of ATR. Taken together these findings and investigation suggest that SchB directly binds to ATR protein kinase, and inhibits the kinase activity in cells following DNA damage. The specific inhibition of ATR by SchB has clinical implications in anti-cancer therapies.



## **Proteome alteration in human myoblasts upon oxidative stress and replicative senescence**

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Age-related decline in skeletal muscle mass during ageing has been attributed at least in part to a reduction in regenerative potential of resident stem cells, also known as satellite cells. Although increased oxidative stress is associated with the impairment of the proliferation and function of these cells, the proteins either involved in the response to oxidative stress or those damaged by oxidation have not yet been identified. In this work changes in the proteome of human myoblasts during replicative senescence and in response to acute oxidative stress have been addressed. A parallel proteomic analysis aimed at identifying differentially expressed proteins as well as those targeted by carbonylation has been performed. The carbonylated proteins identified either during replicative senescence or upon acute oxidative stress are mainly cytosolic and involved in key cellular functions, such as carbohydrate metabolism, cellular motility, cellular homeostasis, protein synthesis and protein degradation. Interestingly, almost half of the proteins identified as increasingly carbonylated were the same under the two different experimental conditions, suggesting a particular susceptibility of some proteins for oxidation. In addition, data mining indicates that these proteins can be grouped in a pathway analysis pointing to skeletal and muscle disorders, cell death and cancer as the main molecular networks altered. We next evidenced the proteins which expression level has changed. A different set of proteins were found to be upregulated or down regulated in both approaches suggesting different effects on the proteome upon the two experimental conditions used. As expected, most of the proteins found to be increased upon oxidative stress were those involved in the antioxidant stress response, however, proteins involved in cell morphology, cell motility and energetic metabolism were found to be differentially expressed during replicative senescence. Taking together, our results indicate that proteins involved in key cellular pathways are affected upon oxidative stress and replicative senescence and the impairment of them may be implicated in cellular dysfunction. In addition, this study underscores the importance of performing proteomic analyses looking at different aspects, such as the expression level and specific post-translational modifications, in order to have a broader view of changes affecting the cellular proteome.

## Importance of the mitochondrial Lon protease in aging and Parkinson disease

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Mitochondrial dysfunction has been implicated in the aging process as well as a number of age-associated diseases such as Parkinson's disease and Alzheimer's disease. The *Saccharomyces cerevisiae* homolog of the ATP-dependent Lon protease, Pim1p, is essential for mitochondrial protein quality control, mitochondrial DNA maintenance and respiration. Here, we demonstrate that Pim1p activity declines in aging cells and that Pim1p deficiency shortens the replicative life span of yeast mother cells. This accelerated aging of *pim1Δ* cells is accompanied by elevated cytosolic levels of oxidized and aggregated proteins, as well as reduced proteasome activity. Our results suggest that defects in mitochondrial protein quality control have global intracellular effects leading to the increased generation of misfolded proteins and cytosolic protein aggregates, which are linked to a decline in replicative potential.

Parkinson disease (PD) is the second most common neurodegenerative disease. Compelling evidence suggests that mitochondrial dysfunction could represent a critical event in the pathogenesis of PD. A still rather unexplored field is the involvement of mitochondrial proteases. The expression and the stability of mitochondrial proteases may therefore modulate the phenotype of several neurodegenerative conditions associated to impaired mitochondrial function. We provide evidence indicating that Lon plays a critical role in the removal of oxidatively modified and dysfunctional protein from the mitochondria isolated from mice treated with MPTP which is a complex I inhibitor.

## Silencing of the different Methionine Sulfoxide Reductases reduces cell tolerance to oxidative stress and induces protein modifications in stress conditions

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The accumulation of proteins damaged by oxidative stress is a process directly involved in aging and in pathologies associated to oxidative stress. Protein oxidation by ROS are of two types: reversible and irreversible. The reversible changes mainly affect sulfur containing amino acids such as methionine, the oxidized form of which, methionine sulfoxide, can be repaired by methionine sulfoxide reductases (Msr). The Msr system plays a major role in the maintenance of protein homeostasis during aging and has also been involved in cellular defense against oxidative stress, by scavenging ROS.

Oxidation of methionine results in the production of two diastereoisomeric forms: S or R methionine sulfoxide that can be reduced by MsrA and MsrB, respectively. Methionine sulfoxide reductase A is localized in the cytosol, the nucleus and mitochondria. The three different MsrB have different cellular localization: MsrB1, also called SelX or SelR, is located in the nucleus and the cytosol, MsrB2 is present in mitochondria and the two isoforms of MsrB3 are found in mitochondria and endoplasmic reticulum. Various studies about the roles of Msrs have shown that overexpression of MsrA in WI-38 fibroblasts (Picot et al., 2005) or of MsrB2 in the mitochondria of leukemic cells (Cabreiro et al., 2008) protects against oxidative stress. In addition, experiments on different animal models have demonstrated their importance in the aging process and neurodegenerative diseases.

The purpose of this work is to clarify the role of the different Msr enzymes using RNA interference, a technique based on post translational repression. MsrA, MsrB1 and MsrB2 genes have been silenced in human embryonic kidney cell lines (HEK 293) via microRNA (miRNA) strategy. Simple and double MsrA/MsrB1 mutants have been characterized by measuring the expression of Msr and their residual activity. A functional characterization was then performed by subjecting the clones to oxidative stress using taurine chloramine, an oxidant known to preferentially target cysteine and methionine residues of proteins. Then, their sustainability was measured in order to determine the effects of the inhibition of one or more Msrs on stress resistance. Some of them exhibited a decreased resistance to oxidative stress compared to controls in which a negative control plasmid was introduced. To identify targets or partners of the different Msrs, 2D-DIGE (2 Dimensional Differential Gel Electrophoresis) experiments were performed in normal and oxidative conditions. All in all, these results are expected to give further insights in the precise role of the Msr system at the cellular level.

Picot, C.R., I. Petropoulos, M. Perichon, M. Moreau, C. Nizard, and B. Friguet, *Overexpression of MsrA protects WI-38 SV40 human fibroblasts against H2O2-mediated oxidative stress*. Free Radic Biol Med, 2005. **39**: 1332-1341.

Cabreiro, F., C.R. Picot, M. Perichon, J. Castel, B. Friguet, and I. Petropoulos, *Overexpression of mitochondrial methionine sulfoxide reductase B2 protects leukemia cells from oxidative stress-induced cell death and protein damage*. J Biol Chem, 2008. **283**: 16673-16681.

## **Accumulation of oxidized protein and the relevance to stress-induced premature senescence in WI-38 human fibroblasts**

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Premature senescence can be induced in proliferating cells under conditions of increased oxidative stress. Several models have been used such as exposure of human fibroblasts to subcytotoxic doses of different stressing agents as hydrogen peroxide or tert-butyl hydroperoxide. In a previous study, we have identified proteins that are targets of oxidation (carbonylation) during replicative senescence of WI-38 human embryonic fibroblasts (Ahmed et al., 2010, *Aging Cell*, 9: 252-272). In this study, we sought to determine if oxidative damage to protein is also implicated during stress-induced premature senescence and, if so, whether specific oxidized protein targets could be identified. Our data shows that exposure of human WI-38 fibroblasts to sub-cytotoxic levels of hydrogen peroxide (500  $\mu$ M) is accompanied by an increased level of carbonylated proteins. In addition, exposure of cells to H<sub>2</sub>O<sub>2</sub> results in a rapid inactivation of the proteasome. Interestingly, this was followed by restoration of proteasomal activities. Carbonylated proteins start to accumulate within 1 to 2 weeks together with a decreased cell proliferation rate and the appearance of the senescence marker beta-galactosidase and P16. A proteomic approach was used to identify oxidized protein targets in stressed and senescent WI-38 fibroblasts. We utilized 2D gel electrophoresis coupled with immunodetection of carbonylated proteins that had previously been derivatized by di-nitro-phenylhydrazine. Evidence is provided that a restricted set of proteins is targeted by oxidation both after oxidative stress and upon appearance of stress-induced premature senescence. Identification of these proteins is expected to give insights on the implication of protein oxidation in the establishment of stress-induced premature senescence.

## Lipid peroxidation and protein tyrosine oxidation are mechanistically-associated events in hydrophobic biocompartments: consequences in biomembranes and lipoproteins

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Oxidative stress encompasses a number of oxidative modifications in biomolecules, many of which are triggered by free-radical dependent processes. Prime examples of these are lipid peroxidation and protein oxidation, which can lead to direct alteration of biological function and/or formation of secondary reactive intermediates/products. In biologically-relevant hydrophobic biocompartments such as biomembranes and lipoproteins, lipid peroxidation and protein oxidation may be intertwined due to the organization of these structures resulting in close proximity among the different participating biomolecules. In the past years<sup>1-4</sup>, we have explored how lipid peroxidation and tyrosine oxidation/nitration are interrelated, using model membrane systems, red blood cell membranes and isolated LDL. Mechanistic studies have been performed by incorporation of a series of hydrophobic tyrosine analogs to the tested lipidic systems and exposing them to a variety of oxidizing systems including peroxytrite, peroxy radical donors and hemin. Overall, we have found that lipid peroxidation and tyrosine oxidation processes in hydrophobic biocompartments are connected *via* the reaction of lipid peroxy radicals (LOO<sup>•</sup>) with tyrosine residues which results in the one-electron oxidation of tyrosine to tyrosyl radicals (Tyr<sup>•</sup>):



The overall proposed mechanism connecting both processes predicts that 1) tyrosine oxidation yields will be influenced by oxygen levels, as molecular oxygen is a critical reagent in the propagation phase of lipid peroxidation<sup>1</sup> and 2) that a fraction of the tyrosyl radicals could combine with lipid peroxy radicals to yield termination adduction products. Indeed, in the different tested systems the levels of oxidized and/or nitrated tyrosine (to 3,3'-dityrosine and/or 3-nitrotyrosine, respectively) were significantly decreased under low oxygen tensions as happened with lipid peroxidation products (lipid hydroperoxides and malondialdehyde). Second, peroxy radical formation from PLPC (13S)-OOH by MeOAMVN (2, 2'-azobis (4-methoxy-2,4-dimethylvaleronitrile) in the presence of BTBE or hydrophobic tyrosine-containing peptides resulted in the formation of novel compounds which were HPLC purified and analyzed by MS and NMR-based techniques and identified as of Diels-Alder type tyrosyl-lipid tricyclic adducts. The data showed herein further support the connection between lipid peroxidation and protein oxidation processes *in vitro* and *in vivo*<sup>5</sup>, revealing a previously unrecognized influence of oxygen levels on tyrosine oxidation and nitration yields and identifying mixed oxidation products which may represent novel biomarkers and mediators of membrane and lipoprotein oxidative damage.

<sup>1</sup>Bartesaghi *et al. Biochemistry*, 45: 6813-6825 (2006); <sup>2</sup>Romero *et al. Chem Res Toxicol*, 20 (11):1638-1648 (2007); <sup>3</sup>Bartesaghi *et al. Chem Res Toxicol*, 23: 821-835 (2010); <sup>4</sup>Shchepin *et al. J Am Chem Soc*, 132:17490-17500 (2010); <sup>5</sup>Stadler *et al. Free Radic Biol Med*, 45(6): 866-74 (2008).

## Pepsinogen is nitrated in the stomach *in vivo*: modulation by NSAIDs, oral bacteria and salivary nitrite

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Dietary nitrate, long believed to be an inert product of nitric oxide ( $\text{NO}$ ) oxidation, is now recognized as a critical mediator of physiological functions in the gastrointestinal tract (GIT) due to its stepwise reduction to nitrite and  $\text{NO}$ . In the stomach,  $\text{NO}$  is engaged in gastroprotection but nitrite can also generate other reactive nitrogen oxides with nitrating capacity, which may have an impact on gastric pathophysiology. Here we studied the impact of dietary nitrite supplementation on the nitration *status* of the healthy and ulcerated rat gastric mucosa. Additionally, the *in vivo* nitration of specific gastric mediators, namely pepsinogen, was screened in connection with the impact of such post-translational modification on pepsin function. Finally, germ-free mice were used to elucidate the role of bacteria in gastric nitration reactions in the stomach.

Wistar rats were divided in two groups: a healthy untreated group and another one experiencing acute gastric inflammation induced by diclofenac ( $30 \text{ mg.Kg}^{-1}$ ). Animals were further divided in two subgroups, one fed with nitrite  $1.38 \text{ mg.Kg}^{-1}$  and the other with water. All compounds were given by oral gavage. Protein tyrosine nitration was evaluated by immunohistochemistry and immunoprecipitation. Nitrotyrosine (NT) labeling was detected in the deep mucosa of untreated rats, suggesting that nitration is a physiological event in the stomach. NT yields increased in the stomach of rats with acute gastric ulceration ( $p < 0.01$ ) and were further enhanced in the subgroup fed with nitrite ( $p < 0.01$ ). NT staining was located within the *lamina propria* and blood vessels but also in cells of the oxyntic glands, where an intense cytoplasmatic staining suggests nitration of specific gastric mediators stored in cytoplasmatic vesicles, such as pepsinogen. Indeed, pepsinogen nitration occurs under basal conditions but increases under gastric ulceration. Unexpectedly, healthy nitrite-fed rats showed reduced levels of both, overall and pepsinogen nitration in respect to untreated rats. Pepsinogen nitration had no effect on activation to form pepsin, but it strongly inhibited the proteolytic activity of pepsin ( $p < 0.001$ ).

Mechanistically, both  $\text{NO}$ -dependent (e.g. iNOS and nitrite-derived  $\text{NO}$ ) and independent (e.g., via myeloperoxidase activity) nitration pathways can be envisaged. In order to address the origin of the nitrating species *in vivo* we used germ-free (GF) mice because these animals lack nitrate-reducing bacteria and, therefore, do not reduce nitrate to nitrite, thereby preventing  $\text{NO}$  formation in the gastric lumen. GF mice showed lower levels of NT under basal conditions than wild-type. When nitrite (but not nitrate) was added to the drinking water ( $1 \text{ mM}$  for 7 days) NT yields decreased ( $p < 0.01$ ) whereas in wild-type mice both, nitrate and nitrite, induced a decrease in NT. No differences in gastric iNOS expression were found between GF and conventional animals. The results support that protein nitration is in part regulated by salivary-derived nitrite, gastrointestinal bacteria and intragastric generation of reactive nitrogen oxides.

We demonstrate that pepsinogen is nitrated under physiological conditions in the stomach and that nitration increases under acute ulceration. Dietary nitrite seems to have a dual role in modulating gastric nitration as it enhances nitration under inflammatory conditions but has the opposite effect in healthy animals. The results also suggest that pepsinogen nitration impairs the proteolytic function of the derived pepsin, thereby impacting on protein function. Expectedly, an inefficient protease would impair the digestion of dietary proteins but, on the other hand would also prevent the breakdown of endogenous proteins (mucins, collagen) vital for gastric integrity, thus preventing peptic ulcer disease.

Overall, these results unravel a role of dietary nitrite in the gastro physiopathology via nitrative modification of local proteins.

## **Nitrotryptophan: A novel post translational modification in naïve and differentiated PC12 cells**

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Neuron growth factor (NGF) signaling in PC12 cell, which is derived from Pheochromocytoma of rat adrenal medulla, induces expression of neuronal nitric oxide synthase (nNOS) and nitric oxide (NO) production. Subsequently, NO causes a differentiation of PC12 cells to neuronal cell with morphological changes, such as neurite extension. In this study, we revealed that 6-nitrotryptophan-including proteins were produced in PC12 cell (naïve PC12 cell) and NGF-induced PC12 cell (differentiated-PC12 cell) by proteomics using anti 6-nitrotryptophan antibody, which we have developed (1). The peptides from five ribosomal proteins, which are 60S ribosomal protein L7 (Trp154), 60S acidic ribosomal protein P1 (Trp43), 40S ribosomal protein S2 (Trp60), 40S ribosomal protein S6 (Trp45), and 40S ribosomal protein S19 (Trp52), were identified as nitrotryptophan residue including proteins. Among them, the tryptophan nitration was observed only in the differentiated PC12 cells for S19 protein, but only in naïve PC12 cells for L7 protein.

The positive signal of nitrotryptophan-including proteins in the western blotting around 16kDa (Band 1), which includes 40S ribosomal protein S19, was suppressed by treatment with NOS inhibitor, L-NAME. The tryptophan nitration of 40S ribosomal protein S19 and S2 were not observed by LC-MS-MS analysis of this sample.

This is first report to identify several specific sites of nitrated tryptophan on proteins not only in viable culture cell but also in a physiological process, cell differentiation.

(1) Ikeda K, Hiraoka BY, Yamakura F, et al. (2007) *Nitric Oxide: Biology and Chemistry*, 16, 18-28.

## Differential effects of a High-Fructose diet or a High-Fat/High-Sucrose diet on the mitochondrial ROS production and respiration in rat liver and skeletal muscle.

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Frequencies of overweight and obese people are constantly rising up. Increase in obesity is associated to a permanent rise in the number of patients with a metabolic syndrome. The aim of this study was to better understand the physiological events involved in the appearance of a metabolic syndrome in obesity. Numerous data clearly suggest that mitochondria could play a major role in these processes through a cross talk with the nucleus. We therefore focused on the study of the alterations of the mitochondrial function (respiration and ROS production) in response to various hypercaloric diets, and their relation with the occurrence of obesity and/or metabolic syndrome.

**Methods.** Male Wistar rats were fed with either a standard diet (Control), High-Fructose (HF) or High-Fat/High Sucrose (HF/HS) for 6 weeks. Indices of oxidative damages and body composition were then determined on plasma and liver and muscle tissues. Mitochondria were isolated from liver and red portions of quadriceps muscle. Oxygen consumption and H<sub>2</sub>O<sub>2</sub> release (as index of mitochondrial ROS production) were measured with various substrates of the respiratory chain (Glutamate+Malate, Succinate, Glutamate+Malate+Succinate, Palmitoyl-Carnitine).

**Results.** Rats under HF/HS displayed increase in body weight, insulin resistance, liver steatosis, and increase in visceral adiposity associated with larger mesenteric and retroperitoneal adipocytes when compared to control. These changes were not seen in HF rats which showed insulin resistance. In HF/HS, plasma TBARs and AOPP as well as the GPx activity were higher compared to control. In muscle, AOPP and catalase and GPx activities were also higher. In HF, SH groups were higher in plasma and liver while AOPP and GPx activity were higher in muscle when compared to control. HF diet was associated to a lower oxygen consumption by liver isolated mitochondria under non phosphorylating conditions whatever the substrate used excepting palmitoyl-carnitine, compared to control (-30 to -60%). We also report a higher citrate synthase activity in liver tissue in the same condition ( $11,5 \pm 0,7$   $\mu\text{mole}/\text{min}/\text{g}$  wet tissue) compared to control diet ( $7,5 \pm 0,6$   $\mu\text{mole}/\text{min}/\text{g}$  wet tissue). No changes were significant in phosphorylating conditions or in muscle mitochondria. After HF/HS diet, oxygen consumption by muscle and liver mitochondria was not significantly different from control. Enzymatic activities of respiratory complexes II and III are lower in HF and in HF/HS compared to control. HF diet altered the H<sub>2</sub>O<sub>2</sub> release by liver isolated mitochondria. It was higher with succinate as a substrate, compared to the control diet. These changes appear dependent on both complex I, through reverse electron flux, and complex III. HF/HS diet had no effect on liver or muscle mitochondrial H<sub>2</sub>O<sub>2</sub> release.

**Conclusion.** These results indicate that a 6-week HF diet is not associated with obesity or higher fat storage although there are oxidative damages. On the other hand, a 6-week HF/HS diet induces insulin resistance associated with obesity but without significant mitochondrial alterations. Obesity is associated with oxidative damages that could be of extramitochondrial origin. HF diet could specifically induce insulin resistance through oxidative stress. Lack of obesity could be related to a decreased mitochondrial activity. Diet composition could have different effects with induction of functional changes under HF and structural changes under HF/HS as suggested by citrate synthase activity. Moreover, HF/HS appears to be less deleterious than HF diet since a compensatory mitochondrial biogenesis could counteract consequences of mitochondrial ROS production.

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## **Boienergetics, free radical peroxidation, and mitochondrial PTP sensitivity in old rat heart mitochondria under effect of precursors of ubiquinone biosynthesis**

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Bioenergetical processes play the central role in regulation of myocardial functional activity. Imbalance in myocardial energy supply regardless of cause may play the main part in development and progression of cardiovascular system's numerous pathologies of various etiologies, as well as in ageing. Mitochondrial permeability transition pore (MPTP) is a mitochondrial channel the opening of which causes mitochondrial membrane potential collapse, which leads to apoptosis. Ubiquinone (coenzyme Q, CoQ) is the key component of cellular bioenergetics as a transporter of protons and electrons in mitochondrial electron-transport chain and is an important lipid-soluble endogenously synthesized antioxidant. Role of CoQ in regulation of MPTP, which is involved in apoptotic pathways, was also demonstrated. CoQ biosynthesis is a multi-step process with subtle regulation mechanisms that are often disrupted under pathological conditions and during ageing. Additional administration of CoQ is often used in the form of CoQ medicals to provide an organism with sufficient amount of this coenzyme, but this may lead to inhibition of its endogenous synthesis. Therefore, search for ways to improve endogenous CoQ synthesis is a prospective field of study. Thus, the aim of the present work was to study the effect of complex precursors and modulator of CoQ biosynthesis, namely vitamin E, on bioenergetics, intensity of free-radical oxidation and sensitivity of MPTP to  $Ca^{2+}$  in old rats' heart tissue.

Male Wistar rats aged 6 – 7 months and male Wistar rats aged 24 months were fed standard fodder for the duration of the experiment. Biologically active substances were administered *per os* daily for 10 days. The animals were divided into 3 groups of 10 rats each: 1<sup>st</sup> group – control adult animals; 2<sup>nd</sup> group – control old animals; 3<sup>rd</sup> group – old animals treated with  $\alpha$ -tocopherol, 4-hydroxybenzoic acid and methionine. Levels of CoQ, vitamin E, conjugated dienes, TBA-reactive products, and products of free radical oxidation of proteins were determined in heart homogenates and mitochondria. Activities of mitochondrial complexes I, II, and IV were assayed in heart mitochondria. MPTP opening was studied using spectrophotometric assay of mitochondrial swelling.

The results of our research demonstrate that CoQ and vitamin E levels in rat heart mitochondria under administration of biologically active substances were 1.5 and 1.8 times these of control old animals. Treatment with precursors and modulator of CoQ biosynthesis normalizes functional activity of enzyme complexes of electron-transport chain, namely NADH-CoQ-oxidoreductase, succinate-CoQ-oxidoreductase and cytochrome *c* oxidase. This may be interpreted as an improvement in respiratory processes in heart tissue. According to works by other authors, ageing is accompanied by intensification of free radical oxidation of macromolecules. Indeed, in our experiments we found elevated levels of products of lipid peroxidation, such as conjugated dienes and TBA-reactive products, and of protein peroxidation in old rat heart. Animals treated with complex of biologically active substances had significantly lower content of mentioned products of lipid and protein free-radical oxidation.  $Ca^{2+}$ -induced opening of MPTP was fully prevented in presence of its classic inhibitor cyclosporine A ( $10^{-5}$  M). This ascertains the fact that MPTP opening was the cause of the observed mitochondrial swelling. Administration of abovementioned complex led to a statistically significant decrease in sensitivity of MPTP opening to  $Ca^{2+}$  in concentrations  $10^{-4}$ – $10^{-7}$  M in comparison to control animals.

Therefore the results obtained may be the basis for further development of the medicals of metabolic type to be used successfully in prophylaxis and treatment of different cardiac pathologies, and in ageing.

## Hydrogen peroxide toxicity in differentiated C2C12 muscle cells: are SERCA2 and Bcl-2 family proteins responsible for differential sensitivity of reserve cells and myotubes to Ca<sup>2+</sup>-mediated apoptosis?

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Age-associated muscle weakness and sarcopenia are often linked to elevated oxidative stress and pro-apoptotic response of senescent tissues. However, data on cell vulnerability to pro-apoptotic stimuli in cell culture models are controversial. This may be in part due to the fact that during myogenic differentiation, tissue or cell culture represents a heterogeneous population of cells at different stages of differentiation, such as myoblasts, reserve (satellite) cells, and myotubes, thus generating diverse integrative responses. In addition, in most studies only plated cultured cells were examined, leaving the pool of detached cells behind the scope of analysis. The aim of our study was to analyze the effect of hydrogen peroxide separately in different populations of differentiated C2C12 murine myoblast cells. Our data show that serum depletion for 4-6 days leads to myoblast differentiation that was validated through monitoring the content of myogenic transcription factors (MyoD, myogenin), and protein markers of muscle differentiation (Caveolin-3, SERCA1). Myoblast differentiation results in formation of at least two distinct cell populations that can be separated based on sensitivity to trypsin, and expressing, respectively, markers of myotubes (muscle specific isoforms SERCA1 and Cav-3) and reserve cells (Pax7 and Cav-1). The levels of anti-apoptotic protein Bcl-2 were significantly elevated after myoblast differentiation and the protein was detected exclusively in reserve cells, representing another marker for this muscle cell type. After serum depletion some cells were spontaneously detached and lost from the dishes. Analysis of protein markers in these floating cells revealed mostly non-differentiated myoblasts on the 2<sup>nd</sup> day of differentiation, and differentiated myotubes on 4<sup>th</sup> and 6<sup>th</sup> days after differentiation onset. All the floating cells displayed diminished Bcl-2 levels, and elevated pro-apoptotic Bad and Bax as well as SERCA2 levels relative to adherent cells, suggesting apoptotic mechanism of cell loss. In differentiated C2C12 cells (after 6 days of serum depletion) the exposure to hydrogen peroxide (0.2-4 mM) in the serum free medium induced significant (over serum-free control) loss of cells from the dishes in an exposure time and hydrogen peroxide concentration manner. Microscopic and protein marker analysis of floating and attached cells demonstrated predominant loss of terminally differentiated myotubes, whereas reserve cells stood attached and exhibited low sensitivity to apoptosis. Importantly, floating cells express significantly higher levels of SERCA2, and lower levels of Bcl-2 and HSP70 than attached cells suggesting potential involvement of Ca<sup>2+</sup>-mediated apoptotic mechanism of muscle cell loss induced by hydrogen peroxide. We have shown earlier that Bcl-2 may exert its anti-apoptotic effect at the level of endoplasmic reticulum (ER) Ca<sup>2+</sup> load via downregulation of SERCA2 [1] and that the other Bcl-2 family proteins and ER chaperone proteins, such as HSP70, can modulate this effect [2]. The implication of this mechanism in the apoptotic responses of differentiated muscle cells in aging tissues is discussed.

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## Involvement of mitochondrial and oxidative mechanisms in glyphosate-induced epidermal cell death

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A deregulation of programmed cell death mechanisms in human epidermis accelerates skin aging. We previously showed that glyphosate provoked cytotoxic effects on cultured human keratinocytes, affecting their antioxidant capacities (1-2) and impairing morphological and functional cell characteristics (3). This glyphosate-induced oxidative stress investigation can provide us a possible way to an interesting *in vitro* skin aging model. The aim of the present study, led on the human epidermal cell line, HaCaT, was to examine the part of apoptosis in the cytotoxic effects of glyphosate and the intracellular mechanisms involved in the apoptotic events. We have conducted different incubation periods to reveal events implied in glyphosate-induced cell death. We observed an increase in the number of early apoptotic cells for a low cytotoxicity level (15%), and then, a decrease, in favor of late apoptotic and necrotic cell rates for stronger cytotoxicity conditions. At the same time, we showed that the mitochondrial membrane potential disruption could be a cause of apoptosis in keratinocyte cultures. It is now apparent that the oxidative imbalance is not only glyphosate dose-dependent, but also directly related to the exposure time; this conceptual advance could concern many other environmental toxic agents. Moreover, an original and complementary study by Atomic Force Microscopy (AFM) allows us to combine imaging and mechanical properties measurements on HaCaT cells for different oxidative stress conditions. Finally, a better understanding of the glyphosate-induced deleterious mechanisms will help us in a relevant choice of antioxidant molecules able to reverse these cellular impairments.

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## **Oxidative stress in a model of Alzheimer's Disease. Paradoxical results**

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Alzheimer's Disease is a neurodegenerative disorder which is associated with ageing. Many clinical studies are performed, but for obvious ethical reasons, the study of organs, particularly brain and liver, requires animal models.

There are two major animal models of Alzheimer's Disease, one is the "double transgenic", i.e., the APP/PSEN1 mice and the other is the "triple transgenic". We have used the double transgenic model because we have observed that amyloid plaques occur only in the old animals and not in the young. We have used three groups of animals of different ages – young (3 to 5 months), adult (10 to 13 months), and old (more than 20 months). Of these ages we have both wild type and transgenic mice. We have analysed oxidative stress parameters, particularly hydrogen peroxide production by mitochondria, protein oxidation (determined by carbonylation), glutathione oxidation (determined as the ratio GSSG/GSH), lipid peroxidation (determined as malondialdehyde, MDA).

All the studies reported here deal with oxidative stress in liver. We started thinking that oxidative stress would increase with age (as postulated by the free radical theory of ageing). However, we have observed that oxidative stress occurs in young animals (where it is normally measured). This difference disappears in mature animals in which parameters of oxidative stress are not distinguishable from wild type and transgenic mice and, finally, the situation is reversed in the old and old controls have higher hepatic oxidative stress than young ones.

We conclude that, at least in this model, oxidative stress does not correlate with the severity of Alzheimer's Disease, but rather with the young model of it in the early stages of the process. The interpretation of these data will be discussed. This work was supported by grants SAF2009-08334; BFU2007-65803/BFI from the MEC; ISCIII2006-RED13-027 from the RETICEF, PROMETEO2010/074 and EU Funded COSTB35. This study has been co-financed by FEDER funds from the European Union.

## **Cytosolic NADP<sup>+</sup>-dependent isocitrate dehydrogenase regulates cadmium-induced apoptosis**

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Cadmium ions have a high affinity for thiol groups. Therefore, they may disturb many cellular functions. We recently reported that cytosolic NADP<sup>+</sup>-dependent isocitrate dehydrogenase (IDPc) functions as an antioxidant enzyme to supply NADPH, a major source of reducing equivalents to the cytosol. Cadmium decreased the activity of IDPc both as a purified enzyme and in cultured cells. In the present study, we demonstrate that the knockdown of IDPc expression in HEK293 cells greatly enhances apoptosis induced by cadmium. Transfection of HEK293 cells with an IDPc small interfering RNA significantly decreased the activity of IDPc and enhanced cellular susceptibility to cadmium-induced apoptosis as indicated by the morphological evidence of apoptosis, DNA fragmentation and condensation, cellular redox status, mitochondria redox status and function, and the modulation of apoptotic marker proteins. Taken together, our results suggest that suppressing the expression of IDPc enhances cadmium-induced apoptosis of HEK293 cells by increasing disruption of the cellular redox status.

## The role of the mitochondrial palmitate/Ca<sup>2+</sup>-induced pore in the adaptation of animals to hypoxia

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Earlier we found that being added to rat liver mitochondria, palmitic acid (Pal) *plus* Ca<sup>2+</sup> opened a cyclosporin A-insensitive pore (PalCaP), which remained open for a short time. Apparently, this pore is involved in the Pal-induced apoptosis and may also take part in the mitochondrial Ca<sup>2+</sup> recycling as a Ca<sup>2+</sup> efflux system. In this paper, we continue studying physiological and regulatory aspects of the pore. It is known that laboratory animals vary significantly in their sensitivity to hypoxic conditions, which is genetically predetermined. In this work we examined the opening of PalCaP in the liver mitochondria of hypoxia resistant, hypoxia-sensitive rats, and hypoxia-adapted animals. In liver mitochondria of the hypoxia-resistant rats, PalCaP opens easier than in the organelles of the hypoxia-sensitive animals. The latter, however, can be adapted to hypoxic conditions, becoming quite tolerant to the inductors of PalCaP. In contrast to PalCaP, the classical MPT pore opens easier in mitochondria of the hypoxia-sensitive rats. These data indicate a difference in the function of these pores and confirm our recent observations that regulation and physiological significance of PalCaP and MPT pore are rather different. The adaptation of hypoxia-sensitive rats to the low-oxygen conditions increases the sensitivity of their mitochondria to PalCaP inductors. We found that the opening of PalCaP in liver mitochondria of the hypoxia-resistant rats leads to the inhibition of ROS production, whereas in the hypoxia-sensitive animals, the production of ROS by mitochondria is stimulated under the same circumstances.

Based on the results of this work, we suppose that a possible mechanism of animal's adaptation to hypoxia is the activation of a Ca<sup>2+</sup> cycle, which is mediated by Ca<sup>2+</sup> uniporter and PalCaP and leads to the mild uncoupling and decreased ROS production in mitochondria.

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## **A comparison of mechanism(s) of action of zinc (II) and selenite on AS-30D hepatoma cells and isolated mitochondria**

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Mitochondria of AS-30D rat ascites hepatoma cells cultivated in vitro are found to be the main target for Zn<sup>2+</sup> and sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>). We have shown that high μM concentrations of Zn<sup>2+</sup> or selenite are strongly cytotoxic, killing the AS-30D cells by both apoptotic and necrotic ways with an involvement of severe mitochondrial dysfunction. Both Zn<sup>2+</sup> and selenite produced strong changes in intracellular generation of reactive oxygen species (ROS) and the mitochondrial dysfunction due to disturbance of the electron transport chain (ETC), dissipation of the membrane potential ( $\Delta\Psi_{\text{mito}}$ ) dissipation and the mitochondrial permeability transition (MPT) pore opening. The study revealed also the significant distinctions in the toxic action of Zn<sup>2+</sup> and selenite on AS-30D cells; in particular, selenite induced a much higher intracellular ROS level (the early event) compared to Zn<sup>2+</sup>, but a lower  $\Delta\Psi_{\text{mito}}$  loss as well seen as a lower decrease of the uncoupled respiration rate of the cells. In the case of Zn<sup>2+</sup>, just the mitochondrial ETC disturbance was the early and critical even in the mechanism of its cytotoxicity. Sequences of events manifested in the mitochondrial dysfunction and cytotoxicity produced by the metal/metalloid under test are compared with those obtained earlier for Cd<sup>2+</sup>, Hg<sup>2+</sup> and Cu<sup>2+</sup>. The compatibility of the data found on isolated mitochondria and in intact cells is also discussed.

## The role of histidine on wound healing using cultured rat intestinal epithelial cells

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**Aim:** Most Japanese people who leads general eating habits has fully been taking in the protein derived amino acid which is important as a nutrient. However, the amino acid balance in the living body will lose a balance for various stresses under pathological condition. The aim of this study was to investigate the functional role of histidine which is the important amino acid in pathological conditions on wound healing using cultured rat intestinal epithelial cells (RIE). **Material & Methods:** A round artificial wound was induced in the center of confluent RIE monolayers. The closure of round wounds were evaluated in all the amino acid addition culture medium (Full culture medium), an amino acid additive free culture medium (Zero culture medium), and histidine lack culture medium. MTT assay was used for the measurement of cellular proliferation. The expression of HSP70, Caspase-3, TGF-beta, PCNA and PDGFR-beta in RIE cultured with various medium were estimated by western blotting. **Results:** In the histidine lack culture medium and the Zero culture medium, the restitution speed of RIE showed delay remarkably. In MTT assay, compared with the Full culture medium, the cell proliferation after 24 hour incubation in the histidine lack culture medium was significantly decreased. In the histidine lack culture medium and the Zero culture medium, the expression of HSP70 and Cleaved Caspase-3 were increased, compared with the Full culture medium. The expression of TGF-beta was decreased time-dependently between 3 groups. Furthermore the expression of TGF-beta and PCNA were decreased in the histidine lack culture medium and the Zero culture medium. **Conclusion:** It was suggested that the induction of heat shock protein, the induction of apoptosis and the reduction of growth factor were involved in the mechanisms of delayed wound healing by lack of histidine.



## Carbon monoxide liberated by CORM-3 uncouples mitochondrial respiration and modulates the production of reactive oxygen species.

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Carbon monoxide (CO), produced during the degradation of heme by the enzyme heme oxygenase, is an important signaling mediator in mammalian cells (1). The development of carbon monoxide-releasing molecules (CO-RMs), compounds that carry and deliver precise amounts of CO to organs and tissues (2), offers a unique opportunity for studying the chemical reactivity of CO with biological components and exploiting its therapeutic potential (3). In the present study we show that CORM-3, a well-characterized water-soluble CO-releasing agent (4), uncouples respiration when applied to isolated cardiac mitochondria. Addition of low micromolar concentrations of CORM-3 (1-20  $\mu$ M), but not an inactive compound that does not release CO (iCORM-3), significantly increased mitochondrial oxygen consumption rate (state 2 respiration) in a concentration-dependent manner. In contrast, higher concentrations of CORM-3 (100  $\mu$ M) suppressed ADP-dependent respiration through inhibition of cytochrome c oxidase. The uncoupling effect mediated by CORM-3 was inhibited in the presence of the CO scavenger myoglobin corroborating that CO is directly responsible for the observed effect. Moreover, this effect was associated with a gradual decrease in membrane potential over time ( $\Delta\psi$ /min) and was partially reversed by malonate, an inhibitor of complex II activity. Similarly, inhibition of uncoupling proteins or blockade of adenine nucleotide transporters attenuated the effect of CORM-3 on both state 2 respiration and  $\Delta\psi$ . Hydrogen peroxide ( $H_2O_2$ ) produced by mitochondria respiring from complex I-linked substrates (pyruvate/malate) was increased by CORM-3. However, respiration initiated via complex II using succinate resulted in a 5-fold increase in  $H_2O_2$  production and this effect was significantly inhibited by CORM-3. These findings disclose a counterintuitive action of CORM-3 suggesting that CO at low levels acts as an important regulator of mitochondrial respiration.

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## Effect of bariatric surgery on inflammatory biomarkers, antioxidants and oral health *status*

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**Objective:** This study evaluated the outcomes of bariatric surgery on levels of vitamins C and E,  $\beta$ -carotene, (diet/blood), inflammatory markers such as myeloperoxidase (MPO), nitric oxide metabolites (NO<sub>x</sub>), C-reactive protein (CPR), as well as oral health *status*, in patients submitted to Roux-en-Y gastric bypass surgery. **Methods:** Prospective single-blinded controlled study, where participants were sorted in two groups: Control Group (CG) and Bariatric Group (BG), both composed by 35 individuals with mean ages of 38.7 $\pm$ 9.4 and 39.6 $\pm$ 9.2 years, and mean body mass index (BMI) of 22.2 $\pm$ 2.1 and 47.6 $\pm$ 9.1 kg/m<sup>2</sup>, respectively. The oral health *status*, and antioxidant and inflammatory markers contents were determined at the basal period, as well as at the 6<sup>th</sup>, 12<sup>th</sup> and 24<sup>th</sup> months after surgery. **Results:** Compared to the basal period after 24 months of surgery, BMI decreased from 47.05 $\pm$ 1.46 to 30.53 $\pm$ 1.14 kg/m<sup>2</sup> ( $P$ <0.001), and also decreases in vitamin C consumption (15.3 $\pm$ 4.5%,  $P$ <0.001), in energy intake (27.07 $\pm$ 5.3%,  $P$ <0.001), increases in levels of  $\beta$ -carotene (17.30 $\pm$ 1.81%;  $P$ <0.001) and vitamin E (607.88 $\pm$ 40.3%,  $P$ <0.001) were found. Also, CPR (89.56 $\pm$ 1.98%,  $P$ <0.001) and NO<sub>x</sub> (24.14 $\pm$ 6.6%,  $P$ <0.001) showed decreased levels and MPO showed increased levels (24 $\pm$ 8.3%,  $P$ =0.014) compared to the basal period. After 12 months increased incidence of vomiting ( $P$ =0.001) and teeth hypersensitivity ( $P$ =0.027) were detected. Increased prevalence of gum bleeding (from 15.4% to 26.9%) and loosen teeth (from 3.8% to 19.2%) were also observed after 24 months post-surgery. Salivary flow increased from 0.4 $\pm$ 0.02 mL/min at the basal period to 1.2 $\pm$ 0.09 after 24 months (17.4 $\pm$ 7.1%,  $P$ <0.001), while after 12 months the buffer capacity was reduced to 5.3 $\pm$ 0.19 ( $P$ =0.004), being classified as moderate.

**Conclusions:** 24 months after the Roux-en-Y gastric bypass surgery decreased levels of vitamins E and C, as well as CPR and NO<sub>x</sub> levels, together with increased MPO levels were found. After such period, a high prevalence of vomiting was also found as an underlying risk factor for hypersensitivity, caries and tooth erosion.

### **Growth of dental pulp stem cells at 3% y 21% O<sub>2</sub>:**

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**Introduction:** It's a common practice to culture stem cells in the presence of ambient oxygen (~21% O<sub>2</sub>), while oxygen level observed in physiological environment is often much lower (3-7%). Furthermore, it has been demonstrated that high concentration of O<sub>2</sub> can cause oxidative stress via production of reactive oxygen species (ROS)-free radicals that can damage lipids, proteins and DNA, altering cell metabolism.

**Aim:** The aim of the present study is to compare the growth of dental pulp mesenchymal stem cells at 3% and 21% of O<sub>2</sub> and a parameter of oxidative stress at both conditions.

**Material and methods:** Dental pulp mesenchymal stem cells were isolated and characterized with confocal microscopy. Posteriorly, they were cultivated at 3% and 21% of O<sub>2</sub>. At days 3, 6 and 9, the cells were counted and the viability of the cells was determined. Samples were also collected to measure oxidized proteins.

**Results:** The dental pulp mesenchymal stem cells were positive for the markers STRO1, OCT4, CD133, NESTIN, CD34 and negative for CD45. The proliferation rate of dental pulp mesenchymal stem cells was higher at 3% O<sub>2</sub> than ambient 21% O<sub>2</sub> while the cell viability was higher than 90% at both conditions. Moreover, the proteins oxidation of cells cultivated at 3% O<sub>2</sub> showed less oxidation than those cultivated at 21% O<sub>2</sub>.

**Conclusion:** The proliferation of dental pulp mesenchymal stem cells is enhanced at physiological oxygen levels which can be related with lower oxidative stress.

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## Selenium, selenoprotein expression and sulforaphane in a model of inflammation-mediated colon carcinogenesis

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Age-related diseases, in particular cancer, are discussed to be associated with selenium-deficiency. Also, a wide range of dietary chemopreventive agents, comprising phytochemicals such as glucosinolates and derived metabolites, have been demonstrated to prevent carcinogenesis via activation of endogenous defense systems. A link between the effects of selenium and chemopreventive phytochemicals could be seen in the activation of the Nrf2 system, which *inter alia* controls the expression of the selenoproteins GPx2 (Banning 2005) and TxR1 (Hintze). However, it is not known whether selenium affects carcinogenesis directly or via expression of specific selenoproteins.

Wild-type and GPx2-KO mice were subjected to inflammation-mediated carcinogenesis (AOM/DSS) and analysed for the expression of selenoproteins, apoptosis, inflammatory parameters and cancer of the intestinal tract. Groups of animals were fed a selenium-poor, -adequate, or -supranutritional diet and treated with the Nrf2 activator sulforaphane (SFN) (3,4). Expectedly, GPx2 and TrxR increased with increasing selenium supply and were induced by SFN, however not uniformly regarding localization and selenium supply. Surprisingly, GPx1, which is not an Nrf2 target, was highly increased in GPx2-KO mice in each selenium status in areas where GPx2 is expressed in wild-type mice (3). A time-dependent increase in intestinal apoptosis was found in GPx2-KO compared to wild-type mice (3,4). Inflammation and cancer multiplicity was decreased by selenium and GPx2. Surprisingly, the effect of SFN was rather detrimental under selenium-restriction but beneficial in both genotypes under selenium-adequacy, revealing that SFN needs a selenoprotein for acting anti-inflammatory and/or anti-carcinogenic. Since the expression of Nrf2 targets GPx2 and TrxR did not strictly correlate with the anti-inflammatory/anti-carcinogenic effects of SFN, other selenoproteins, possibly GPx1, support the chemopreventive action of SFN in an as yet unclear way. This hypothesis complies with the observation that spontaneous carcinogenesis is only observed in mice deficient in both, GPx1 and GPx2 (5). In short, the interaction of Nrf2 activators and selenium is appreciably more complex than anticipated. Up to now it only appears justified to state that a diet balanced in selenium and phytochemicals is likely protective. Whether this interpretation can be extended to longevity remains to be elucidated.

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**Tyrosol, one of the main phenolic compounds in virgin olive oil, increases lifespan and delays aging in the nematode *Caenorhabditis elegans*.**

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Plant polyphenols are known to induce protection in a wide range of pathologies including cancer, cardiovascular disease and neurodegenerative disorders. Recently, several phenolic compounds have also been shown to increase life expectancy and stress resistance in simple model organisms. Although not long ago these effects were believed to be solely due to the antioxidant and anti-inflammatory properties present in many of these compounds, growing evidence points towards an antioxidant-independent action, most likely involving changes in gene expression patterns and affecting different signaling cascades.

Tyrosol and Hydroxytyrosol, in simple forms or in conjugates, are the main phenolic compounds present in Virgin Extra Olive Oil. Numerous studies have shown that phenolic compounds of olive oil have effects potentially beneficial to human health such as inhibition of LDL oxidation and platelet aggregation. Moreover, a recent report has linked tyrosol with longevity and cardioprotection through its effects on SIRT1 expression. Nevertheless, the potential effects of these compounds on the lifespan of a whole organism have not been studied before.

In order to investigate the effects of tyrosol on longevity, we decided to use the nematode *Caenorhabditis elegans*, since it is a well characterized model organism to study the aging process which facilitates survival assays and molecular analyses.

Our results showed that one of the specific tyrosol concentrations assayed was able to induce a 20% increase in the median lifespan of *C. elegans*. This phenol also delayed the onset of a typical marker of aging in these nematodes. Since longevity in *C. elegans* is related to heat stress resistance, we also investigated the effect of tyrosol in the nematode thermotolerance. We found that tyrosol induces a two-fold increase in the survival of adult nematodes to heat stress and a 4-fold increase in the expression of a *small Heat Shock Proteins* (sHSP) family gene. These genes code a wide range of chaperones playing a crucial role in the stress response, both in nematodes and in humans. Interestingly, these proteins expression in *C. elegans* is under the regulation of the *Insulin/igf-1* (IIS) signaling pathway, known to regulate longevity in this and other organisms.

In conclusion, this study demonstrates for the first time that a single phenolic compound from virgin olive oil is able to enhance longevity in an animal model. Our results suggest that this effect may be related to the ability of tyrosol to induce the expression of specific genes directly involved in key longevity regulation pathways.

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We thank the *Caenorhabditis* Genetic Centre, which is funded by the National Institutes of Health National Centre for Research Resources, for supplying the strain used in this study.

### **The effect of soy consumption on longevity of OF-1 male mice:**

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INCLIVA.

The role of soya feeding and in particular of soy isoflavones on health and longevity has been the subject of intense research in recent years. The major conclusion being that increasing the intake of soy will prevent age-associated diseases and even prolong life span.

In fact, to our knowledge, the role of soya feeding on longevity in control populations of mammals has not been performed. Thus the major aim of this study was to determine whether soya feeding prolongs life span in mice kept in specific pathogen free housing. We used 157 mice and they were divided into two groups, one fed with a soy-free diet (similar to the Western diet for humans) and another with a soya-rich diet (equivalent to the Eastern type diet). All along the longevity curve we controlled weekly their weight and food intake. We also performed a number of behavioural tests like the novel object recognition test, the grip strength, the neuromuscular coordination (tightrope test) and the oral glucose tolerance test. Groups of five mice were sacrificed in each group at the beginning of the study, and at 80, 50, and 10 per cent of the survival. These animals were used to obtain blood and organs to measure parameters of oxidative stress. Hepatic mitochondrial peroxide production, lipid peroxidation (determined as malondialdehyde) and oxidised proteins (measured as carbonylation) were determined in blood and in several organs of these animals. Also, the expression of longevity-related genes, such as manganese superoxide dismutase, and glutathione peroxidase were measured.

We also measured the blood concentration of genistein, daizdein, and equol, the major metabolites of genistein.

Results show the following: the soy-rich diet resulted in an increase of genistein and its metabolites in plasma, which is significant and which is similar to that obtained in human nutrition when persons eat a soya-rich diet. We also found a significant increase in the expression of antioxidant enzymes, namely superoxide dismutase and glutathione peroxidase. The most relevant result is that long life soya feeding does not prolong longevity in well kept animals.

The interpretation of the results in the light of the free radical theory of ageing will be discussed.

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## Identification and characterization of novel compounds affecting chronological lifespan in *S. pombe*

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The identification of compounds that affect lifespan and the characterization of their molecular targets is an important aspect of aging research. These studies lead to the discovery of new compounds and aging pathways and establish the foundation for treatments that might delay cellular aging or shorten lifespan of cancer cells.

Simple organisms such as yeast have contributed a large part to the molecular fundamentals of aging. Studies in yeast elucidated several important molecular aging mechanisms and discovered novel longevity genes that proved to be conserved also in multicellular eukaryotes.

The aim of the project is to identify compounds that affect cellular aging of the model organism *S.pombe*. We are seeking to identify small molecules that affect the chronological lifespan. For this purpose, we established a high-throughput screen which enables the detection of small molecules from large natural compound libraries that delay or accelerate the aging of stationary phase cells.

In a next step, we will characterize the molecular targets of the identified compounds. For this purpose, we will narrow down their site of action by a yeast three hybrid screen, genetic and biochemical analyses.

In first screens we have identified several compounds that affect chronological lifespan in *S.pombe*. Preliminary results will be presented.

## Moderate ethanol increases life span in drosophila melanogaster

**Gambini J, Edo R, López-Grueso R, Bonet-Costa V, Abdelaziz KM, El Alami M, Ingles M, Dromant M, Olaso G, Escriva C, Borrás C and Viña J.**

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The French paradox, indicates that populations who drink red wine tend to live longer than those who do not in spite of the fact, that many of these populations have high intake of saturated fat. The majority of the beneficial effects of moderate red wine intake are associated with the concomitant ingestion of isoflavones present in red wine. However, the effect of ethanol has been somewhat overlooked. Moreover, ethanol which is metabolised via ethanol dehydrogenase, to give rise to acetaldehyde, results in an increase in NADH formed in this reaction. The fact, sirtuins are NAD-dependent and modulate DNA expression, resulting in an alleged increase in life span prompted us to study the effect of the NADH/NAD ratio in the regulation of these interesting enzymes.

To test this, we measured the NADH/NAD ratio in cytoplasm by determining the lactate/pyruvate ratio. Following the ideas of such pioneers as Theodore Bucher and Hans Krebs, who in the 60's and 70's showed that the redox ratio of nicotinamide coenzymes cannot be determined directly, because they are bound to hydrogenases enzymes and because significant differences occur between mitochondria and cytosol.

Previous work, from our laboratory has shown that increasingly NADH/NAD ratio results in an increased expression of sirtuins in cultures cells, in drosophila melanogaster and exercise in mammals.

The aim of this work, was to test whether these changes associated with ethanol intake result in an increased longevity in control populations of drosophila melanogaster.

Ethanol was added to the drosophila food and the NAD/NADH ratio was determined from the lactate and pyruvate values, considering the equilibrium reaction of lactate dehydrogenase.

The main result we have observed is that moderate ethanol increases longevity by itself and not because of the wine component associated to it. This is due to an increased expression of sirtuins. Of course, our results do not preclude the fact that other beneficial compounds such as phytoestrogens may be associated in one beverage, red wine and others. So, the main conclusion here is that ethanol itself in a moderate amount, is beneficial to promote longevity, at least in invertebrates.

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## **The effect of exercise and SIRT activation on brain function and ROS defense in rats artificially selected to high or low running capacity**

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Sirtuins are NAD<sup>+</sup>-dependent protein deacetylases. Resveratrol, an activator of SIRT1 has been shown to reduce plaque pathology in Alzheimer disease. Regular exercise enhances performance of the brain, and can cause an elevated level of ROS, which could result in oxidative challenge. Among the several defense systems against this kind of oxidative challenge we became interested in OGG1 mediated DNA repair.

Aim of the study: We tested the effects of resveratrol on rats selectively bred over 24 generations for intrinsic aerobic high running capacity (HRC) or low running capacity (LRC). The animals were administered with resveratrol daily for 4 months. Some of the groups were subjected to treadmill running at 70% of the VO<sub>2</sub>max. We measured the protein levels (SIRT1, SIRT4, SIRT6, PBEF, OGG1, acetylated lysin) of the brain tissue with western blot and used RT PCR to examine changes in hippocampal mRNA. We also made immunohistochemical staining to determine acetylated OGG1 levels. In addition, we used HCT116 cells test OGG1-SIRT1 interaction by siRNA of SIRT1 and applying activators/inhibitors of SIRT1.

Results: In passive avoidance test the HRC animals had better performance in the long term memory. Histochemical staining shows decreased acetylated OGG1 levels in these groups. WB assays show that acetylated lysine and OGG1 levels were decreased in the resveratrol-fed animals. The data obtained from cell culture suggest that SIRT1 could deacetylate OGG1, which decrease the activity of OGG1.

Conclusion: SIRT1 appears to down-regulate the activity of OGG1, which could result in accumulation of 8-oxoG in the DNA.

## **Estrogen Replacement Therapy (ERT) requires a “critical time” for its beneficial effects**

**López-Grueso R, Bonet-Costa V, Edo R, Abdelaziz KM, El Alami M, Ingles M, Dromant M, Olaso G, Escriva C, Gambini J, Borrás C and Viña J.**

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**INTRODUCTION:** The life expectancy of the population has been increasing steadily over the twentieth century in both genders although the survival of women has always been higher compared to men. These differences in longevity are reproduced in other animal species such as rats. There must be some biological basis to support the differences and this natural phenomenon could be explained on the basis of the mitochondrial theory of aging. Mitochondria are a major source of free radicals in cells and generate less amount of hydrogen peroxide from females than those of males. Differences can be explained by the effects of estrogens because ovariectomy cancels out the benefits shown in females compared to males.

**AIM:** Our aims were to study the oxidative stress and antioxidant parameters in young female Wistar rats and to evaluate the effect of ovariectomy and/or the initiation of estrogen replacement therapy (ERT).

**MATERIAL AND METHODS:** We used female Wistar rats, divided into young (4–7 months), young control (Sham) and ovariectomized (3 or 6 weeks) groups. There were groups which were given 1 mg/kg/day subcutaneously of 17 $\beta$ -estradiol for three weeks, starting the day after surgery (OVX 3E) or three or six weeks later, for the same period of three weeks (OVX 3+3E and OVX 6+3E). We determined hydrogen peroxide levels (H<sub>2</sub>O<sub>2</sub>), glutathione peroxidase (GPx) and catalase enzymatic activity in mitochondria and an oxidative damage to lipids (malondialdehyde, MDA) in plasma, isolated hepatic mitochondria and liver.

**RESULTS:** The ovariectomized rats generate higher H<sub>2</sub>O<sub>2</sub> levels than Sham but are diminished only if estrogen replacement therapy (ERT) is initiated immediately. The same pattern occurs with GPx and Catalase enzymatic activity in mitochondria and there was a recovery of MDA in plasma after the negative effects produced by ovariectomy.

**CONCLUSIONS:** Ovariectomy causes a decrease in antioxidant enzymes and an increase of oxidative stress parameters. There is a “critical time” to initiate the ERT after ovariectomy for mimic similar effects compared to control rats.

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## Effects of exhaustive exercise on expression of RCAN1 isoforms in rat muscles: involvement of oxidative stress.

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**Introduction:** The *RCAN1* gene (also called *Adapt78* or *DSCR1*) generates a series of RCAN1 protein isoforms including RCAN1-4, RCAN1-1L, and RCAN1-1S whose expression is regulated by oxidative stress and calcium. RCAN1 proteins are natural cellular regulators of calcineurin, a Ca<sup>2+</sup>-dependent protein phosphatase involved in several crucial physiological processes and pathological conditions such as skeletal myocyte differentiation, cardiac hypertrophy, Alzheimer’s disease and cancer. Although exhaustive exercise is known to involve oxidative stress, very little is known about its effect(s) on RCAN1 protein expression or distribution patterns in muscle. **Objectives:** In this study, we analyzed the effects of exhaustive exercise, and the potential involvement of oxidative stress, on RCAN1 proteins in various rat muscles (gastrocnemius, soleus and EDL). **Methods:** Wistar male rats were divided into two groups: control (C) and exercise (E). Animals were sacrificed at 0h, 1h, 2h, 6h and 24 hours after an exhaustive exercise on a treadmill. RCAN1-4, RCAN1-1S and RCAN1-1L expression were evaluated in muscles by Western blot analysis. F2-isoprostane concentrations were measured by LC-MS. Vitamin E contents were quantified by HPLC, and antioxidant capacities were estimated by spectrofluorimetry. **Results:** We show that RCAN1-1S initially increased, then decreased 6 and 24 hours after exhaustive exercise. This reduction in RCAN1-1S levels might be dependent on oxidative stress given the concomitant changes in Vitamin E content in all muscles. Moreover, antioxidant defences seem to be decreased in EDL. These results suggest that exhaustive exercise regulates RCAN1-1S, possibly by an oxidative stress-dependent mechanism.

## Characteristic of mitochondrial biogenesis of training sensitive and training resistance rats

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### Introduction

It is well-known everybody adapted with different magnitude to the same exercise stimuli. Twin studies shown that trainability is partly dependent on genetics. We studied rats which as been shown to react differently to aerobic exercise training (Keller, Vollaard et al. 2011). Mitochondrial biogenesis is one of the most important processes in skeletal muscle, which is responsible for the degree of adaptation to endurance training. Therefore, we were interested in the effect of exercise in the mitochondrial biogenesis in training sensitive and training resistance rats.

### Methods

27 selective breeding 11<sup>th</sup> generation for low response to training (LRT) and high response to training (HRT) rats were training for 3 months at the 70% of  $VO_{2max}$ . Animals were divided into low response, training and control groups (LRTE and LRTC) and high response training and control groups (HRTE and HRTC). Treadmill training duration was constant (30 min) and the speed started at 15 m/min and reached 25m/min on the last weeks. The changing of  $VO_{2max}$  was measured by a specific treadmill. The biochemical analysis was assessed by Western-blot. Statistical significance was assessed by one-way ANOVA, followed by Tukey's post hoc test and correlation. The significance level was set at  $p < 0.05$ .

### Results

The maximal oxygen uptake and the running distance were significantly higher in both of the exercise groups and training sensitive rats had more massive increase in  $VO_{2max}$ . The level of nuclear respiratory factor-1 (NRF-1) and peroxisome proliferator-activated receptor gamma coactivator alpha-1 (PGC-1) decreased in LRTE group but increased in HRTE group compared to control groups by effect of exercise. Content of mitochondrial transcription factor 1 (mtTFA) changed similarly to NRF-1 and PGC-1. Exercise resulted decreasing level of mitofusin-1 (Mfn1) and increasing level of mitochondrial fission-1 (Fis1). We measured significant increasing the levels of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) in LRTC and HRTE groups.

### Discussion

Our data suggest that one of the reasons of different response to aerobic exercise training is due to the different activation of proteins that are involved in the biogenesis of mitochondria. We aim to identify the key proteins that are responsible for different adaptation to aerobic exercise training.

Keller, P., N. B. Vollaard, et al. (2011). "A transcriptional map of the impact of endurance exercise training on skeletal muscle phenotype." *J Appl Physiol* 110(1): 46-59.

## Markers of mitochondrial biogenesis are affected by aging and exercise training in rat skeletal muscle

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### Introduction

Aging is associated with impairment of mitochondrial network and function in the skeletal muscle, which could be impacted by mitochondrial biogenesis and quality control of mitochondrial proteins.

### Methods

We assessed the effects of six weeks moderate intensity regular exercise on mitochondrial biogenesis and on proteins that play an important role in quality control of mitochondrial proteins.

### Results

Exercise training attenuated the age-associated decline in SIRT1 activity, AMPK, pAMPK and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), UCP3 and Lon protease contents in the gastrocnemius muscle of rats. On the other hand, it prevented the age-related increase in NRF1, TFAM, Fis1, Mfn1 and polynucleotide phosphorylase (PNPase) levels. We could not detect significant changes in the level of reactive oxygen species and HSP78 levels as a result of aging or exercise training.

### Summary

This revealed that exercise training counteracts with the aging process of skeletal muscle in those proteins which are involved in the biogenesis of mitochondria and quality control of mitochondrial proteins.

## **The effect of endurance exercise and resveratrol activation on physiological performance on rats artificially selected to high or low running capacity**

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### Introduction:

Sirtuins are NAD<sup>+</sup> dependent protein deacetylases and suggested regulators of aging, fat and sugar metabolisms, DNA repair, mitochondrial biogenesis, brain function and fiber type differentiation of skeletal muscle. In the present study we were interested in the effect of regular training and resveratrol supplementation on the mitochondrial biogenesis on rats with different intrinsic aerobic capacity.

### Methods:

We have trained selectively bred 24 generations for intrinsic aerobic high running capacity (HCR) or low running capacity (LCR) rats and some groups were supplemented by resveratrol for 4 month. Exercised animals were subjected to treadmill running for 16wk, 5/wk, 1hr/day at 70% of the VO<sub>2</sub>max.

### Results:

Twelve weeks exercise training significantly increased the VO<sub>2</sub>max in both groups (in the HCR Rats 26% than at LCR 15%). Resveratrol selectively effected HCR rats and resulted in significant improvement of aerobic endurance capacity. The supplementation increased the gripping performance by 300% in HCR compared to control animals. The accumulation of free radicals increased in all trainer groups, while decreased in resveratrol supplemented groups. The acetylation of lysine residues were significantly higher in the gastrocnemius of LCR than HCR rats. Significant increase was not found in the content of SIRT1 and NAMPT. On the other hand, the level of OGG1, SIRT4, SIRT6 and FOXO1 showed running capacity dependent alterations. Mitochondrial biogenesis related protein content such as PGC1 $\alpha$  and NRF1 not showed significant differences, but TFAM increased in trainer and resveratrol-fed groups in case of both genotype rats (HCR and LCR). Fis1 and Mfn1 were significantly higher in muscle of control HCR rats compared with control LCR, but there were no any changes in Fis1 content after treatments, while in Mfn1 content increased significantly in LCR group as a result of training and resveratrol treatment.

### Discussion:

One of the objectives of the study was to test whether regular training could overcome the “LCR genetics” associated low aerobic capacity and metabolic problems. Our data shows, that regular exercise is a powerful tool to compensate disadvantages of unfavourable genetics setup, and prevent genetics associated metabolic alterations. Moreover, our data revealed that resveratrol significantly enhances endurance power at HCR trained rats. Moreover, it appears that exercise training and resveratrol treatment significantly decreases the differences in the mitochondrial biogenesis associated proteins between HCR and LCR groups. Overall, in conclusion it appears that life-style modification, physical activity and/or nutrition beneficially effects physiological performance and prevents genetics-related physiological malfunction of selectively bred rats.

## Age-dependent 8-oxoguanine-DNA-glycosylase activity is modulated by adaptive responses to physical exercise in human skeletal muscle

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### Introduction

8-Oxo-7,8 dihydroguanine (8-oxoG) accumulates in the genome over time and is believed to contribute to the development of aging characteristics of skeletal muscle and various aging-related diseases.

### Results

Here, we show a significantly increased level of intrahelical 8-oxoG and 8-oxoguanine DNA glycosylase (OGG1) expression in aged human skeletal muscle compared to young ones. In response to exercise, 8-oxoG level is lastingly elevated in sedentary young and old subjects, but it returned rapidly to pre-exercise levels in DNA of physically active individuals independent of age. 8-oxoG level in DNA inversely correlated with the abundance of acetylated OGG1 (Ac-OGG1) but not with total OGG1, apurinic/apyrimidinic endonuclease (AP)-1 or Ac-APE1. Ac-OGG1 level is linked to conditioning of muscle to exercise-induced oxidative stress as shown by decreased levels of lipidperoxides and expression of Cu,Zn-SOD and Mn-SOD and interplays between acetyl transferase p300/CBP and deacetylases SIRT1 and SIRT3 but not SIRT6 expression.

### Conclusion

Together these data suggest that not OGG1 level but its acetylated form is responsible for removal of 8-oxoG from the DNA of human skeletal muscle and Ac-OGG1 level is dependent on adaptive cellular processes to physical activity, but is age independent.

## Selenium supplementation specifically stimulates the repair of oxidative DNA damage

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**Scope:** Epidemiological studies have demonstrated an inverse relationship between selenium (Se) level and cancer incidence and/or mortality. However, the molecular mechanisms underlying the cancer chemopreventive activity of Se compounds remain largely unknown. To test the hypothesis of a role of DNA repair, we investigated the activation of DNA repair systems in response to 4 different genotoxic agents in cultured cells pre-treated by Se.

**Methods and results:** LNCaP human prostate adenocarcinoma cells were either untreated or grown in the presence of low concentrations of Se compounds (30nM sodium selenite, or 10mM selenomethionine) and exposed to UVA, H<sub>2</sub>O<sub>2</sub>, methylmethane sulfonate or UVC. Cell viability as well as DNA damage induction and repair were evaluated by the alkaline Comet assay. Overall, Se was shown to be a very potent protector against cell toxicity and genotoxicity induced by oxidative stress. Furthermore, Se-treated cells exhibited increased oxidative DNA repair activity, indicating a novel mechanism of Se action.

**Conclusion:** Our study establishes that supplementation of cells with low, physiologically relevant concentrations of Se protects LNCaP cells against the cytotoxic and genotoxic effects of oxidative stress, but not of agents leading to DNA alkylation and photoproducts formation. The protection against oxidative genotoxicity is likely explained by the enhanced repair of oxidative DNA lesions. The benefits of Se may be explained by a combination of antioxidant activity and the enhancement of DNA repair capacity.

The short-term outlooks will be to investigate the relative gene expressions and to evaluate the protein expression of several DNA repair enzymes, in cells treated or not with Se compounds. Finally, we will examine the major seleno-dependent pathways of DNA repair, by host cell reactivation assay (HCR). We will carry out an *in cellulo* kinetic study, to evaluate the Se-mediated modulation of repair activities.



## **Sperm head defects and disturbances in spermatozoal chromatin and DNA integrities in idiopathic infertile subjects: Association with cigarette smoking**

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**Objectives:** To evaluate sperm chromatin and DNA integrities in idiopathic infertile men and determine the possible association(s) of cigarette smoking on oxidative stress markers, antioxidant capacity and semen quality.

**Subjects and methods:** Semen samples from men referring to the andrology laboratory were categorized into 3 groups: fertile non-smokers (n=16), infertile non-smokers (n=36), and infertile smokers (n=34). Semen analysis was performed according to WHO criteria. The percentage of sperm DNA fragmentation index (%DFI) and the percentage of sperm with abnormally high DNA stainability (HDS%; immature spermatozoa) were determined by SCSA using the metachromatic properties of acridine orange. Lipid peroxidation, superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) levels in seminal plasma and spermatozoa were measured by spectrophotometric assays.

**Results:** The classical semen parameters were negatively correlated with lipid peroxidation in spermatozoa; motility and morphology were negatively correlated with %DFI (p<0.05). HDS % was also negatively correlated with above markers except for morphology (r=-0.352, p=0.081). DFI% and HDS% were significantly higher in the infertile smokers group than in infertile non-smokers (p=0.032; p=0.001 respectively). Cigarette smoking was significantly associated with DFI%, HDS%, TBARS and the fraction of “round-headed” sperm (r=0.796, p=0.0001; r=0.371, p=0.033; r=0.606, r=0.591, p=0.001 respectively), and decreased SOD levels (r=-0.545).

**Conclusion:** DFI%, HDS% and round-head sperms are increased in idiopathic infertile men; this increase is associated with cigarette smoking. These defects may be attributed to increased oxidative stress and insufficient scavenging antioxidant enzymes in the seminal fluid of infertile patients

## Decreased pulmonary antioxidant defenses and increased oxidative stress in ageing.

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### Background:

During the course of aging there is increased oxidative stress and decreased antioxidant defenses in the body, leading to a wide range of damage to cellular structures, including protein oxidation, enzyme (in) activation and damage to the DNA. Given the current thinking that some pulmonary diseases could be viewed as accelerated lung ageing, we set out to characterize normal lung ageing with respect to (anti)oxidant gene expression, protective protein S-glutathionylation and damaging protein carbonylation. We therefore compared these various parameters in the lungs of male C57BL/6 mice of 12 and 99 weeks of age.

### Results:

Using quantitative real time PCR it was determined that the mRNA expression of the mitochondrial antioxidant gene peroxiredoxin 3, the cytosolic antioxidant gene glutaredoxin 1 and redox sensitive chaperon PARK 7 decreased significantly in lung tissue of 99 week old mice compared to 12 week old mice. Another mitochondrial antioxidant gene peroxiredoxin 2 increased in lungs at 99 weeks compared to lungs at 12 weeks. The pulmonary expression of peroxiredoxin 5, thioredoxin 1, NQO1 and Nrf2 was not affected by ageing, but iNOS expression, not eNOS and nNOS expression, in lungs of 99 week old mice also decreased compared to 12 week old mice. When assessing protective protein S-glutathionylation using a modified DTNB assay we found that it significantly decreased in lung tissue of the older mice. On the other hand, damaging protein carbonylation assessed by oxyblot was very high in lungs of 99 week old mice compared to young mice. Because ageing is mainly studied in brain tissue we also evaluated protein oxidation in the brain of these 2 groups of mice. Surprisingly, protein S-glutathionylation was not significantly attenuated here at 99 weeks, and the increase in protein carbonylation was not as pronounced as in the lung tissue.

### Conclusion:

Pulmonary ageing in mice was found to be associated with decreased mRNA levels of the antioxidant genes GRX 1, peroxiredoxin 3 and PARK 7, as well as of iNOS. Furthermore, a shift from a protective protein oxidation S-glutathionylation to protein carbonylation was observed. Taken together, as the lung ages, there is evidence of decreased antioxidant capacity and increased oxidative stress. Lastly, as it is continuously exposed to air and its pollutants, the lung might be more susceptible to oxidant-induced protein modifications with age compared to the brain.

## Effects of *Balanites aegyptiaca* on manganese superoxide dismutase expression on Adriamycin induced cardiac toxicity in mice

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Adriamycin is an anthracycline antibiotic that is widely used as a chemotherapeutic agent. However, usefulness of this agent is limited due to its cardiotoxic effects. Increased oxidative stress and antioxidant deficit have been suggested to play a major role in adriamycin induced cardiomyopathy and congestive heart failure due to multiple treatments with adriamycin. The aim of this study was to investigate the potential protective effects of natural antioxidants as *Balanites aegyptiaca* against adriamycin-induced cardiotoxicity. Adult male albino mice were intraperitoneally administered with adriamycin (2.5 mg/kg bw). Adriamycin elevated plasma LDH, CPK, and GOT in heart tissue and elevated the cycle threshold (CT) of MnSOD mRNA level ( $28.49 \pm 0.79$ ). Pre-co-treatment with *B. aegyptiaca* (400 mg/kg bw/oral) extract significantly ( $p < 0.05$ ) prevented these alterations and restored the enzyme activities to near normal levels. Also pre-co-treatment with *B. aegyptiaca* extract resulted in decrease ( $p < 0.05$ ) CT of MnSOD mRNA level ( $30.70 \pm 1.33$ ). Application of *B. aegyptiaca* extract with adriamycin drug either significantly reduced or completely prevented its toxic effects. So, these findings demonstrate the cardio protective effect of *B. aegyptiaca* on myocardial marker enzymes and on expression level of MnSOD mRNA during adriamycin induced cardiac damage in mice. Therefore it could be recommended for further investigation in this potentially new indication for clinical application.

## Allergy-Preventive Effects of Chlorogenic Acid and Iridoid Derivatives from Flower Buds of *Lonicera japonica*

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We previously reported that Kinginka tea (flower buds of *Lonicera japonica* Thunb.), if consumed daily, could reduce the risk factors of metabolic syndrome by improving circulatory system characteristics, such as peripheral blood flow and blood pressure.<sup>1)</sup>

In this study, we found allergy-preventive activity of Kinginka tea in the 35% EtOH extract (LJ) by *in vivo* assay. The assay system uses monitoring of the decrease in blood flow (BF) in the tail vein of mice subjected to sensitization with hen-egg white lysozyme (HEL). Bioassay-guided fractionation of the 35% EtOH extract led to isolation of chlorogenic acid (**1**) and three known iridoid derivatives, loganin (**2**), secoxyloganin (**3**) and sweroside (**4**), all of which inhibited the BF decrease. This suggested that Kinginka tea and compounds isolated from them have allergy-preventive properties.

The structure-activity relationship of iridoid derivatives, morroniside (**5**), geniposide (**6**), asperuloside (**7**), aucubin (**8**) and catalpol (**9**), were also tested using the same bioassay method. Compounds **2-5** and **9** having the sp<sub>3</sub> atom at C-8 showed an allergy-preventive effect, while compounds **6**, **7** and **8** having a double bond at C-7, C-8 did not.

1) Oku H., Ogawa Y., Iwaoka E. and Ishiguro K., *Clin. Exp. Pharmacol. Physiol.* **34**, 40-42 (2007).

## Oxidative stress markers in patients after two years of *Roux-en-Y* gastric bypass

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**Objective:** To evaluate the effect of Roux-en-Y gastric bypass on blood markers of oxidative stress such as catalase (CAT), reduced glutathione (GSH),  $\beta$ -carotene, vitamins C and E, ferric reducing antioxidant power (FRAP) and thiobarbituric acid reactive substances (TBARS).

**Methods:** A prospective-controlled clinical study, with subjects distributed in two groups: a control group (CG, n=35), assessed at a single initial time, and the bariatric group (BG, n=35), assessed in the basal period, and at 6<sup>th</sup>, 12<sup>th</sup> and 24<sup>th</sup> months post-surgery.

**Results:** After two years of surgery the body mass index fell from  $47.05 \pm 1.46$  kg/m<sup>2</sup> to  $30.53 \pm 1.14$  kg/m<sup>2</sup> ( $p < 0.001$ ), and 25.7% of patients regained weight between 12 and 24 months. Six months after surgery blood concentrations of vitamin C ( $61.5 \pm 16.1\%$ ,  $p = 0.007$ ),  $\beta$ -carotene ( $1044.4 \pm 537.7\%$ ,  $p = 0.833$ ), vitamin E ( $6.3 \pm 6.3\%$ ,  $p = 0.939$ ) and FRAP ( $8.4 \pm 5.0\%$ ,  $p = 0.728$ ) were increased while CAT activity ( $62.3 \pm 22.8\%$ ,  $p = 0.01$ ) was decreased. Concentrations of GSH ( $14.4 \pm 6.4\%$ ,  $p = 0.005$ ) and TBARS ( $10.0 \pm 16.2\%$ ,  $p = 0.148$ ) were decreased at six months in relation to the basal period. After 12 months of surgery there was an increase in vitamin C ( $217.3 \pm 23.8\%$ ,  $p < 0.001$ ), CAT ( $35.8 \pm 12.0\%$ ,  $p = 0.052$ ), and FRAP ( $6.34 \pm 4.3\%$ ,  $p = 0.487$ ), although  $\beta$ -carotene ( $61.9 \pm 58.6\%$ ,  $p < 0.001$ ), vitamin E ( $20.3 \pm 4.6\%$ ,  $p < 0.001$ ), GSH ( $14.6 \pm 5.4\%$ ,  $p = 0.002$ ) and TBARS ( $71.6 \pm 2.9\%$ ,  $p < 0.001$ ) showed decreased values in relation to basal time. After 24 months, the concentrations of vitamin C ( $31.9 \pm 4.6\%$ ;  $p < 0.001$ ),  $\beta$ -carotene ( $360.7 \pm 368.3\%$ ,  $p < 0.001$ ), vitamin E ( $22.8 \pm 4.1\%$ ,  $p < 0.001$ ), GSH ( $6.6 \pm 5.2\%$ ,  $p = 0.009$ ), CAT ( $12.7 \pm 5.6\%$ ,  $p = 0.029$ ) and FRAP ( $1.2 \pm 3.8\%$ ,  $p = 0.085$ ) were all decreased compared to basal values. The concentrations of TBARS were decreased after 24 months ( $30.4 \pm 6.2\%$ ,  $p < 0.001$ ) in relation to basal, although they were increased compared to 12 months after surgery ( $195.0 \pm 28.2\%$ ,  $p < 0.001$ ).

**Conclusions:** After two years of surgery there was a persistent oxidative stress detected in blood, which could be explained by the imbalance between pro-oxidants and antioxidants and/or by the weight regained.

## Proinflammatory and oxidative stress markers in patients submitted to Roux-en-Y gastric bypass after one year follow-up

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**Objectives:** This study evaluated the effect of weight loss after *Roux-en-Y* bypass gastroplasty on energy intake, levels of vitamin C,  $\beta$ -carotene and vitamin E (diet/blood), nitric oxide metabolites (NOx), myeloperoxidase (MPO), thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and activity of catalase (CAT).

**Methods:** Prospective controlled study with a Control Group (CG) assessed one time and a Gastroplasty Group (GG) assessed in the basal period and also after 3, 6 and 12 months post-gastroplasty, both composed of 5 men and 31 women (n=36 each group). Mean age was 38.7 $\pm$ 9.4 and 39.6 $\pm$ 9.2 years and Body Mass Index (BMI) was 22.2 $\pm$ 2.1 and 47.6 $\pm$ 9.1 kg/m<sup>2</sup>, respectively.

**Results:** The percentage of weight loss at the 12<sup>th</sup> month was 35.8 $\pm$ 1.0% ( $P$ <0.001) lower than that of the basal period. At the basal period subjects of GG showed higher levels of NOx ( $P$ =0.007) and TBARS ( $P$ <0.001) and lower levels of vitamins C and E ( $P$ <0.001) compared to subjects from CG. At the 3<sup>th</sup> month MPO activity was decreased ( $P$ <0.001), and after 6 months of surgery GSH levels were decreased ( $P$ =0.037) while CAT activity was increased ( $P$ =0.029). At the 12<sup>th</sup> month levels of NOx ( $P$ =0.004), TBARS ( $P$ <0.001),  $\beta$ -carotene ( $P$ <0.001) and vitamin E ( $P$ <0.001) were decreased while those of vitamin C ( $P$ <0.001) were increased.

**Conclusion:** After one year *Roux-en-Y* bypass gastroplasty attenuated pro-inflammatory and oxidative stress markers, but an antioxidant supplementation seems necessary to compensate the systemic oxidative stress.

## Aldehyde reductase deficiency prolongs pentobarbital induced hypnosis in mice

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Aldehyde reductase (ALR, EC 1.1.1.2) is a monomeric oxidoreductase which belongs to the aldo-keto reductase superfamily. It is mainly distributed in the liver and kidney and catalyzes the reduction of various types of aldehydes to their corresponding alcohols in an NADPH-dependent manner. Because aldehyde compounds are deleterious, ALR is regarded as a detoxification enzyme. However, ALR has a broad range of substrate specificity, and the precise physiological roles of ALR remain to be determined. Recently we generated ALR knockout (KO) mice and demonstrated that ALR has the activities of glucuronate reductase and glucuronolactone reductase, which are involved in ascorbic acid biosynthesis (Takahashi et al, submitted for publication). Ascorbic acid is a cofactor for Cu(I)-dependent monooxygenases and Fe(II)-dependent dioxygenases, and participates in a variety of physiological phenomena. Thus, ALR is suggested to have the direct enzymatic activity of substrate reduction and also exert detoxification and antioxidation activities via ascorbic synthesis.

In this study, we investigated ALR detoxification activity for pentobarbital (PTB) by using ALR-KO and ALR over-expression (Tg) mice. PTB sodium (75mg/kg) was injected to wild type (Wt), ALR-Tg, and ALR-KO mice intraperitoneally. PTB-induced sleeping time of Wt, Tg, and KO mice were 66min 56sec±388sec, 45min 30sec±287sec, and 93min 44sec±831sec, respectively. The serum PTB concentrations at 30min after intraperitoneal injection were significantly higher in KO mice than those in Wt and Tg mice. The serum PTB concentrations of awaking time were almost the same in all three genotypic mice, suggesting that the sensitivity of PTB receptors was not modified by ALR expression. Then, to investigate the effect of ascorbic acid synthesis for PTB metabolism, Wt, Tg, and KO mice were fed an ascorbic acid-deficient diet. The ascorbic acid level in the tissue of each genotype group corresponded approximately to the amount of ALR protein. However, PTB-induced sleeping time of the mice fed the ascorbic-deficient diet was not significantly different from the mice fed a standard diet in all three genotypes. Thus, the ascorbic acid is suggested not to be involved in the mechanism of PTB metabolism. Taken these data together, ALR appears to play a role in PTB detoxification by accelerating clearance of PTB from serum in an ascorbic acid-independent manner.

### Human Keratoconus Tissue and 4-Hydroxy-nonenal implication.

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**Purpose:** The purpose of this study was to determine whether oxidative stress-related challenges cause a dysfunction in the antioxidant corneal defences in the keratoconus (KC) versus healthy corneas and that the presence of 4-hydroxy-nonenal (4-HNE), a lipid peroxidation product, may play a role in the pathogenesis of KC.

**Methods:** A total of 8 healthy and 11 ectatic corneas were studied. The research reported was conducted in compliance with "Declaration of Helsinki". Different oxidative stress-related markers were determined to assess their implication in the KC pathophysiology. Total antioxidant capacity and total nitrites present in the samples were determined. Furthermore, lipid peroxidation and glutathione contents were determined by ELISA and the presence of 4-HNE was detected by immunohistochemistry.

**Results:** The antioxidant capacity and glutathione content in KC tissue were significantly decreased ( $p < 0.05$ ) when compared to healthy corneas. Moreover, the total nitrites and lipid peroxidation were significantly elevated in the corneas with KC when compared to controls ( $p < 0.05$ ). There was a statistically significant difference ( $p < 0.05$ ) in the amount of HNE-positive cells in KC corneas when compared with healthy corneas by immunohistochemistry.

**Conclusions:** The increased levels of oxidative stress markers and the decreased antioxidant capacity and antioxidant defences in KC corneas confirm previous data that oxidative stress is involved in the development of this disease (*Buddi et al., J Histochem Cytochem 50:341-351,2002*) and these results may provide new etiopathogenic insights for the prevention and treatment of this disease in the future.



## **Accumulation of lipid peroxidation products in Organ Culture storage media prior to human corneal transplantation**

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**Purpose:** Organ Culture medium is currently used as storage media for donor corneas. Little is known about potential alterations that may occur in the media during corneal storage prior to transplantation. The purpose of the study was to examine the level of the oxidative stress marker malondialdehyde (MDA) in the upper and lower layers of the storage media, during storage of corneas for transplantation.

**Methods:** Sixty human corneas were stored in Organ culture medium, for 7-21 days prior to transplantation. At different times (0, 7, 14 and 21 days) medium was taken out from the upper and lower layers of the storage containers, and MDA were measured by HPLC.

**Results:** Measurement of the lipid peroxidation product MDA in the upper and lower layers of organ culture medium, showed higher amounts of MDA in lower layers when compared to upper layers ( $p < 0,05$ ), as well as increasing levels of MDA at longer storage time ( $p < 0,05$ ).

**Conclusion:** Measurements of MDA in Organ Culture medium after 0, 7, 14 and 21 days revealed increasing levels during storage and a higher accumulation in the lower layers of the medium. This observation calls for a closer examination of donor cornea storage systems devices and their influence on cell damage during storage.

**Diversity in antioxidant defenses in human epidermis: comparison of melanocytes and keratinocytes suggests that pigment cells are adapted to oxidative stress induced by melanin related compounds.**

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**SUMMARY**

Melanin and its chemical intermediates can both generate and scavenge reactive species. Melanocytes are thus targets of a peculiar endogenous oxidative stress, especially when exposed to sunlight. For instance oxidative stress induced by UV radiation from a solar simulator (SSUV: 300-400 nm or UVA: 320-400 nm) in cultured human melanocytes was stronger when melanogenesis was stimulated. In fact, photoinduced DNA breakage detected using the comet assay and fluorescence of the redox probe Dihydro-Rodhamine123 were enhanced upon UV exposure when melanin content was increased. Antioxidant status in human melanocytes and keratinocytes from same donors also appeared different: (i) reduced glutathione content was higher in keratinocytes, (ii) basal expression of NQO1 (mRNA and protein) was higher in melanocytes, (iii) when Nrf2 was stimulated (by sulforaphane, lipoïc acid or by silencing of Keap1), HO1 (mRNA and protein) and modulatory subunit of  $\gamma$ -glutamyl-cysteine-ligase (GCLm, mRNA) were mainly induced in melanocytes whereas catalytic subunit of GCL (GCLc) was over expressed in keratinocytes, (iii) in a microarray assay, HO1, ferritin, catalase as well as genes from NQO or GST family displayed a stronger basal expression in melanocytes whereas genes from GPX family were mainly expressed in keratinocytes. Melanogenesis could influence such differences through endogenous generation of quinones and hydrogen peroxide. These data are of importance in order to better understand how skin can cope with environmental oxidative stress.

## **Comparison study of morphological changes and antioxidant activity in tissues in case of photorejuvenation and photoepilation procedures and possibility of correction using DNIC**

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**Introduction:** Intensive pulse light (IPL) is widely used in cosmetology. The most common therapeutic application of intensive pulse light is photorejuvenation and photoepilation. The feature of this therapeutic action is transfer of moderate dosage (10-300 J/cm<sup>2</sup>) of IPL within very short time (2-5 msec). IPL action leads to appearance of biological markers of tissue damage. Level of nitric oxide production is an important indicator of any pathological process. The understanding of these processes appears to be very important not only from the point of existing imagination about IPL effects mechanisms but also prospective in creation of the methods of correction of undesirable effects of phototherapy and creation of preventive methods of their development.

**Materials and methods:** definition of morphological features of skin tissues, antioxidant level in blood serum and tissues and nitric oxide level of tissues (skin, liver, heart) in case of photorejuvenation and photoepilation with using IPL experimental animals - white lab rats, divided into following groups: 1-control (n=10), 2-photoepilation (photorejuvenation) 1 day (n=10), 3-photoepilation (photorejuvenation) 14 days (n=10), 4-photoepilation (photorejuvenation) 30 days (n=10). Antioxidant level and oxygen specieses with hemiluminescence method. Tissue destruction was observed by morphological study. We used solution of dinitrosyl iron complexes for correction of disorders applying to animal skin.

**Results:** Antioxidant activity decreases after the procedures with stable level of lipid hydroperoxides. Morphological destructive changes were defined in skin. It was mostly stressed after one month. IPL in spite of being visibly safe and widely used in cosmetology, induces changes of nitric oxide level in tissues (skin, liver, heart), decreasing of antioxidant level and morphological destruction of skin tissues, that needs for developing preventive methods of correction. Using of DNIC significantly improved both morphological picture and antioxidant parameters in all experimental groups.

## The effects of UV-B-induced prooxidative status in human skin cell cultures. The role of some natural antioxidants

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**Introduction and objectives:** Melanocytes and keratinocytes form in the skin a close association, named melanocyte-epidermal unit, capable to secrete a wide range of signaling molecules, including cytokines, neuropeptides and neurotransmitters in response to different stimuli, like stressors or UV radiation. The aim of our study was to evaluate the *in vitro* effects of UV-B-induced oxidative stress in various skin cell combinations and the potential protective role of a natural compound with antioxidant properties.

**Materials and methods:** Normal keratinocyte cell line (HaCat) and melanocytes were cultivated alone and in co-culture in specific media and supplements. In order to evaluate the effects of UV-B irradiation on cells' viability we used two doses of UV-B (100 and 500 mJ/cm<sup>2</sup>) and MTT cell proliferation assay was performed. The effects of a red grape seed extract (*Vitis vinifera*, variety Burgund Mare), as a natural source of polyphenols was evaluated by treating the cells prior irradiation with this product from the non-toxic range (37.5 µg/ml). Metalloproteinases (MMP2 and MMP9), with well documented implication in oxidative stress, were determined with gel zimography in the supernates of the cells, after 1 and 2 days from irradiation, in order to assess the effects of UV-B irradiation on the integrity of the cells' collagenous extracellular matrices.

**Results:** Irradiation of the individual or co-cultivated cells with the lower dose of UV-B radiations (100 mJ/cm<sup>2</sup>) resulted in a noticeable decrease in kertinocytes' viability, melanocytes being non- and co-cultures slightly affected. The application of the higher dose of UV-B (500 mJ/cm<sup>2</sup>) caused more severe decrease in keratinocytes and co-cultures' viability (p<0.0001), melanocytes, as it was expected, remained resistant. The administration of the red grape seed extract improved statistically significant the viability of the keratinocytes (p<0.001), after the application of the higher dose of UV-B, without a noticeable effect on other cell combinations. Our study on the metalloproteinases also demonstrated UV-B radiation-induced alterations. The inactive form of MMP2 increased in all cell types and combinations upon irradiation, in both time points of evaluation (p<0.05). MMP9 increased only in keratinocytes, with the appearance of the active form of the enzyme after 2 days from irradiation. These finding suggest that dermal damage induced by UV-B irradiation might not only be due to specific direct effects but also from indirect participation of the epidermal cytokines, produced mainly by keratinocytes.

**Conclusions:** There was a noticeable difference between the responses to UV-B irradiation of the analyzed epidermis cells alone or in co-culture, the most sensitive being keratinocytes alone, but the harmful effects was protected in some extent by this natural antioxidant. The increased amounts of MMP2 and MMP9 upon irradiation suggested that UV-B radiations altered the integrity of the collagenous extracellular matrices of the cells, most likely indirectly, via cytokines produced by keratinocytes.

## **Influence of duration of streptozotocin-induced diabetes on oxidant stress**

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**BACKGROUND:** Oxidant Stress (OS), the imbalance between reactive oxygen species production and breakdown by endogenous antioxidants, has been implicated in the onset and progression of diabetes-associated complications (Oberley, 1988). Elevated oxidant stress in diabetes is partly due to prolonged/repeated hyperglycaemia. However, literature data are conflicting concerning the consequences of diabetes on oxidant stress: some studies reported increased or not OS markers, and others noted decreased, unchanged or increased antioxidant enzymes activity (Wohaieb and Godin, 1987). These discrepant data could be explained by differences in protocols such as diabetes induction (streptozotocin vs alloxan), the dose of the drug used and the study delay after diabetes induction (ie diabetes duration).

The purpose of this study was to determine if diabetes-induced alterations on oxidant stress (isoprostanes and SOD activity) could be influenced by diabetes duration. We also focused our attention on glycaemia and weight changes, which reflect metabolic alterations induced by diabetes.

**METHODS:** At the beginning of the experiment, 40 males Wistar rats, 9 weeks old, were randomly assigned to control (C, n=20) or diabetic (STZ, n=20) group. Diabetes was induced by streptozotocin injection (45mg/kg), whereas controls received citrate buffer only. 6 weeks later (T1), 10 rats in each group were sacrificed (C-T1 and STZ-T1). The remaining 10 diabetic rats and 10 control rats were sacrificed 9 weeks after diabetes induction (T2). At T1 and T2, tissues were removed after pentobarbital anesthesia and rapidly frozen to -80°C until analysis. Body weight and glycaemia of each rat were reported. Isoprostanes and SOD activity were determined in skeletal muscle (gastrocnemius), as markers of lipoperoxidation and activity of antioxidant system, respectively.

**RESULTS:** At T1 and T2, diabetes was characterized, in comparison with controls at the same time, by higher glucose levels (T1:536±32 vs 145±4 mg/dL, T2:562±25 vs 127±6mg/dL) and lower body weight (T1:315±33 vs 505±11g, T2:249±16 vs 500±11g.). Moreover, body weight in STZ-T2 group was significantly lower than in STZ-T1, indicating that 3 additional weeks of diabetes induced a more severe weight loss.

6 weeks after diabetes induction, no differences in isoprostanes levels were observed between control and diabetic groups (T1: 28,4±1.3 vs 40,1±1.8pg/mg prot., respectively). But 3 weeks later, isoprostanes levels were significantly higher compared to controls (T2: 63,4±13.1 vs 31,9±3 pg/mg prot.), and to those of diabetics at T1.

SOD activity was not influenced by the diabetic state, either at T1 or T2 (T1:23,1±1,9 vs 23,1±2,3 U SOD/mg prot., T2: 33,9±6,9 vs 33,7±3,5 U SOD/mg prot.). Increase in SOD activity between T1 and T2 being similar in diabetics and controls, it could be induced by aging rather than diabetes itself.

**CONCLUSION:** Six weeks after its induction, diabetes was characterized by hyperglycaemia and weight loss, without change in pro/anti-oxidant balance. Three additional weeks 1)- accentuated weight loss, and increased isoprostanes levels in diabetics 2)- increased SOD activity in diabetics and controls, indicating that this latest adaptation didn't result from diabetic state. These differences in adaptive responses according to diabetes duration highlighted the fundamental importance of this parameter in further studies.

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## **Alloxan radical induces reactive oxygen species in the reaction system of alloxan with glutathione or ascorbate**

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Alloxan is widely used to produce experimental diabetes. Although the diabetogenic action of alloxan is thought to be initiated by generation of reactive oxygen species (ROS), the mechanism of alloxan toxicity is not fully understood. Glutathione reduced form (GSH) and ascorbate can be an antioxidant in a predominantly aqueous environment, such as plasma, extracellular fluids and intracellular. We have investigated the generation of ROS in the interaction system of alloxan with GSH or ascorbate. Rapid oxygen consumption was rapidly initiated after the addition of alloxan to reaction medium in the presence of GSH or ascorbate. These oxygen consumptions were suppressed by addition of SOD and catalase. The consumption was not observed by alloxan, GSH or ascorbate alone. These results suggest that superoxide anion radical and hydrogen peroxide could be generated in the both reaction systems of alloxan with GSH or ascorbate, and the ROS generations were induced by the interaction of alloxan with GSH or ascorbate. The generation of alloxan radical ( $a^H = a^N = 0.045$  mT,  $g$  value = 2.0052), an electron reductance of alloxan was observed in the system of alloxan with GSH using ESR. The ESR signal intensities of alloxan radical were proportional to the concentrations of GSH in the reaction system. Alloxan radical and ascorbate free radical (AFR;  $a^H = 0.168$  mT,  $g$  value = 2.0054), an electron oxidant of ascorbate, were generated in the reaction system of alloxan with ascorbate. The signal intensities of AFR were proportional to the concentrations of alloxan in the reaction system. Under anaerobic conditions, the ESR signal intensity of alloxan radical was significantly increase in comparison with an aerobic conditions, whereas the intensity of AFR was decreased. These results suggest that alloxan radical and AFR were generated by the interaction of alloxan with ascorbate in the reaction system, and that the alloxan radical, but not AFR, reacted with molecular oxygen, resulting in the generation of ROS.

**Prevention of free fatty acid-induced lipid accumulation, oxidative stress, and cell death in primary hepatocyte cultures by a *Gynostemma pentaphyllum* extract**

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Hepatocytes of a primary cell culture that are exposed to high glucose, insulin, and linoleic (LA) acid concentration respond with lipid accumulation and oxidative stress up to cell death [Müller et al.]. Such alterations are typically found in patients with non-alcoholic fatty liver disease (NAFLD). We used this cellular model to study the effect of an ethanolic *Gynostemma pentaphyllum* (GP) extract in NAFLD. When hepatocytes were cultured in the presence of high insulin, glucose, and LA concentration the extract completely protected the cells from cell death. In parallel, the extract prevented accumulation of triglycerides (TG) and cholesterol as well as oxidative stress within the hepatocytes. Our data further demonstrate that the extract from GP stimulates the production of nitric oxide (NO) in hepatocytes and affects the molecular composition of the mitochondrial phospholipid cardiolipin (CL). We conclude that GP is able to protect hepatocytes from cell death, lipid accumulation, and oxidative stress caused by diabetic-like metabolism and lipotoxicity. Therefore, GP could be beneficial for patients with diabetes mellitus and NAFLD.

Müller C, Gardemann A, Keilhoff G, Peter D, Wiswedel I, Kropf S, Schild L. Palmitate protects hepatocytes from oxidative stress and triacylglyceride accumulation by stimulation of nitric oxide synthesis in the presence of high glucose and insulin concentration. Free Radic Res. 2010;44:1425-34.

## Protective effects of the complex between manganese porphyrins and catalase- poly(ethylene glycol) conjugates against hepatic ischemia/reperfusion injury *in vivo*

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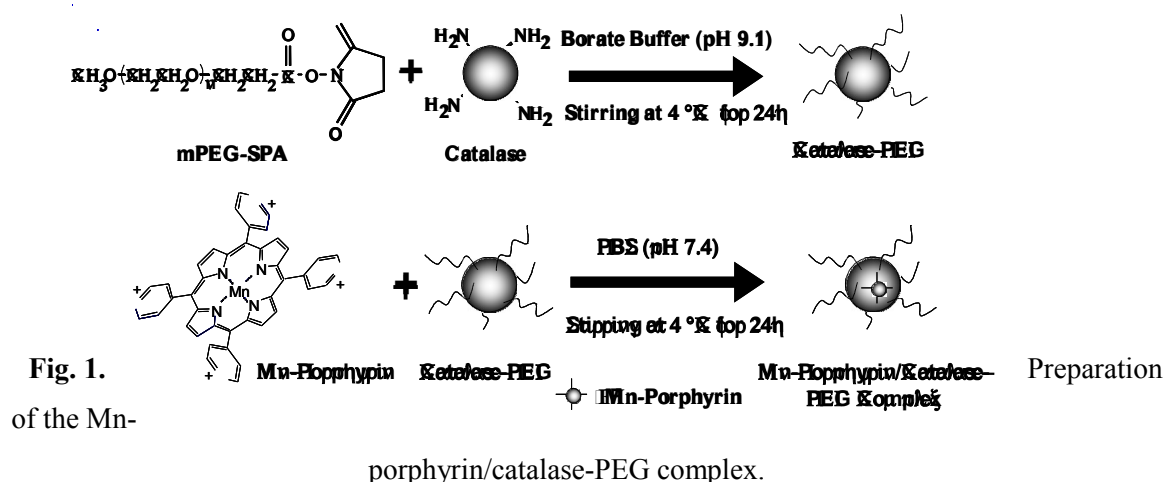
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The complex between manganese (Mn) porphyrins and catalase-poly(ethylene glycol) (PEG) conjugates has been designed for the protective effect against hepatic ischemia/reperfusion injury *in vivo*. The resulting Mn-porphyrin/catalase-PEG complex with dual enzymatic activity of superoxide dismutase (SOD) and catalase enhanced the blood circulation. The spin reduction rate in the rats treated with the Mn-porphyrin/catalase-PEG complex was significantly higher than that in the untreated rats and almost equal to that in the sham group rats. Furthermore, the Mn-porphyrin/catalase-PEG complex significantly decreased the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. These results suggest that the Mn-porphyrin/catalase-PEG complex exhibited the antioxidative activity to protect hepatic ischemia/reperfusion injury *in vivo*.



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## Oxidative stress in pediatric patients after kidney transplantation

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Oxidative stress plays an important role in pediatric patients with end-stage renal disease and contributes to their increased risk of cardiovascular disease. There are a few reports indicating that oxidative stress is still present after restoration of renal functions by successful kidney transplantation. This oxidative stress may be associated with post-transplant complications like endothelial dysfunction. Possible causes of this oxidative stress could be inflammation, immunosuppressive drugs or dyslipidemia.

In our study we compared parameters of oxidative stress in pediatric patients (n = 32; age: 36-216 months; median: 145 months) which were 10-121 months (median: 44,5 months) after kidney transplantation with an age-matched control group (n = 15; age: 50-207 months; median 176 months). As a marker of lipid peroxidation plasma F<sub>2</sub>-isoprostane levels were measured and the concentrations of the antioxidants vitamin E and uric acid were analysed. In addition in the group of transplanted patients these parameters were compared with parameters of the clinical condition.

The concentration of plasma F<sub>2</sub>-isoprostanes was higher in patients after kidney transplantation compared to the control group ( $0.22 \pm 0.01$  vs.  $0.18 \pm 0.01$  ng/ml;  $p < 0.05$ ; mean  $\pm$  SE). The concentrations of the lipophilic and hydrophilic antioxidants vitamin E and uric acid were increased in patients after kidney transplantation compared to the control group (vitamin E:  $11.47 \pm$  vs.  $8.98 \pm$  mg/dl;  $p < 0.05$ ; uric acid:  $6.87 \pm 0.32$  vs  $4.87 \pm 0.38$  mg/dl;  $p < 0.01$ ). Hyperhomocysteinemia was detected in the patients after kidney transplantation ( $16.82 \pm 1.34$   $\mu$ mol/l homocysteine vs.  $7.96 \pm 0.51$   $\mu$ mol/l;  $p < 0,001$ ). There was a trend for a positive correlation between the systolic blood pressure and plasma F<sub>2</sub>-isoprostanes ( $r = 0.33$ ;  $p = 0.070$ ).

In this study we demonstrated that an increased lipid peroxidation measured as plasma F<sub>2</sub>-isoprostanes was even present in a situation with an increased concentration of the lipophilic antioxidant vitamin E. The increased concentration of homocysteine may have contributed to the oxidative stress. The trend of a positive correlation between plasma F<sub>2</sub>-isoprostanes and the systolic blood pressure may indicate a contribution of lipid peroxidation to the clinical condition of pediatric patients after kidney transplantation.

## Oxidative stress in infantile autism

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**Background:** Infantile autism is defined by speech and cognitive delay, qualitative impairment of communication, social interaction and limited interests with ritualistic behaviour. The origin of autism results from the interaction of environmental and genetic factors.

**Patients and Methods:** We examined 49 patients with infantile autism and 24 healthy controls for parameters of oxidative stress and antioxidant factors. The measured parameters for oxidative stress included plasma for lipid peroxides, oxidized LDL, antibodies against LDL in blood and oxidized DNA in lymphocytes using the comet assay. Simultaneous analysis of antioxidant factors comprised serum copper, manganese, selenium, zinc, the copper/zinc ratio, total cholesterol, ceruloplasmin, vitamins C and E ( $\alpha$  and  $\gamma$ -tocopherol), co-enzyme Q10,  $\beta$ -carotene, thiol proteins, reduced and oxidized glutathione, glutathione peroxidase and superoxide dismutase. Statistical analysis used the non-parametric Kruskal-Wallis test with a significance level at  $p=0.05$ .

**Results:** When compared to healthy controls, the patients with autism had elevated oxidative stress parameters for the antibodies against oxidized LDL and oxidized DNA ( $p<0.05$ ), whereas no significant differences were found for the lipid peroxides and oxidized LDL. In the group with autism, copper, ceruloplasmin, copper/zinc ratio and SOD activity were significantly increased, while  $\beta$ -carotene was significantly decreased.

**Conclusions:** Elevated levels of oxidized DNA have never been reported in infantile autism at our knowledge. Further research efforts will be necessary to better understand the aetiology of deranged copper and  $\beta$ -carotene metabolism.

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## Automated method for large-scale screening of oxidative stress related biomarkers and processes in serum.

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### Introduction.

Oxidative stress and redox status have been related to many chronic diseases and ageing. To test whether biomarkers of oxidative stress really can describe the underlying pathological processes, large-scale screening is recommended in epidemiological cohorts. In addition many components of the oxidation and antioxidant system must be determined simultaneously to obtain an overall view of these processes.

In this study we present an automated method for the determination of a large set of biomarkers of both oxidative processes and antioxidant status, which is suitable for large-scale screening of serum samples.

### Materials and methods.

The auto-analyser used for the measurements of oxidative stress and antioxidant biomarkers is the LX-20 Pro (Beckman-Coulter). The kits used on this auto-analyser are from various companies and have been adjusted to the format and measurement procedures of the LX-20. Serum samples are required for this set of biomarkers.

With this procedure 100-200 samples can be measured per day by one technician, depending on the number and kind of biomarkers. This technique requires only small amount of serum, including dead volume. Although only single measurements are performed, the inter assay variation is usually below 5 %.

### Results.

On the auto-analyzer the following processes with representative biomarkers can be measured in large series:

Lipid oxidation	ROM, TOS
Protein oxidation	total thiol groups
Glycation	fructosamine
Inflammation	HS-CRP, homocysteine
Tissue toxicity	GGT, ALAT, cystatin C, amylase
Oxidation	iron, ferritin, ceruloplasmin, copper
Total antioxidants	FRAP, TAS, BAP
Anti oxidant enzymes	paraoxonase, myeloperoxidase, $\alpha$ 1-antitrypsin
Hb protection	haptoglobin,
Serum antioxidants	creatinine, bilirubin, uric acid, zinc

If needed this set can be extended by more oxidative stress related parameters using other techniques, such as HPLC or GC with various detectors and ELISA's: vitamin B6, vitamin C, 25OHVitD, vitamin E, carotenoids, malondialdehyde, glutathion status, superoxid dismutase, glutathion peroxidase, fatty acid patterns, interleukines, adipokines, etc.

## The Antioxidant Properties of Erythrocytes

**Dr Ross Richards.**

Professor Isaac Ginsburg.

We have demonstrated independently<sup>1,2</sup> as have a number of other investigators<sup>3,4</sup> over the last ten years that erythrocytes have enormous antioxidant capacity in the form of catalase, glutathione and a number of expendable structures including the haemoglobin molecule itself and the plasma membrane, and are able to act altruistically to protect other tissue from oxidant damage.

Most of the work done describing the antioxidant properties of blood has been done on plasma which contains low-molecular weight antioxidants and albumin. This work takes no account of the powerful antioxidant properties of erythrocytes and other blood cells and raises the question as to whether this reflects true antioxidant capacity.

Additionally, it has been shown that dietary polyphenols are adsorbed to the surface of erythrocytes and other blood cells and act synergistically with the endogenous cellular antioxidant activity<sup>5</sup>. This may explain the difficulty in detecting polyphenol activity in plasma since it is separated from the plasma along with the cells.

We submit that measurement of whole blood antioxidant capacity rather than that of plasma alone is important if a true reflection of antioxidant capacity is required.

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## Comparison among of biomimetic lipid aggregates as *in vitro* models to assess antioxidant capacity against peroxidation

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The oxidation of unsaturated lipids present in biomembranes is generally induced by an overproduction of reactive oxygen species (ROS), which may disrupt the structure and functional role of lipid bilayers. The development of high-throughput methods to assess the ability of compounds to counteract the lipid oxidative damage in a biomimetic system is of utmost importance to pharmaceutical, nutritional and biomedical research. In this context, the ideal method for determination of antioxidant properties should assess the effect of a compound/sample in reaction conditions that mimic those found when oxidative stress is induced *in vivo*. At the present moment there is no standard methodology to fulfill this task [1].

Therefore, the objective of this work was the comparison of different lipid aggregates as biomimetic models of biological membranes for assessment of antioxidant capacity against peroxidation. Hence, several lipid model systems were tested, namely micelles of hexadecylphosphocholine and liposomes of L- $\alpha$ -phosphatidylcholine. Different types of liposomes were prepared, comprising multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs). All lipid model systems were characterized concerning their size distribution and zeta-potential by dynamic light scattering. Antioxidant assessment was based on the intensity of fluorescence decrease of the target/probe (fluorescein) along time under reproducible and constant flux of peroxy radicals, generated from the thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). In the presence of a compound/sample that contains chain-breaking antioxidants, the decay of fluorescence is inhibited.

The antioxidant capacity of Trolox (soluble analogue of Vitamin E), ascorbic acid, glutathione and uric acid was evaluated in the presence of each structure in a competitive scheme. Preliminary results indicate that the lipidic organization in different aggregates influences the value of antioxidant activity, with higher sensitivity for micelles, followed by MLVs and LUVs. Current experiments are targeted towards application to food samples.

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## **EPR-spectroscopic and chemiluminescence tests in blood serum as control bioradical parameters**

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While carrying out most of clinical researches control group choice has essential importance, results of investigation of its members may be accepted as normal. Usually these are donors. But received parameters of bioradical oxidation and antioxidant system activity in them have inaccuracies. This is associated with incorrect taking account the probable influence on these parameters of age, sex, donorship length and quantity of blood taking in them.

The purpose of research – to optimize approaches to control groups forming for bioradical parameters researching in patients.

Materials and methods – by EPR-spectroscopy ceruloplasmine (CP), transferrine (TF) levels in blood serum and intergral parameter of antioxidant system AOS CP/TF were determinated; by chemiluminescence technique peroxides lipids (PL) and antioxidant capacity (AOC) levels were investigated – in period from 1991 to 2010. Dependence of obtained data on age, sex of donors and quantity of blood taking per year was studied.

Results. It was found donorship length, sex are not to influence on the level of bioradical parameters. Some parameters (CP) were dependent on age. Most importantly that increasing of taking blood quantity in donors more than 2 per year causes AOC decreasing.

CP level in donors blood in period 1991-2006 progressively decreased (from 79 to 56 rel. un.), in period 2006-2009 did not practically changed (58 rel. un.). During TF researching the similar dynamics was observed, TF average meanings decreased from 76 to 58 rel. un. in period 1991-2006 and in 2006-2009 TF parameter became 59 rel. un. Intergral parameter AOS CP/TF in period 1991-2006 rised (from 0.91 to 1.13), while in period 2006-2009 slightly decreased (to 1.05). PL level of blood serum in period 1991-2009 increased from 55 to 66 rel. un., AOC level in period 1991-2006 rised from 38 to 48 rel. un., but in period 2006-2009 decreased to 35 rel. un.

Resume. In control group donors with blood taking frequency not more than 2 per year may be included. Current meanings of oxidant-antioxidant system parameters don't depend on age, sex and donorship length. Control groups renewal should be performed every 2-3 year in realtime with simultaneous clinical research, that is conditional on change of nutrition character and social stress level in practically healthy men population.

## Carbon-Based Free Radical Sensors for Local MRI Oxymetry *In Vivo*

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MRI oxymetry method is suggested based on newly synthesized carbon microparticles with oxygen-sensitive magnetic susceptibility  $\chi$ . Changes in  $\chi$  caused by molecular oxygen effect relaxation characteristics ( $T_2$ ) of surrounding bulk water protons in tissue providing a new avenue for local MRI oxymetry *in vivo*.

For several years we have been synthesizing and studying the magnetic properties of a new class of carbon char particles. These microparticles are strongly paramagnetic, and their electron spin relaxation is a reproducible function of the partial pressure of oxygen ( $PO_2$ ) to which they are exposed. The carbons have reduced detection limits for molecular oxygen by orders of magnitude over the most sensitive existing methods. Newly synthesized carbon-based sensors, answer both the sensitivity and bio-reduction problems encountered with nitroxides. The particle/oxygen effects persist when the particles are suspended in water/gel, and water proton relaxation is influenced by their presence.

The influence of  $PO_2$  on electron spin relaxation ( $T_{1e}$ ,  $T_{2e}$ ) in carbon microparticles continues to be a subject of lively interest. Extremely narrow EPR line observed in new materials is a result of a strong Heisenberg electron exchange between paramagnetic free radicals. We have shown that adsorbed molecule oxygen progressively destroys a Heisenberg exchange between paramagnetic centers. This reduction in spin exchange will alter the magnetic susceptibility of the particles, which can be observed by changes in proton  $T_2$  relaxation of water diffusing past the particles.

In aqueous char suspensions we experimentally observed changes in water proton relaxation that we believe are the result of an oxygen dependence of the magnetic susceptibility of the carbon particles suspended in the water. Magnetic field inhomogeneities at the surface-liquid interface of several newly synthesized carbon chars suspended in water have been analyzed by using  $H^1$  NMR spectroscopy and transverse relaxation times ( $T_2$ ) as well as SQUID magnetic susceptibility data. Calculations show a good agreement with experimental data and indicate that the inhomogeneities are due to the difference in susceptibilities  $\Delta\chi$  between water molecules and the solid surface.

We analyzed Hahn Spin Echo and Carr-Purcell-Meiboom-Gill (CPMG) data as well as asymmetric CPMG data for magnetically heterogeneous systems. The sensitivity of the proton chemical shift to the hydrogen bond structure of water gives us the opportunity to investigate the proton relaxation changes both on the surface of char particles and in their micropores.

We have shown that the sensitive carbon particles act as transducers, transferring the oxygen effect from the particles to water protons. Oxygen effect was clearly observed at 14.7T MRI scanner (Oxford/Varian Inova-600 microimaging system) utilizing proton  $T_2$ -weighted spin-echo images of "oxygen-free" and oxygenated samples of carbon microparticles suspended in water and gel.

## **Development and validation of a liquid chromatography coupled to tandem mass spectrometry method for the quantification of 8-isoprostane in human plasma**

**Jordan Gonçalves, Rémi Cabane, Marlène Thierry, Julie Ledoux, Claude Nivet and Xavier Morge.**

A LC-MS/MS method was developed and validated in order to quantify 8-Isoprostane in human plasma samples collected during clinical trials. 8-Isoprostane is generally well acknowledged as the most significant biomarker of oxidative stress.

8-Isoprostane and the internal standard (8-Isoprostane-d4) were isolated from the biological matrix by solid phase extraction on MAX cartridges. Reconstituted samples were analysed by LC-MS/MS. Chromatography was performed on XBridge C18 column in gradient mode using as mobile phase a mixture of bidistilled water/acetonitrile spiked with 0.25% of ammonium hydroxide. The analytes were ionised using a Turbo Ion Spray source in negative mode and detected in the multiple reaction monitoring (MRM) scan mode with an API 4000 mass spectrometer.

The method was successfully validated according to the FDA guidelines by testing the linearity of the instrument response according to the concentration, the selectivity, precision and accuracy of the method, matrix effects and the stability of the compound in the matrix (long-term, short-term, freeze and thaw). The lower limit of quantification was 10.0 pg/mL.



## Plasma malondialdehyde as a marker of frailty in humans:

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**Introduction:** Frailty is a geriatric term used as a marker of vulnerability, identifying individuals with a diminished capacity to respond to external stressors. Those who are frail are at increased risk of death, institutionalization and worsening disability. With the phenotype better defined, attention has shifted to pathophysiology, trying to identify some possible biomarkers, such as malondialdehyde (MDA)

**Aims:** In this study we aim to define whether circulating oxidative stress correlates to frailty in terms of lipidic peroxidation.

**Methods:** In 158 elderly outpatients (from 60 to 99 years old), classified as frail (52), prefrail (53) and non- frail (52) according to Fried's criteria, we measured the levels of MDA in plasma, following the method described by Wong et al. (Wong and Knight, 1987). This method is based on the hydrolysis of lipoperoxides in plasma and subsequent formation of an adduct thiobarbituric acid (TBA) and MDA (TBA- MDA<sub>2</sub>). This adduct was detected by high performance liquid chromatographic techniques (HPLC) in reverse phase and quantified at 532nm. The device model used is ULTIMATE 3000 of DIONEK and the chromatographic technique was carried out in isocratic conditions being the mobile phase a mixture of KH<sub>2</sub>PO<sub>4</sub> 50 mM (pH 6,8) and acetonitrile (70:30) .

**Results:** A significant increase in MDA levels was shown in frail patients when compared to non- frail (p=0,023). However, there was no significant increase of MDA with the age of the patients.

**Conclusions:** MDA can be considered as a biomarker that strongly predicts the frailty conditions, more than aging, and seems to be reliable and easily measurable in the context of the multidimensional analysis of elderly patients.

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## Method for Assessing Hydroxyl Radical Scavenging Activity

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Hydroxyl radical ( $\bullet\text{OH}$ ) is the most reactive species of reactive oxygen species (ROS); therefore,  $\bullet\text{OH}$  is one of the important player in oxidative stress. The  $\bullet\text{OH}$  can be measured using electron paramagnetic resonance (EPR) spin-trapping method. Generally,  $\bullet\text{OH}$ -scavenging-effect of an anti-oxidant has been estimated by a method adding the anti-oxidant and a spin-trapping agent to a  $\bullet\text{OH}$  generation system. Fenton reaction system or irradiating UV to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) has been often used as the  $\bullet\text{OH}$  generation system. Using Fenton reaction system for estimating  $\bullet\text{OH}$ -scavenging-effect, iron-chelating effect of the anti-oxidant and/or reaction of  $\text{H}_2\text{O}_2$  with the anti-oxidant must be considered to estimate the  $\bullet\text{OH}$ -scavenging-effect correctly. For using UV+ $\text{H}_2\text{O}_2$  reaction system, reaction of  $\text{H}_2\text{O}_2$  with the anti-oxidant and absorption of UV by the anti-oxidant must be considered for correct estimation of the  $\bullet\text{OH}$ -scavenging-effect. X-ray irradiation to water can also generate  $\bullet\text{OH}$ . In this simple system, estimation of the  $\bullet\text{OH}$ -scavenging-effect of an anti-oxidant can be much easier, while reduction of spin adduct with the anti-oxidant must be considered. In this study, we are trying to propose an efficient method for estimating  $\bullet\text{OH}$ -scavenging-effect of an anti-oxidant.

A reaction mixture containing 30 mM of a spin-trapping agent (DMPO) and an arbitrary concentration of an anti-oxidant was prepared using 100 mM phosphate buffer (pH7, containing 0.05 mM DTPA). X-ray was irradiated to the reaction mixture and then amount of the  $\bullet\text{OH}$ -spin-adduct (DMPO-OH) was measured immediately using X-band EPR spectrometer. A reaction mixture containing 60 mM was prepared and irradiated by X-ray, then the anti-oxidant was added to estimate reduction of DMPO-OH by the anti-oxidant.

X-ray irradiation to the reaction mixture containing DMPO generates DMPO-OH in the reaction mixture dose dependently. Even doing the experiment under  $\text{N}_2$  gas atmosphere, no difference was obtained for amount of DMPO-OH generated compared to the experiment in the air. The DMPO-OH obtained in the reaction mixture was from  $\bullet\text{OH}$  generated by X-ray. When anti-oxidant 1 was added to the reaction mixture, amount of X-ray induced DMPO-OH in the reaction mixture was decreased depending on the dose of the anti-oxidant 1. To determine whether the anti-oxidant 1 was really scavenging  $\bullet\text{OH}$ , reduction of DMPO-OH caused by the anti-oxidant 1 was estimated. The anti-oxidant 1 was added to the reaction mixture containing DMPO-OH, which was previously generated by X-ray irradiation. The reduction rate of DMPO-OH by the anti-oxidant 1 was enough slow; therefore, the reducing DMPO-OH amount by adding anti-oxidant before X-ray irradiation was mainly due to scavenging  $\bullet\text{OH}$  by the anti-oxidant 1. Anti-oxidant 2 also showed the reducing DMPO-OH amount, when the anti-oxidant 2 was added to reaction mixture before X-ray irradiation. Anti-oxidant 2, however, showed a rapid reduction of DMPO-OH, when the anti-oxidant 2 was added after X-ray irradiation. Scavenging  $\bullet\text{OH}$  by anti-oxidant 2 can not estimated directly from the result of the experiment; however, it is possible to think that anti-oxidant 2 was a strong reductant which can be reduce relatively stable nitroxyl radical (DMPO-OH in this case). Using the method in this experiment, anti-oxidative property can be estimated using scavenging  $\bullet\text{OH}$  and/or reducing DMPO-OH.

## Antioxidant Therapy of Aging: Systems Reliability-Theory Insight

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*In vitro*, antioxidants inhibit free-radical chain oxidation reactions of fatty acids, etc. *In vivo*, however, neither natural antioxidants, like vitamin E and flavonoids, nor synthetic antioxidants like butylated hydroxytoluene (BHT) are able to operate as simple free radical scavengers. Efficiency of such the scavengers of oxygen radicals in cells is negligibly low as compared with the enzyme antioxidant systems, SOD et al. As for radical OH<sup>•</sup>, it reacts with any organic molecules so rapidly that there is no antioxidant to intercept this radical *in vivo*. Meanwhile, “antioxidants” can provide the systems preventive protection from free radicals. Moreover, such antioxidant prophylaxis can be afforded by magnetic isotope of magnesium, <sup>25</sup>Mg, as the nutrition additive. Among three stable isotopes, <sup>24</sup>Mg, <sup>25</sup>Mg and <sup>26</sup>Mg with natural abundance 79, 10 and 11%, only <sup>25</sup>Mg has a nuclear spin and, hence, its atomic nucleus is magnetic. We have revealed that cells of *Escherichia coli*, the standard cell model, demonstrate essentially higher viability when they grow on media supplied with the magnetic isotope, <sup>25</sup>Mg, by comparison to media supplied with the nonmagnetic <sup>24</sup>Mg or <sup>26</sup>Mg (Koltover et al., 2011). Magnetic <sup>25</sup>Mg works as much more effective cofactor of oxidative phosphorylation in cells in comparison with nonmagnetic <sup>24</sup>Mg and <sup>26</sup>Mg. It transpires the down-grading production of O<sub>2</sub><sup>•-</sup> and its reactive products. Thus, <sup>25</sup>Mg can produce the preventive beneficial antioxidant effect *in vivo*, like BHT, for example. [Supported by Russian Foundation for Basic Research, projects # 10-03-01203, 10-04-90408-Ukr\_a].

**Vascular and cardiac modifications during the early stage of L-NAME-induced hypertension: modulation by (-)-epicatechin.**

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Hypertension is associated to vascular dysfunction and cardiac modifications. Changes in dietary habits are studied as possible strategies to prevent and/or attenuate the progression of hypertension and the secondary effects. In this work, an (-)-epicatechin (EC) enriched diet was evaluated during the initial stage of an experimental model of hypertension induced by L-NAME. Sprague-Dawley rats were divided into 3 groups: Control (C), L-NAME (L: 40 mg kg<sup>-1</sup> day<sup>-1</sup>), L-NAME+epicatechin (L+EC: 40 mg kg<sup>-1</sup> day<sup>-1</sup> and diet supplemented with 4 g EC per kg of diet). At the end of the 4-days treatment, L-NAME induced a significant increase in blood pressure (+42±6 mm Hg) associated to a diminished level of plasma NO metabolites (-45%). The presence of EC in the diet avoided the increase in blood pressure and restored NO level in plasma. In order to advance on mechanistic studies, aortas were obtained at the end of the experimental period. L group showed increased activity of NADPH-dependent superoxide anion production in aortic tissue, in association with increased p47<sup>phox</sup> expression (+92% and +73% compared with C). Expression of eNOS was not different among the three experimental groups, but eNOS activation (detected as relative amount of Ser1177 phosphorylated) was significantly lower in L group and reestablished in L+EC (C=0.55±0.06; L=0.23±0.02\*, and L+EC=0.43±0.06 AU, \*p<0.05 respect to C and L+EC groups). These results suggest that the antihypertensive effect of EC may be associated to the increase in NO bioavailability through eNOS activation and decreased superoxide anion production. During this initial stage of hypertension, cardiac tissue from L animals did not show any difference in weight, malondialdehyde content, and metalloprotease activity, but present enhanced NADPH-dependent superoxide anion production, decreased MnSOD activity and expression, and increased GPx activity. All those modifications were reverted by the presence of EC in the diet. These results are suggesting that dietary administration of EC would be able to increase NO bioavailability in a model of L-NAME induced hypertension preventing both vascular and cardiac modifications secondary to NO-deficiency.

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## Procyanidins modulate membrane-associated signals in intestinal cells

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Large procyanidins, although not-absorbed protect intestinal cells from proinflammatory conditions. We tested the hypothesis that large procyanidins interact with intestinal epithelial cells, and based on these interactions prevent the activation of different events initiated at the membrane lipid rafts, e.g. calcium mobilization, oxidant formation, and the activation of select cell signals. Using liposomes with different lipid composition and Caco-2 cells we investigated the effects of an extract enriched in large procyanidins extracted from cocoa. Large procyanidins have a capacity to prevent detergent-mediated disruption of liposomes that increase with the content of glycolipids (main components of lipid rafts) in the liposomes. In Caco-2 cells large procyanidins inhibited NF- $\kappa$ B activation initiated by pro-inflammatory compounds; presenting the higher inhibition for events initiated at the lipid rafts, i.e. tumor necrosis factor and deoxycholate. In addition, large procyanidins inhibited deoxycholate-induced calcium mobilization, oxidant production, and the activation of mitogen activated protein kinases. It can be concluded that large procyanidins can interact with synthetic and biological membranes and protect them from different insults. This interaction seems to be favored in certain zones of the membranes, e.g. the lipid rafts.

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**Pyruvate imbalance mediates metabolic reprogramming and mimics lifespan extension by dietary restriction in *Caenorhabditis elegans*.**

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Dietary restriction (DR) is the most universal intervention known to extend animal lifespan. DR also prevents tumor development in mammals, and this effect requires the tumor suppressor PTEN. However, the metabolic and cellular processes that underly the beneficial effects of DR are poorly understood. We identified *slcf-1* in an RNAi screen for genes that extend *Caenorhabditis elegans* lifespan in a PTEN/*daf-18*-dependent manner. We showed that *slcf-1* mutation, which increases average lifespan by 40%, mimics DR in worms fed ad libitum. An NMR-based metabolomic characterization of *slcf-1* mutants revealed lower lipid levels compared to wild-type animals, as expected for dietary-restricted animals, but also higher pyruvate content. Epistasis experiments and metabolic measurements support a model in which the long lifespan of *slcf-1* mutants relies on increased mitochondrial pyruvate metabolism coupled to an adaptive response to oxidative stress. This response requires DAF-18/PTEN and the previously identified DR effectors PHA-4/FOXA, HSF-1/HSF1, SIR-2.1/SIRT-1, and AMPK/AAK-2. Overall, our data show that pyruvate homeostasis plays a central role in lifespan control in *C. elegans* and that the beneficial effects of DR results from a hormetic mechanism involving the mitochondria. Analysis of the SLCF-1 protein sequence predicts that *slcf-1* encodes a plasma membrane transporter belonging to the conserved monocarboxylate transporter family. These findings suggest that inhibition of this transporter homolog in mammals might also promote a DR response.