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SFRR-Europe 2013 Oral Presentations

PLENARY LECTURE – SFRR-E

Are age-related changes in redox biology important contributors to weakness in old age?

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The potential role of free radicals and other reactive oxygen species in human ageing has been studied for 80 years and while a great deal of scientific advance has been made, little practical benefit to older people has derived from such studies. Physical frailty is a major factor reducing quality of life in the elderly and is driven by weakness caused by loss of skeletal muscle mass and function. A paradox for research in this area was that skeletal muscle generates superoxide and nitric oxide as part of normal metabolism and this is increased by exercise, but these species were also implicated in oxidative damage during ageing. The key to this paradox was realisation that reactive oxygen and nitrogen species mediate homeostatic adaptations to contractions in muscle through redox signaling. Thus, exercise leads to increased expression of cytoprotective proteins, mitochondrial biogenesis, improved metabolic control and other beneficial effects through redox-sensitive pathways. Our work demonstrated that key adaptations to contractile activity are attenuated during ageing and that overexpression of key products of these pathways (e.g. HSP10 or HSP70i) preserved muscle function in mice during ageing. In order to understand the mechanisms by which these key adaptive pathways to exercise are attenuated during ageing, we studied their redox control and observed that key pathways are chronically activated with ageing, but refractive to stimulation by exercise. The chronic stimulation results from an oxidising environment in ageing muscle that prevents further activation by exercise. Current studies examining the mechanisms underlying the oxidising environment in ageing muscle indicate that this may be driven by the presence of denervated fibres within the muscle bulk. Understanding the relationship of changes in muscle innervation to the attenuated exercise responses of aged muscle holds substantial promise in indicating approaches to preservation of muscle mass and function in the elderly.

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PLENARY LECTURE – C. PASQUIER

A protective stratagem against oxidative injury: Role of the Mitochondrial Lon protease in aging and Neurodegenerative diseases

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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. Failure of protein maintenance systems is considered a critical component of the aging process. Pioneering studies have shown that mitochondrial matrix contains the Lon protease that degrades oxidized, dysfunctional, and misfolded protein. In addition, age-dependent decreases in the level of Lon are associated with the appearance of dysfunctional aconitase and the buildup of oxidatively modified protein. Recent evidence indicates that Lon expression may be regulated by pro-oxidants in a manner consistent with the removal of oxidatively modified protein. The *Saccharomyces cerevisiae* homolog of the ATP-dependent Lon protease, Pim1p, is essential for mitochondrial protein quality control, mitochondrial DNA maintenance and respiration. We have demonstrated that Pim1p activity declined in aging cells and that Pim1p deficiency shortened the replicative life span of yeast mother cells. This accelerated aging of *pim1τ* cells is accompanied by elevated cytosolic levels of oxidized and aggregated proteins, as well as reduced proteasome activity. Our results suggested 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. Compelling evidence suggests that mitochondrial dysfunction could represent a critical event in the pathogenesis of Parkinson disease (PD). A still rather unexplored field is the involvement of mitochondrial proteases in PD. We provided evidence that Lon played a critical role in the removal of oxidatively modified and dysfunctional protein from the mitochondria isolated from dopaminergic

neurons of mice model of PD. The specific role of Lon in these processes, physiological substrates, and mechanisms of pro-oxidant induced activation must be established.

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PLENARY LECTURE - DSM

Novel and old adipokines in relation to obesity, metabolic syndrome, oxidative and ER stress

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This plenary lecture will review published and novel evidence on the link between obesity and metabolic syndrome, oxidative and ER stress mediated by novel and well known adipokines.

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PLENARY LECTURE – ELSEVIER

Determination of biological age in humans: Results from the EU FP7 MARK-AGE project

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The MARK-AGE Consortium, comprising 26 partners from 14 European countries, has received funding from the European Commission (during the time period 2008-2013) to conduct a population study (3,300 subjects) aiming at the identification of a set of biomarkers of ageing that could serve as a measure of biological age. Two larger groups of subjects have been recruited, i.e. (i) randomly recruited age-stratified individuals from the general population covering the age range 35-74 years and (ii) subjects born from a long-living parent belonging to a family with long living sibling(s) already recruited in the framework of the GEHA project. For genetic reasons such individuals (termed GEHA offspring) are expected to age at a slower rate. They have been recruited together with their spouses as controls, thus allowing initial validation of the biomarkers identified. (iii) A small number of patients with progeroid syndromes have also included in the study. A wide range of candidate biomarkers were tested, including (a) classical ones for which data from several smaller studies have been published; (b) new ones, based on recent preliminary data, as well as (c) novel ones, based on recent research on mechanistic aspects of ageing, conducted by project participants. Bioinformatic analyses have been performed to extract a robust set of biomarkers of human ageing from the large amounts of data generated. Based on our results we have developed a strategy to determine biological age of men and women, respectively.

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SESSION1

Oxidative stress, endothelial biomarkers and non-invasive vascular function in the general population in health and disease

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Endothelial dysfunction (ED) in the setting of cardiovascular risk factors such as hypercholesterolemia, hypertension, diabetes mellitus, chronic smoking as well as in patients with heart failure has been shown to be at least in part dependent on the production of reactive oxygen species (ROS) such as superoxide and the subsequent decrease in vascular bioavailability of nitric oxide (NO). Methods to quantify endothelial dysfunction include forearm plethysmography, flow-dependent dilation of the brachial artery, finger-pulse plethysmography, pulse curve analysis, and quantitative coronary angiography after intracoronary administration of the endothelium-dependent vasodilator acetylcholine. Superoxide sources include the NADPH oxidase, xanthine oxidase, and mitochondria. Superoxide produced by the NADPH oxidase may react with NO released by the endothelial nitric oxide synthase (eNOS) thereby generating peroxynitrite ($ONOO^-$), leading to eNOS uncoupling and therefore eNOS-mediated superoxide production. While a number of preclinical lines of evidence support the concept that vascular disease is a consequence of increased oxidative stress, and despite the results of many studies suggesting a beneficial impact of antioxidant drugs on endothelial function, large clinical trials have failed to demonstrate a benefit of antioxidants on cardiovascular outcomes. Studies exploring the possibility that classical antioxidants such as vitamin C, vitamin E, selenium, or folic acid may improve the prognosis of patients with cardiac disease have substantially reported neutral-and occasionally negative-results. In contrast, medications such as statins, ACE inhibitors, certain β-blockers, or angiotensin I receptor blockers, which possess indirect 'ancillary' antioxidant properties, have been associated with beneficial effects in both preclinical studies and large clinical trials.

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Homocysteine and coronary atherosclerosis: from folate fortification to recent clinical trials

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Homocysteinemia had been considered as an emerging cardiovascular risk factor in the late 90s, since its plasma levels had been consistently linked with increased cardiovascular risk in both primary and secondary prevention. In experimental studies as well as in small mechanistic clinical trials, treatment with folates appeared to improve endothelial function and other “soft” markers of atherosclerosis. In agreement to these observations, the introduction of folate fortification programme as a means to prevent neural tube deficiency in North America, was associated with a simultaneous reduction of the incidence of cardiovascular diseases. Importantly, in mechanistic studies it was demonstrate

that further to the reduction of homocysteine, folates exert direct effects on vascular redox signalling in experimental settings, supporting further the concept that folate treatment may actually reduce cardiovascular risk in humans. However, in randomised clinical trials conducted over the last 5-10 years, we have shown that treatment with low-dose folate (400μg/d) leads to overloading of the human vascular cells with 5-methyl-tetrahydrofolate (5-MTHF), the active metabolite of folic acid, and any further pharmacological treatment beyond that fails to increase its intracellular levels in humans. Similarly, the large clinical trials using folate/b-vitamins failed to lower cardiovascular risk in either primary or secondary prevention. Therefore, it is now widely accepted that treatment with folates does not modify cardiovascular risk, when it is administered without any specific indication. However, the clinical benefit of this treatment for patients with moderate and/or severe homocysteinemia remains to be clarified. Given that severe homocysteimemia triggers acute thrombosis, homocysteine-lowering strategies should remain a rational therapeutic intervention in these patients, until results of large randomised clinical trials targeting this population, become available.

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Vitamin E and K interactions in the heart and the brain

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Vitamin E (α-tocopherol) was discovered almost 100 years ago, but its roles in the brain and heart are still not understood. Another key nutrient is present in high concentrations in the brain is vitamin K, as menaquinone-4 (MK-4), while phylloquinone (PK) is the main form of vitamin K in the heart. To study vitamin E metabolism in rats, we used α-tocopherol injections (100 mg/kg body weight) to massively increase hepatic α-tocopherol concentrations. They decreased brain MK-4 concentrations by half, whether the vitamin was provided as dietary PK or menadione. Remarkably, heart was unaffected by excess vitamin E. To investigate whether vitamin E increases metabolism of vitamin K, we investigated the initial catabolic step of vitamin E and K metabolism, the ω-hydroxylation by human cytochrome P450 4F2 (CYP4F2). We found that although CYP4F2 discriminates between various tocopherols in vitro, α-tocopherol does not appreciably increase PK ω-hydroxylation. Thus, excess vitamin E does not increase vitamin K metabolism. To further investigate the mechanism for the interaction between the vitamins, rats were fed deuterium-labeled (d_4)-phylloquinone (d4-PK) for 17 d and injected subcutaneously with saline, vehicle, or α-tocopherol (100 mg/kg BW) daily for the last 7 d. α-Tocopherol-injected livers contained high α-tocopherol and α-carboxy ethyl hydroxy chroman (α-CEHC) concentrations, while brains had only elevated α-CEHC levels. Brain, kidney, and lung were especially vulnerable to depletion of d_4-PK, PK, and d_4-MK-4; however, the conversion of d_4-PK to d_4-MK-4 was not affected by vitamin E treatment in any tissue, except the heart, where high PK concentrations protected vitamin K status. Based on the observations in the brain, the mechanism for decreased vitamin K status in α-T intoxicated rats appears to be dependent upon high α-CEHC concentrations. How α-CEHC interferes with regulation of vitamin K conversion of PK to MK-4 remains under investigation.

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Enhanced oxidative stress and ER stress on cardiovascular system: role of vitamin E

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Hypercholesterolemia is the major risk factor for atherosclerosis and the development of cardiovascular diseases such as heart failure. During atherosclerosis, lipoproteins such as LDL become trapped at the site of lesion and are converted to oxLDL which provokes a cascade of maladaptive inflammatory responses. There is good evidence that the clinical progression of advanced atherosclerosis involves both endoplasmic reticulum (ER) and oxidative stress. Increased oxidative stress has been shown to induce autophagy, apoptosis, mitochondrial dysfunction which contributes to several chronic disease processes. Ox-LDL alters the activity of the autophagy through the LC3/beclin-1 pathway. When the proteasome is impaired, autophagy provides a possible alternate pathway for clearing aggregated proteins. Incidentally, the ratio of the protein expression of membrane associated LC3-II to cytosolic LC3-I (LC3II:I) is often used to assess autophagic activity. Beclin-1 is negatively regulated by its interaction with the anti-apoptotic protein Bcl-2 under normal conditions. However, increased oxidative stress activates the ubiquitin-proteosome system, which functions to degrade Bcl-2. This allows for beclin-1 activation subsequently resulting in autophagic cell death. It has revealed that cholesterol induces upregulation of LC3-II and ox-LDL activates the autophagic lysosome pathway through the LC3/beclin1. This results an increase in the formation of autophagosomes and autolysosomes, leading to the degradation of ox-LDL. In the present study the effect of enhanced oxidative stress and ER stress in cardiovascular system of hypercholesterolemic rabbits have been investigated. In addition we focused on the role of vitamin E in this model. The results of molecular components of ER stress, autophagy and apoptosis (such as ATF6, BiP, Grp94, LC3 II/I, beclin-1, Bcl-2, Bax, Aif, Caspase-9 and Caspase-3) indicate that hypercholesterolemia increases oxidative stress and ER stress and this controls cell death and contributes to progression of atherosclerosis and cardiac failure.

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SESSION 2

Combating 'inflammaging' through a Mediterranean whole diet approach: the NU-AGE project rationale

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The development of a chronic, low grade, inflammatory status named "inflammaging" is a major characteristic of aging which plays a critical role in the pathogenesis of age-related diseases. The NU-AGE rationale is that a one year Mediterranean whole diet (considered by UNESCO a heritage of humanity), newly designed to meet the nutritional needs of the elderly, will reduce inflammaging in fully characterized subjects aged 65-79 year of age, and will have systemic beneficial effects on health status (physical and cognitive). Before and after the dietary intervention a comprehensive set of analyses, including omics (transcriptomics, epigenetics, metabolomics, metagenomics) will be performed to identify the underpinning molecular mechanisms. NU-AGE will set up a comprehensive database as a tool for a systems biology approach to inflammaging and nutrition. NU-AGE is highly interdisciplinary, includes leading research centres in Europe on nutrition and aging, and is complemented by EU multinational food industries and SMEs, interested in the production of functional and enriched/advanced traditional food tailored for the elderly market, and European Federations targeting policy makers and major stakeholders, from consumers to EU Food & Drink Industries.

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Diet-microbiota-health interactions in older subjects

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The microbiota associated with the human body is now intensively studied as an environmental risk factor for disease, and a modulator of health. The development of culture-independent methods for microbiota analysis has allowed identification of alterations in the microbiota associated with the extremes of life, with functional gastrointestinal diseases, with endocrine disease, with antibiotic therapy, and with habitual diet, and evidence is accumulating for linkages other conditions and syndromes. In a baseline analysis of the faecal microbiota composition of 161 older persons, we previously reported a core microbiota and aggregate composition that was distinct from younger persons (Claesson *et al.*, PNAS 2011). We also identified significant inter-individual variation at phylum level, the reasons for which were then unclear. To investigate further, we analyzed the microbiota composition of 178 elderly subjects, none receiving antibiotics, and for whom we had collected dietary intake information. The data revealed distinct microbiota composition groups. Clustering of subjects was also distinguishable by analysis of faecal metabolites and shot-gun metagenomic data. Major separations in the microbiota correlated with selected clinical measurements. Novel constellations of microbiota subtypes were identified. Correlations in the data between diet, microbiota and health status suggest a causative axis (Claesson *et al.*, Nature 2012). However, distinction between cause and consequence requires a large-scale intervention and prospective analysis of subjects for microbiota-related pathophysiologies. The NuAge project combines multinational participation to take account of ethnic and geographical factors, with a defined nutritional intervention of a well characterized cohort at baseline, to identify diet-microbiota-health causative linkages.

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Oxidative stress and metabolic diseases associated with aging: a metabolomic approach

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Increased oxidative stress has been associated with aging processes characterized among others, by a decline in physiological functions and an increased risk of developing metabolic diseases such as type 2 diabetes and its associated cardiovascular events. Metabolic diseases are linked to intrinsic such as genetic predisposition but also to extrinsic factors such as behavioral and environmental aspects for instance physical activity and nutrition. For many years, their diagnostic has been based on a limited set of clinical and biological parameters when clinical signs have already appeared. The development of high-throughput technologies which permit generating large-scale analyses, can now offer the possibility of characterizing global alterations associated with disease conditions or nutritional exposure. Metabolomics can be described as a global analysis of small molecules present in a biofluid, produced or modified as a result of a stimulus. This field has been driven by major advances in analytical tools, chemometrics and bioinformatics. We have recently demonstrated during an overfeeding protocol that metabolomics allows detecting subtle changes in metabolism and potential early metabolic dysfunctions that were not evidenced by classical biochemical parameters presently used by clinicians. The data acquisition and retrieval may be achieved by targeted analysis (e.g lipidomics) or untargeted analysis without prior selection of pathways. Most of the data published were case-control studies but utilization of metabolomics for risk assessment of pathologies is now tested. A recent cohort nested case-control study in the Framingham Offspring study suggested that metabolic profiling at baseline could predict the risk of developing T2D. This was recently confirmed using the EPIC Postdam Study that also identified choline containing phospholipids to be independently associated with the risk of T2D. Such an approach should allow a better understanding of the metabolic transitions associated with aging leading to appropriate nutritional strategies for better aging and the development of personalized nutrition.

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Pro-inflammatory stimuli induce Nrf2/ARE signalling in macrophages mediated by ROS

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Objectives: Oxidative stress has been implicated in a variety of inflammatory diseases. Nuclear factor-erythroid 2 (NF-E2)-related-

factor-2 (Nrf2) is a transcription factor maintaining cellular defence against oxidative stress. This study investigates the effects of pro-inflammatory stimuli on the Nrf2/ARE signalling cascade in macrophages.

Methods: Nrf2 activation in response to different pro- and anti-inflammatory stimuli was studied via promoter studies using RAW 264.7 cells and primary murine macrophages. Therefore we used LPS, peptidoglykan, zymosan, TNF-α, IL-1β and IL-6 as pro-inflammatory, and IL-4 and IL-10 as anti-inflammatory stimuli. Kinase inhibitors, antioxidants and diphenyliodonium chloride as NADPH-oxidase inhibitor were used to elucidate signal transduction. Expression of the Nrf2 target genes HO-1 and NQO1 was studied by qRT-PCR. Western Blot analysis for HO-1 was performed. ROS production in stimulated cells was investigated using the H_2DCF-DA and lucigenin reagents.

Results: Treatment of macrophages with pro-inflammatory stimuli showed increased Nrf2 activity but not the treatment with the anti-inflammatory cytokines IL-4 and IL-10. Nrf2 induction was dependent on NADPH-oxidase, ERK and p38 kinase activity. Treatment mediates an increase of HO-1, which is known to act anti-inflammatory due to carbon monoxide production and NFκB inhibition.

Conclusion: These data demonstrate an anti-oxidative and anti-inflammatory role of Nrf2 during inflammatory processes. Pro-inflammatory stimuli seem to promote the resolution of inflammation at an early stage of acute inflammation by Nrf2 induction. The inhibitor studies revealed ROS as a potential signalling molecule in this Nrf2 activation.

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SESSION 3

Regulatory Mechanisms for Proteolytic Systems in Oxidative Stress

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The two major proteolytic enzymes responsible for the selective degradation of oxidized proteins, across an enormous range of biodiversity from *Archaea* to *homo sapiens*, are the (cytoplasmic, nuclear, and ER) Proteasome and the mitochondrial Lon protease. Starting in 1989, we were one of the first laboratories to show that cells can transiently adapt to oxidative stress (and de-adapt when the stress moderates), by up-regulation of more than 50 protective genes, and down-regulation of a similar number of housekeeping, and proliferative genes. In pursuing this phenomenon, we have found that both the Proteasome and Lon are highly inducible. In mammalian cells, Lon, 20S Proteasome and its Pa28 (or 11S) activator, and the Immunoproteasome (a 20S Proteasome variant with three substituted subunits) are all strongly induced during adaptation to oxidative stress; all of these proteins contribute to the adaptive response, as demonstrated by antisense mRNA, siRNA, gene knock-out experiments, and site-directed mutagenesis. We have performed similar experiments at the organismal level in the nematode worm, *Caenorhabditis elegans* and the common fruit-fly, *Drosophila melanogaster*; which are both widely used in model studies of aging because of their short lifespans, well-studied genetics, and easily-manipulated gene expression profiles. Our experiments show that stress-induced synthesis of the 20S Proteasome and its PA28 (11S) regulator is controlled by the Nrf2 signal transduction pathway in mammalian cells, and that orthologs of Nrf2 in *C. elegans* (SKN-1) and *Drosophila* (CNC-C) also control stress-induction of the 20S Proteasome. The three mammalian immunoproteasome subunits are also induced by oxidative stress, but are not under control of the Nrf2 system; we propose that the Irf1 and/or NFκB signal transduction pathways may regulate immunoproteasome synthesis. In contrast with these transcriptional control pathways for proteasome, the mitochondrial Lon protease appears to undergo translational induction during stress adaptation. We have previously reported that the proteolytic capacity of both Proteasome and Lon decline with age in human beings, partly due to enzyme inhibition by aggregates of oxidized and cross-linked proteins. Now we find that the inducibility of both Proteasome and Lon declines with age, making older worms, flies, and people much less able to adapt to oxidative stress.

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Proteasome activation as a novel anti-aging strategy

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Aging and longevity are two multifactorial biological phenomena whose knowledge at molecular level is still limited. We have studied proteasome function in replicative senescence and cell survival (Mol Aspects Med, in press, 2013). 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; J Biol Chem 284, 30076-30086, 2009). Our current work proposes that proteasome activation is an evolutionary conserved mechanism, as it can delay aging in various in vivo systems. Moreover, additional 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).

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Pathophysiological importance of protein aggregation

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Proteins which are oxidatively modified are degraded by the 20S proteasome. If this degradation is insufficient protein

aggregates are formed. Originally it was thought, that these protein aggregates are merely waste products of cellular metabolism, but mounting evidence demonstrates an active participation of such protein aggregates in several physiological and pathophysiological cellular responses. So aggregates are able to induce macrophagy, produce via incorporation of metals reactive oxygen species and are able to inhibit the proteasomal system.

Processing of these initial protein aggregates is leading to the formation of lipofuscin. In the current literature, the lysosomal system is considered to be essential in the intracellular formation and accumulation of lipofuscin. In contrast to that, our experimental results demonstrated that both autophagosomes and the lysosomal system are not mandatory for the formation of lipofuscin. An inhibition of these systems is not leading to a decline in the lipofuscin formation, but rather in an enhanced toxicity of the formed protein aggregates.

Interestingly, an inhibition of the proteasomal system by protein aggregates is accompanied by an up-regulation of several heat shock proteins (Hsps), including Hsp27, Hsp70 and heme oxygenase-1 (HO-1). It was earlier demonstrated that the induction of classical Hsps, such as Hsp27 and Hsp70, is dependent on an HDAC6-dependent mechanism. Our data demonstrate that also HO-1 induction is mediated by HDAC6 via p38MAPK deacetylation and Nrf-2 activation.

Furthermore, the activation of Ap-1 can also achieved by proteasome inhibiting via protein aggregates, pointing towards a multiple involvement of protein aggregates in the modulation of cellular stress response.

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Regulation of protein turnover by heat shock proteins

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Protein turnover is a multifactorial process with the involvement of many different proteolytic systems. Among the others, proteasomal system takes a wide place and plays important role in the degradation of oxidatively damaged proteins. Proteasome system includes the core particle and also regulatory particles attached to this core, such as 19S and 11S. These regulatory particles may change the potential and also the way of action of the proteasome. Besides these particles, several other molecules are also believed to have roles in the proteolytic degradation.

In this study, we focused on the heat shock proteins as the regulators of protein turnover regarding the overall proteolysis, proteasomal degradation and protein aggregation. Heat shock proteins are involved in the chaperone system which have several different roles in the protein folding and degradation. This brings the new ideas that heat shock proteins may be involved in the accumulation of proteins during aging. Therefore recent studies have been carried out in this field.

In the experiments, young (PD:25) and senescent (PD:58) skin fibroblast cells were used. The effect of aging and heat stress (42°C, 1h) were tested in terms of HSP70 and 90 expressions. Heat stress application changed the expressions of HSPs in a different manner in young and senescent cells when tested in two different time points. Overall proteolysis and protein aggregation have been tested by liquid scintillation counting and proteasomal degradation have been assayed by fluorometric and native gel electrophoresis assays. The role of HSPs in the protein turnover has been confirmed via silencing and inhibitor experiments. Obtained results may bring new ideas for the involvement of HSP70 and HSP90 in the age related diseases which should be further tested.

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SESSION 4

Peroxynitrite-mediated damage in the artery wall and its consequences

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Background: The extracellular matrix (ECM) of the vascular basement membrane is vital for maintaining the functional and mechanical properties of arteries. Matrix components, including laminin, perlecan and fibronectin interact with growth factors and proteins to regulate endothelial and smooth muscle cell adhesion, proliferation and migration. These interactions are potentially perturbed in cardiovascular disease and atherosclerotic lesions, where activated leukocytes generate oxidants, including peroxynitrous acid (ONOOH) that may alter ECM composition and function, and contribute to lesion development and rupture. *Hypothesis:* that exposure of ECM materials (laminin, perlecan, type IV collagen, fibronectin), either individually or in intact matrices laid down by cells, to ONOOH results in changes to ECM structure and function and results in modulated cell adhesion and proliferation. *Results:* Exposure of either isolated ECM components, or ECM synthesized by human coronary artery endothelial cells (HCAECs), to ONOOH (but not decomposed oxidant) at concentrations > 1 μM results in a loss of antibody recognition of perlecan, collagen IV, laminin and fibronectin, including critical cell binding sites on laminin and fibronectin. These changes are associated with the formation of nitrated epitopes on tyrosine and tryptophan residues, thiol oxidation, formation of aggregated and fragmented materials, altered cell and growth factor binding, and changes in inflammation-associated genes in HCAECs exposed to the oxidant-treated ECM. Immunohistochemical studies have provided evidence for co-localisation of 3-nitrotyrosine with ECM proteins, in advanced human atherosclerotic lesions, consistent with the occurrence of ECM damage *in vivo*. *Conclusions:* Peroxynitrous acid (ONOOH) modifies extracellular matrix materials by inducing structural and functional changes to matrix molecules, particularly perlecan, laminin and fibronectin, which are important in endothelial cell activity and function. These data suggest a mechanism through which oxidants generated by activated leukocytes modify the arterial basement membrane in atherosclerotic lesions, and thereby contribute to lesion development and rupture.

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Oxidized phospholipids generated by primary innate immune cells

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Primary innate immune cells including neutrophils, platelets and monocytes/macrophages represent the first line of defense against bacteria and blood loss during acute tissue injury. Their rapid activation in response to pathophysiological stimuli includes significant remodeling of the lipid compartment. Over the last 6 years, we have demonstrated using a targeted lipidomic approach, that this occurs in concert with enzymatic oxidation of membrane phospholipids (phosphatidylethanolamine (PE) or phosphatidylcholine (PC)). Several new families of related lipids are generated that are gaining interest for their biological actions of relevance to innate immune defense. All are generated within 2-5 min of cell activation by either cyclooxygenases (COX) or lipoxygenases (LOX) oxidizing either free or esterified unsaturated fatty acid. They remain cell associated and their generation is coordinated by receptor-dependent signal transduction pathways, including src tyrosine kinases, calcium mobilization and phospholipases. Platelets generate several families, including six 12-hydroxyeicosatetraenoic acid (HETE)-PE/PCs, four 14-hydroxydocosahexanoic acid (HDOHE)-PEs, via 12-LOX, and eight lipids comprising prostaglandins E2 (PGE2) or PGD2 attached to PE via COX-1, in response to thrombin, collagen or ionophore. Human monocytes generate four 15-HETE-PEs and four 15-ketoeicosatetraenoic acid (KETE)-PEs via 15-LOX, while human neutrophils generate three 5-HETE-PEs and one 5-HETE-PC via 5-LOX, in response to bacterial peptides. Last, murine peritoneal macrophages generate four 12-HETE-PEs and four 12-KETE-PEs via 12/15-LOX. The lipids were generated and screened for biological functions. Activities identified to date include: enhancing coagulation factor activity, inhibiting Toll-like receptor function, enhancement of neutrophil chemokine and superoxide generation, and low affinity PPARγ ligand binding activity. Current studies aim to determine the role of the lipids in initiation and progression of atherosclerosis and development of autoimmunity in vivo, in both human and murine disease.

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Mechanisms of cellular dysfunction induced by the myeloperoxidase-derived oxidant hypothiocyanous acid (HOSCN)

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Myeloperoxidase (MPO) is a haem enzyme released by activated phagocytes under inflammatory conditions, which forms reactive oxidants including hypochlorous and hypothiocyanous acids (HOCl and HOSCN). HOCl is a potent oxidant that has been linked to tissue damage and the progression of many diseases, including atherosclerosis. In contrast, the role of HOSCN in disease is poorly characterised, despite HOSCN accounting for a major proportion of the MPO oxidants produced under physiological conditions, particularly in smokers, who have elevated plasma thiocyanate (SCN^-). In this study, we examined the mechanisms and cellular targets of HOSCN with macrophage-like cells (J774A.1), which play an important role in the development of atherosclerosis. Exposure of macrophages to HOSCN resulted in the rapid inactivation of a number of thiol-dependent intracellular enzymes, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and creatine kinase (CK). Inactivation occurred via reversible modification of the active site thiol residue and the formation of sulfenic acid intermediates. A proteomics approach with the thiol-specific probe, 5-iodoacetamidofluorescein, showed that in addition to GAPDH and CK, multiple proteins involved in metabolism and glycolysis, including fructose bisphosphate aldolase and triosephosphate isomerase, together with a number of chaperone, heat shock and structural proteins were modified in macrophages treated with HOSCN. Treatment of cells with HOSCN also led to perturbations in mitochondrial function, mitochondrial membrane depolarisation and cell death via apoptosis. The selectivity of HOSCN for protein thiols, and the ability to react in a reversible manner, via the formation of protein-bound sulfenic acids, supports a novel role of HOSCN as a modulator of metabolic and stress response pathways. This pattern of reactivity is quite different to that observed in with HOCl, and can result in a greater extent of cellular dysfunction. These results may have important implications for the development of atherosclerosis and other pathologies exacerbated by smoking.

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Slow release hydrogen sulfide donors and inflammation: A novel therapeutic opportunity

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Hydrogen sulfide (H_2S) is emerging as important mediator in disparate physiological and pathophysiological processes [1], highlighting the therapeutic potential for pharmacological manipulation of H_2S. The development and characterisation of appropriate H_2S donors are crucial as commonly used sulfide salts such as NaSH or Na_2S, generate H_2S (and Na^+) as an instantaneous bolus rather than model the slow and sustained enzymatic generation of H_2S from CSE, CBS or 3-MST [1]. In addition, commercial preparations of NaSH invariably have a purity of only $>60\%$, which should preclude its use. As with NO and CO, it is likely that the tissue and/or cellular responsiveness to H_2S may well be dependent upon the manner in which cells/tissues are exposed to this gas [1]. Indeed, our recent work using a 'first generation' slow release H_2S donor GYY4137, reduced blood pressure in genetically and experimentally induced hypertension and inhibited tissue damage, oedema and inflammatory signalling in sepsis and arthritis *in vivo* whereas NaSH was pro-inflammatory. Although clearly biologically active *in vivo*, the generation of H_2S from GY4137 is inefficient. We have attempted to overcome this limitation and synthesised several novel compounds capable of generating H_2S at different rates (e.g. AP67, AP72, AP105; analogous to the NONOates in the nitric oxide field). These compounds will further enable researchers to characterise the physiology and pharmacology of H_2S in a variety of *in vivo* models. More recently we have synthesised novel dithiolethione (e.g. AP39) or thiohydroxybenzamide (e.g AP123) derivatives containing a triphenylphosphonium moiety enabling specific delivery of sub-nanomolar concentrations of the compounds to mitochondria.

The anti-hypertensive, anti-inflammatory, cytoprotective and anti-proliferative effects *in vitro*, *ex vivo* and *in vivo* of these compounds in comparison to other H_2S donors will be discussed. These studies highlight the potential for H_2S manipulation and mitochondrial delivery of H_2S to regulate vascular and inflammatory signalling in health and disease.

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[1]*Whiteman M, Le Trionnaire S, Chopra M, Fox B, Whatmore J. Emerging role of hydrogen sulfide in health and disease: critical appraisal of biomarkers and pharmacological tools. Clin Sci (Lond). 2011;121(11):459-88.*

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SESSION 5

The False Dichotomy of Antioxidant Defense and Redox Signaling

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Although antioxidant defense is often thought to be due to non-enzymatic scavenging of free radicals and hydroperoxides, the predominant mechanisms for their removal involve enzymatic catalysis. The nucleophile in such reductions is most often a thiol, particularly glutathione (GSH) or thioredoxin (Trx). In contrast, conjugation reactions of thiols with electrophiles are often non-enzymatic with glutathione S-transferases accelerating the reactions only slightly under physiological conditions. Over the past decade, increasing evidence has shown that the predominant signaling pathways in which redox reactions play a key role also involve cysteine modification. As with antioxidant defense, redox signaling involving hydroperoxides most often involves enzymatic reactions with GSH and Trx or closely related thiol proteins, including glutaredoxin as nucleophiles in the redox-dependent activation or inactivation of signaling proteins. On the other hand, sensing of electrophiles, including the quinones formed from polyphenols or the ubiquitous lipid peroxidation product 4-hydroxy-2-nonenal, involve reaction with particularly reactive protein thiolates. Thus, there is really little difference in the underlying biochemistry involved in antioxidant defense and redox signaling; enzymatic reactions of thiolates and oxidants and non-enzymatic alkylation of thiolates. The major difference in signaling by electrophiles however, is that target protein cysteine in electrophilic signaling must be in an unusual microenvironment where it can be assisted in its nucleophilic attack on the electrophile. This same chemistry will however, make the signaling molecule a likely target in oxidative stress. Examples of thiol modifications involved in antioxidant defense or redox signaling include the activation of Nrf2 through alkylation of Keap1, the activation of Src kinases, the inactivation of protein tyrosine phosphatase 1b, the activation of the JNK pathway, and protein disulfide isomerase oxidation.

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Redox proteomics - focusing the mass spectrometer on protein oxidation

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Protein oxidation is one of the most challenging post translational modifications to be analysed by mass spectrometry. This is mainly due to: a) a great diversity of the origin and chemical structure of protein oxidation products and 2) extreme low abundance of oxidised proteins in biological systems. Mass spectrometry is an ideal tool for studying protein modifications because covalent addition or loss of a chemical moiety from an amino acid leads to an increase or decrease in the molecular mass of that residue. For example, the oxidation of a methionine residue (131 Da) increases its mass to 147 Da by the addition of single oxygen atom (16 Da). Through the observation of a discrete mass increment or decrement of intact protein or peptide it is possible to assign a respective modification. Additionally, the tandem mass spectrometry allows the site-specific assignment of modifications at the resolution of individual amino acids in proteins. Modified proteins exist in cells and tissues at very low levels. Therefore analytical strategies involved very often require modification-specific detection and enrichment techniques combined with electrophoretic and microfluidic separations and advanced mass spectrometry. Analysis of oxidized proteins is exceptionally challenging because there are many different types of modifications of proteins that are induced by ROS. Those modifications can be introduced in different amino acids and can co-exist in oxidized proteins together making the analysis even more challenging. Due to the different properties of the different oxidative modifications to proteins several dedicated approaches specific for particular type of modification have been developed. This presentation will summarise the main challenges of redox proteomics and will introduce the newest developments in the field. Specifically methods for the quantitative analysis of cysteine oxidation and carbonylation will be discussed.

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"Multi-omics" techniques in oxidative stress research: Connecting protein and lipid oxidation

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Overproduction of reactive oxygen and nitrogen species (ROS/RNS) results in oxidation of different biomolecules, such as proteins, lipids, nucleic acids and carbohydrates, in large amounts. The oxidation products often represent reactive secondary oxidants capable to modify other molecules or interfere with signaling and regulatory pathways. For instance, lipid peroxidation results in various carbonylated compounds with high reactivity towards nucleophilic motifs in proteins, other lipids and nucleic acids. To understand the complexity and biological significance of oxidative stress it is important to consider all types of oxidations

and study them in systems biology environment. We combined different "omics" technologies to study protein and lipid oxidation in different *in vitro* models and *in vivo* samples. A new highly specific method to detect and identify carbonylated lipid peroxidation products (oxoLPP) was developed based on hydrazide derivatization followed by shotgun and LC-MS/MS based lipidomics. This allowed us to identify over 200 various reactive oxoLPP generated from different lipid classes after *in vitro* or *in vivo* oxidation in a single analysis. In order to understand the role of reactive oxoLPP as reactive secondary oxidants their reactivity towards proteins and nucleophilic lipids was studied using a library of reactive oxoLPP prepared in house. Thus, we could identify these modifications at nucleophilic amino acid residues in proteins using a bottom-up LC-MS/MS proteomics approach. This combined "multi-omics" workflow was successfully applied to study oxidative modifications of lipids and proteins in plasma samples from patients with type 2 diabetes and obesity. Additionally, we could access quantitative changes of the lipidome (fatty acids, phospholipid nitration), proteome (cysteine modifications by nitrated fatty acids) and metabolome (glutathione nitroalkylation) for a cardiomyocyte model of nitrosative stress. Thus, we could study the interplay between different biomolecules oxidation in complex cellular environment for the first time.

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Redox and stress sensing by vimentin

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Electrophilic lipids are endogenous mediators that may exert both cytotoxic and cytoprotective actions through the covalent modification of cellular proteins. The cytoskeletal protein vimentin has been found to be the target for the covalent addition of various electrophilic species, including HNE and cyclopentenone prostaglandins, as well as for redox-induced modifications, like glutathionylation. The target for these modifications is the single cysteine residue of the protein, cysteine 328, which is highly conserved between species. Although alterations in the dynamics of vimentin network have been observed under oxidative or electrophilic conditions, the contribution of the direct modification of vimentin to these effects remains a matter of controversy. We have studied the response of various vimentin WT or C328S constructs in vimentin-deficient cells. Our results show that the presence of cysteine 328 is essential for the response of the vimentin network to various reactive species including HNE and the oxidant diamide. HNE induces a juxtanuclear accumulation of vimentin with loss of peripheral filaments, which is attenuated in C328S vimentin. In turn, diamide induces a drastic disassembly of the vimentin network into dots or aggregates which is completely prevented in C328S vimentin. Morever, C328S vimentin shows limited performance at the initial steps of filament formation and in supporting various cellular functions. These results highlight the role of vimentin as a sensor for oxidative and electrophilic stress, in which cysteine 328 plays a key role.

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Acute hypoxia signals mediated by reactive oxygen species: superoxide caught in the act

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Oxygen is a key reagent for cell metabolism. Eukaryotic cells sense the reduction in oxygen availability (hypoxia) and trigger a series of cellular and systemic responses in order to adapt to hypoxia, including the limitation of oxygen consumption. There has been a thorough debate on the paradoxical increase in reactive oxygen species (ROS) production when oxygen concentration in cells is reduced, and its implication in the modulation of responses via the PHD-HIF pathway. We have applied complementary methodologies for measuring superoxide in short time of hypoxia, using specific probes. We have observed that when different cell types are subjected to acute hypoxia, there is an increase in the superoxide production for about 10 minutes, probably originated in the mitochondria. These results help to explain the apparently divergent results found by different groups that have not taken into account the time scale of hypoxic ROS production. By using novel thiol redox proteomics techniques we have previously identified several proteins that are specifically oxidised in cysteine residues in endothelial cells after two hours of hypoxia, which may mediate different adaptations to hypoxia, before the HIF transcription programme is fully activated. We propose that cells produce an initial superoxide burst when they are subjected to hypoxia, which can be translated in later times into oxidative signals contributing to hypoxic adaptation and preconditioning.

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SESSION 6

Redox-derived damage-associated molecular patterns: Ligand function of lipid peroxidation adducts

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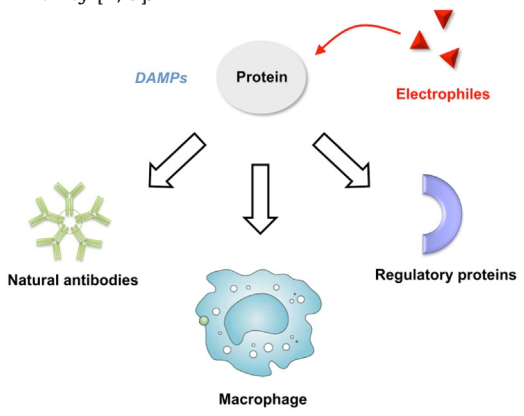
Endogenous electrophiles, such as α,β-unsaturated aldehydes and ketones generated during lipid peroxidation, exhibit a facile reactivity with proteins, generating a variety of intra- and intermolecular covalent adducts. It has been postulated that these host-derived, modified proteins with electrophiles, which constitute the products of diverse classes of oxidative reactions, represent damage-associated molecular patterns (DAMPs). The DAMPs, that occur *in vivo*, can be a ligand of multiple proteins, which in turn, may lead to the profound innate and adaptive immune responses and mediate homeostatic functions consequent to inflammation and cell death.

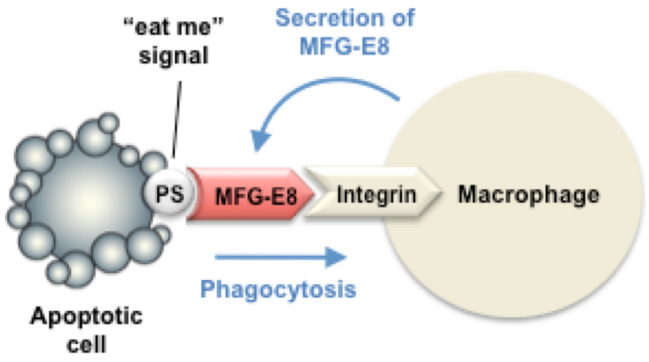
In our recent study on an apoptosis-associated mammary protein, milk fat globule epidermal growth factor factor 8 (MFG-E8), we found that the deficiency of MFG-E8 led to an enhanced production of IgM natural antibodies. In addition, we revealed that the IgM antibodies present in high levels in the MFG-E8$^{-/-}$ mice, specifically recognized the modified protein with 4-oxo-2-nonenal (ONE), a highly reactive aldehyde originating from the peroxidation of ω6 polyunsaturated fatty acids. Following the identification of ONE as the major source of epitopes, we generated several ONE-specific IgM monoclonal antibodies from the MFG-E8$^{-/-}$ mice and characterized their specificity toward lipid peroxidation-derived protein ligands. Furthermore, we examined the ability of the ONE-specific IgM to bind to apoptotic and necrotic cells. These data demonstrate that the impairment of the phagocytic clearance of apoptotic/necrotic cells through MFG-E8 can lead to the generation of natural antibodies, which may play a critical role in removing multiple damage-associated molecules, including lipid peroxidation-modified proteins.

Introduction: Electrophiles, in addition to being noncovalently bound to a protein, have the potential to undergo nucleophilic substitution or addition reactions with the protein. The important endogenous electrophiles that give rise to the modification of a protein may be represented by α,β -unsaturated aldehydes and ketones, such as 2-alkenals, 4-hydroxy-2-alkenals, and 4-oxo-2-alkenals, generated during lipid peroxidation [1-3]. The α,β -unsaturated carbonyl, now conjugated to the diene, forms a powerful electron-withdrawing group. This moiety is labile to react with available nucleophiles, such as protein thiol or histidine residues, via Michael addition, generating a variety of intra- and intermolecular covalent adducts (Fig. 1) and conferring an altered cellular distribution, conformation and catalytic activity. Moreover, the adduction of α,β -unsaturated carbonyls to apolipoprotein B in low-density lipoproteins (LDL) has also been strongly implicated in the mechanism by which LDL is converted to an atherogenic form that is taken up by macrophages, leading to the formation of foam cells. The biological functions of lipid peroxidation adducts generated within those modified proteins are still largely unknown. However, several recent studies have shown that the lipid peroxidation modification of proteins can be directly related to the innate immunity [4, 5].



The innate immunity is stimulated by danger signals called damage-associated molecular patterns (DAMPs), which represent endogenous danger molecules as a group that is separated from pathogen-derived pathogen-associated molecular patterns. DAMPs include endogenous or self-molecules, such as the high-mobility group box 1 and heat shock proteins [6]. DAMPs also refer to a much broader group of oxidatively-modified biological molecules, including oxidized LDL [4]. In the extracellular space, DAMPs can bind to pattern recognition receptors (PRRs) (Fig. 2), which recognize conserved molecular patterns that distinguish foreign organisms, or to specialized receptors to elicit an immune response by promoting the release of pro-inflammatory mediators and recruiting immune cells to infiltrate the tissue [7]. DAMPs, possessing an exposed epitope, are also accessible for recognition by the soluble PRRs, such as natural antibodies and regulatory proteins [4, 8]. DAMPs stimulate the adaptive immunity and participate in autoimmune responses and tissue repair. It has been suggested that the DAMPs-mediated activation of the innate immune system has an important role in the pathogenesis of various immune and inflammatory diseases [9].

The key question is whether the lipid peroxidation modification of proteins plays a role in the innate immunity, especially, if any specific lipid peroxidation-derived adducts could function as DAMPs. Some of the adducts have been recently identified as a candidate ligand of PRRs, leading to downstream inflammation (Fig. 3). West et al. [10] reported that ω-(2-carboxyethyl)pyrrole and other related pyrroles, generated upon the reaction of proteins with unesterified hydroxy-ω-oxoalkenoic acids, are recognized by the Toll-like receptor 2, possibly in a complex with the Toll-like receptor 1/6, and promote angiogenesis *in vivo*, thereby contributing to accelerated wound healing and tissue recovery (Fig. 4). Kumano-Kuramochi et al. [11] recently demonstrated that the 4-hydroxy-2-nonenal (HNE)-histidine Michael adduct is formed as the major product in the oxidized LDL and that it has a significant affinity to one ofthe PRRs, i.e., the lectin-like oxidized LDL receptor-1 (LOX-1) (Fig. 5). Notably, the HNE-histidine adduct has been detected in the *in vitro* oxidized LDL as the major product (about 6 molecules per LDL molecule), suggesting the possibility that LOX-1 recognizes the adduct as the ligand in the oxidized LDL. LOX-1 has also been identified as a potential binding protein for other lipid peroxidation adducts, such as the 4-oxo-2-nonenal-lysine and 2-nonenal-lysine adducts [12, 13]. On the other hand, Weismann et al. [14] identified the plasma complement factor H as a soluble PRR that could bind malondialdehyde (MDA)-modified proteins and block both the uptake of the MDA-modified proteins by macrophages and MDA-induced pro-inflammatory effects *in vivo* (Fig. 6). These studies suggest that the innate immunity has a pivotal role in providing homeostatic responses against lipid peroxidation-specific DAMPs.



Lipid peroxidation plays a role in the pathogenesis of many types of tissue injuries and especially in the tissue damage induced by several toxic substances. In addition, lipid peroxidation has been implicated in the pathogenesis of numerous diseases including atherosclerosis. Several lipid peroxidation products, such as oxidized phosphatidylcholine, cardiolipin, and phosphatidylserine, have been identified as DAMPs [8]. They could be generated in the oxidized LDL and have been suggested to function as a ligand of the PRRs. However, a limited number of lipid peroxidation-derived adducts has been characterized as DAMPs. This may be a matter of primary concern, which represents an important direction to pursue involving lipid peroxidation in redox biology.

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Physiological and pathophysiological importance of advanced glycation end-products

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Biological ageing is induced by the gradual accumulation of cellular and molecular faults. An important cause of faults is intense stress like oxidative or glycogenic stress. Genes influencing longevity are mostly associated with anti-oxidant capacity and/or repair functions. Damage combined with an age-dependent decline in the defense systems result in disturbed homeostasis leading to aging and diseases. Whereas high stress induces premature aging, low stress can induce the genetic repair/defense systems leading to increased life span. An example for such a stressor is advanced glycation endproducts (AGEs). AGEs can induce inflammation, oxidative stress, protein dysfunction and cell death. They are considered as biomarkers of ageing and are associated with cardiovascular diseases. Besides endogenous formation, significant amounts of AGEs are taken up with food. Although nutritional AGEs are considered as undesirable, proinflammatory agents, they may also enclose potentially beneficial antioxidants. We used rodent cardiac cells to evaluate if food AGEs, present in bread crust, can modify the cellular antioxidant defense. Mice were fed with bread crust containing diet to prove the in-vivo relevance for the heart. In mouse cardiac fibroblasts, bread crust extract induced a moderate elevation of ROS production causing an activation of p42/p44MAPK, p38MAPK and NF-κB, followed by increased expression of antioxidative enzymes. Preconditioning studies demonstrated that this was sufficient to protect cardiac fibroblasts and rat adult cardiac myocytes against severe oxidative stress. Furthermore, mice, fed a bread crust containing diet, exhibited a similarly improved cardiac expression of antioxidative defence genes. The consumption of AGEs can therefore contribute to an improved antioxidant status of the heart, thus exhibiting cardioprotective effects in case of severe oxidative stress as in ischemia reperfusion injury.

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Strategies to prevent post-translational protein modifications

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Parametabolic posttranslational protein modifications, adversely affecting protein functions, accumulate with aging, are augmented in various diseases and contribute to their pathogenesis. E. g., protein carbonylation is elevated in a plethora of diseases involving oxidative stress. Enhanced protein nitration has been found to be elevated in various neurodegenerative diseases; enhanced nitration and chlorination accompanies inflammation. Advanced glycoxidation and lipoxidation end products (AGEs and ALEs) contribute to the development of not only diabetic complications, but also of other pathologies. It can be expected that prevention/removal of posttranslational protein modifications may ameliorate the development of various diseases and contribute to life extension and healthy aging. Different strategies have been employed for this purpose, as exemplified for AGEs and ALEs: prevention, inhibition and removal. Antioxidants and metal chelators are useful for prevention of oxidative stress, which generates or facilitates AGEs and ALEs formation. Carbonyl quenchers i. e. compounds trapping reactive carbonyl derivatives, both non-enzymatic and enzymatic, are also effective on the prevention stage. The second level of intervention consists in acceleration of the catabolism of already formed AGEs/ALEs and includes both potentiation of activities of endogenous proteolytic systems and using xenobiotics able to catalytically degrade AGEs/ALEs. The third level of intervention concerns blocking the biological response to AGEs/ALEs, mediated by receptors to these substances (RAGEs), and covers the use of antagonists of such receptors and inhibition of signaling pathways initiated by activation of RAGEs. Natural compounds effective at any level of intervention can be employed as nutraceuticals; new synthetic compounds can become useful drugs.

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Intracellular redox state modulates the T cell surface proteome – relevance to ageing

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During ageing an altered redox balance has been observed in both intracellular and extracellular compartments, primarily due to glutathione depletion and metabolic stress. Maintaining redox homeostasis is important for controlling proliferation and apoptosis in response to specific stimuli for a variety of cells. For T cells, the ability to generate specific response to antigen is dependent on the oxidation state of cell surface and cytoplasmic protein-thiols. Here we describe the effects of depleting intracellular glutathione concentration for T cell exofacial expression of thioredoxin 1 and IL-2 production, and have determined the distribution of Trx1 with ageing. Using buthionine sulfoximine to deplete intracellular glutathione in Jurkat T cells we show using Western blotting that cell surface thioredoxin-1 is lowered and that the response to the lectin phytohaemagglutinin measured by ELISA as IL-2 production is also decreased. Using flow cytometry we show that the distribution of Trx1 on primary CD4+ T cells is age-dependent, with lower surface Trx1 expression and greater variability of surface expression observed with age. Together these data suggest that a relationship exists between the intracellular redox compartment and exofacial surface. Redox imbalance may be important for impaired T cell function during ageing.

Acknowledgments

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SESSION 7

Lipid metabolism, genes, and their regulation by natural compounds

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Obesity is a worldwide public health problem. Millions of people are affected, both in industrialized and developing countries. Obesity is caused by multiple genetic and environmental factors and it is associated with a number of secondary pathologies, such as diabetes, chronic inflammatory processes, hypertension and degenerative diseases. Understanding the molecular mechanisms involved in adipogenesis and body weight regulation is a major effort in a number of laboratories. Genes involved in lipid metabolism have been found to be under the control of major signaling events embedded in AMPK, PGC1-alpha, mTOR, Sirtuins and Sestrins. These signaling "switches" can respond both to environmental conditions, physical exercise as well as to biofactors. Prevention and therapeutic intervention on obesity can take advantage of the present molecular knowledge related to fat metabolism, its genetic regulation and its modification by life style changes.

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Effects of graded caloric restriction on metabolomic signatures in mice

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Caloric restriction is one of the only environmental manipulations that is known to influence both the median and maximal lifespan. Among the postulated causes of the extended lifespan are a reduction in free-radical production, enhanced protection against free-radicals and hence reduced oxidative damage. Among small rodents the effect of CR seems to be linearly related to the extent of restriction – at least to a level of about 65% reduction in calorie intake. This linear relationship between the level of restriction and the extension of lifespan suggests that significant insights can be gained into the molecular mechanisms that underpin the anti-ageing effect by exploring differences between animals kept at different levels of restriction. We have studied the impact of graded caloric restriction (from 0 to 40%) for 3 months on male C57BL/6 mice. Our studies have explored a wide range of outputs including behaviour, physiology, cellular, transcriptomic and metabolomic effects. The current talk will focus on the metabolomic impacts. Using fourier transform mass spectrometry of serum samples we discovered over 5500 metabolic 'features; in the blood of mice under CR, which we will henceforth call 'metabolites'. However not all metabolites were found in all individuals and restricting the analysis to just those metabolites identified in all individuals reduced the total to 2840. We screened these using ANOVA to identify putative compounds linked to the experimental treatment. We then used principal components analysis to reduce the dimensionality of the data and found that the major axes of the PC analysis were linked to the extent of restriction. Extracting the dominant loadings on the significant PCs allowed us to identify around 60 important metabolites that change in relation to the extent of the level of restriction. These compounds are primarily linked to 3 pathways : the steroid metabolism pathway, the aracadonic acid pathway and the mitochondrial energy metabolism pathway.

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Autophagy and Mitochondrial Biology Play a Central Role in Aging: Effects of short-term late-life-onset interventions combining moderate caloric restriction and resveratrol

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Life-long calorie restriction (CR) has been shown to be highly effective in improving overall organ function and reducing the pathophysiological signs of aging in several organs such as the heart, nerves and muscle. In striking contrast, late-age-onset CR interventions have not been extensively studied. Furthermore, the molecular mechanisms of CR-induced cytoprotective effects remain elusive, with recent evidence suggesting the critical involvement of a cellular digestion process called *autophagy* in mediating its beneficial effects. We therefore investigated whether pharmacological or nutritional enhancement of basal autophagy levels will provide protection against oxidative stress in a mouse cardiomyocyte (HL-1) and a human ventricular cardiomyocytes AC16 cell line as well as in old hearts. In Vitro: In cell lines, we mimicked mitochondrial oxidative stress conditions by using a drug called Antimycin A (AMA), which increased mitochondrial superoxide generation, decreased mitochondrial membrane potential, enhanced cell death, increased DNA/RNA oxidative damage and decreased mitochondrial respiration, all commonly observed during aging. Pretreatment of cells (24 hours) with the mTOR inhibitor rapamycin or resveratrol (pro-autophagy compounds) lead to a strong induction of autophagy and had a protective effect against the cytotoxic effects of AMA. Mechanistic genetic and pharmacological manipulations blocking autophagy, attenuated the cytoprotective effects of the pro-autophagy compounds. In Vivo: We furthermore investigated whether a lateage-onset (starting at 24-months), short term CR (1.5 month) intervention of a moderate CR dose (20%) or with the plant polyphenol resveratrol (5 or 50mg/kg/day RESV) alone, or in combination (CR + RESV) can induce autophagy in the hearts of the old F344xBN rats. We also investigated whether such interventions are protective against oxidative stress induced by doxorubicin, a known oxidant generator. Our findings show that 20% CR or RESV alone do not induce autophagy, but the combination of CR+RESV stimulated autophagy in the hearts of the old rats and provided cardioprotection.

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Upregulation of microRNAs by centenarians

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Longevity is not the only feature of centenarians as they also compress morbidity. The reason why they reach such an old age is because they maintain homeostasis. We postulated that these subjects are molecularly well regulated so we analysed their microRNA expression which directly affects gene expression regulation.

Comparison of microRNA expression profiles of young subjects, octogenarians, and centenarians, measured by studying 15,644 mature microRNAs and, 2,334 snoRNAs and scaRNAs in peripheral blood mononuclear cells, was carried out.

Analysis of such parameters demonstrated that the microRNA expression profiles of the oldest group did not differ much from that of the young group; however, it did differ from that of the octogenarians. In addition, the expression of 102 microRNAs was up-regulated in centenarians when compared with octogenarians. Our results also showed that six microRNAs (miR21, miR130a, miR494, miR1975, miR1979 and SCARNA17) are specific for centenarians.

In sum, centenarians up-regulate the expression of microRNAs and scaRNAs which may explain why they can maintain homeostasis when they are so very old. www.nature.com/scientificreports12-02982-T provides a comprehensive explanation of these views.

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SESSION 8

EU-ROS, A European COST Action (BM 1203) on Sources, Targets and Translational Relevance of Reactive Oxygen Species

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Life requires oxygen. This runs the risk that, when oxygen leaks out from normal metabolism, reactive oxygen species (ROS) are formed, which – when too high – trigger disease. With the idea to overcome this, antioxidants are heavily marketed, yet without proof of their effectiveness. Rather, worrying evidence suggests adverse effects. This paradox is due to the fact that ROS are not only 'bad', but – in tightly regulated amounts – also act as essential signalling molecules. Unravelling the fine balance between ROS acting as a friend or a foe is fundamental to understand aerobic life. To advance this important area of biology and medicine, highly synergistic approaches combining diverse and scattered disciplines are needed. For this, COST provides an ideal framework. EU-ROS brings together multi-disciplinary experts to enhance the competitiveness of European research. By applying fundamentally new approaches it will generate advanced knowledge and translate this into novel applications ranging from medicine to crop science. With its dynamic structure, EU-ROS will support capacity building of future European research in this potentially important area and overcome the fragmentation of European R&D on oxygen/ROS research while its translational components will contribute to European societies' economic growth and wellbeing. Its working groups focus on sources, targets, mechanisms, biomarkers, imaging, drugs and translation. Researchers are invited to join the management committee (restricted to 2 + 2 substitutes per country) or working group meetings (by invitation and based on content). Please consult www.cost.eu/domains_actions/bmbs/Actions/BM1203 for more information and updates. Examples for our mission are the discovery of NADPH oxidases and apo-sGC as molecular targets in stroke and diabetic atherosclerosis and nephropathy, both now in clinical development or even market entry.

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ROS Signaling in Mucosal Immunity

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One of the goals of the new COST action EU-ROS is achieving a better understanding of the origins of reactive oxygen species (ROS) in prokaryotes and eukaryotes, their relationship with antioxidants and modification of signaling systems. My laboratory is particularly interested in the regulation of NADPH oxidases and their contribution to immune functions. ROS play a role in mucosal defense, yet how they are induced and the consequences for pathogens are not clear. The lung epithelium expresses mainly the NADPH oxidases Duox1 and Duox2, while the gastrointestinal mucosa harbors Nox1 and Duox2. Duox2 has been linked to gut host defense in model organisms, but its role in lung infections or in mammalian hosts is not well characterized. Further, independently of the ROS source, it is not clear if and how extracellular pathogens will be affected by continuous release of low H_2O_2 concentrations. New aspects of how H_2O_2 plays a role in the host immune response, on how oxidases are activated in the host and the consequences for pathogens will be presented. These observations place epithelial NADPH oxidases as an early antibacterial defense system in the intestinal and airway mucosa that may act as a general virulence modifier.

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Multiscale molecular and functional imaging and detection of oxidative stress or ROS

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We are aiming to present imaging methods dealing with parameters or species implicated in oxidative stress and regulation, such as ROS, O_2 …. From cell detection to *In vivo* imaging. The presentation will show part will development results and problems appearing in Electron Paramagnetic Resonance (EPR) imaging and spectroscopy a unique, non invasive methods to specifically detect and quantify paramagnetic species in living organisms.

Problems limited those modalities will be discussed and taken into account. Example of application of the multidisciplinary approach for EPR imaging will be discussed.

Two problems limit its applications: The anatomic location of the EPR image in the animal body, and the relative instability of the EPR probes due to their possible metabolism during image acquisition. High resolution images has been obtained by using for the first time EPR and X-ray micro-computed tomography, and by taking into account the disappearance kinetics of the EPR probe during image acquisition to reconstruct the image.

This multi-disciplinary approach opens the way to high resolution imaging and local metabolism studies of various radical species in vivo in different physiological and pathological situations. Different applications under evaluation will be presented.

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Inhibition of NOX NADPH oxidases as a potential treatment for neuroinflammation

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Neuroinflammation and oxidative stress are common features of multiple neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS). Chronic activation of microglia and production of proinflammatory and cytotoxic factors including reactive oxygen species (ROS) contribute to progressive degeneration of neurons. In a transgenic model of ALS, NADPH oxidase 2 (NOX2) expression is strongly increased and NOX2 represents a major source of ROS generation during the progression of the disease. Thus inhibition of NOX2 may represent a new promising strategy for the treatment of neurodegenerative disorders. However, no specific and potent NOX2 inhibitor is currently available. In order to identify small molecules NOX2 inhibitors, we performed a screen of a NINDS library using PMA-activated neutrophils and luminol-enhanced luminescence as a read-out. We identified a group of compounds of the phenothiazine family. Based on their potency of NOX2 inhibition we selected the following compounds: prochlorperazine ($IC_{50}=2.4 \pm 0.4$ μM), promazine ($IC_{50}=7.8 \pm 2.2$ μM), perphenazine ($IC_{50}=3.9 \pm 0.7$ μM), thioridazine ($IC_{50}=2.2 \pm 0.2$ μM). We tested the effect of selected compounds on ROS production in mouse microglial RA2 cells stably expressing SOD1 G93A and activated with LPS and found that thioridazine and perphenazine decrease production of $O_2^{\bullet}$ and H_2O_2 in this model. Analysis of hydroethidine oxidation products by LC/MS showed that $O_2^{\bullet}$ production is increased in the spinal cords of SOD1 G93A mice as compared to WT littermates. *In vivo* tests of selected compounds showed that thioridazine has a modest protective effect in SOD1 G93A mice by increasing their survival by 7 days ($p = 0.028$). In conclusion, our preliminary results demonstrate that inhibition of NOX2 is a promising strategy for the treatment of neuroinflammation in neurodegenerative disorders such as ALS.

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SESSION 9

Hydroxynonenal induced activation of redox signalling pathways in vascular endothelial cells adapted to physiological oxygen tension

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4-hydroxynonenal (HNE) is a lipid hydroperoxide end product formed from the oxidation of n-6 polyunsaturated fatty acids. The relative abundance of HNE within the vasculature is dependent not only on the rate of lipid peroxidation and HNE synthesis but also on the removal of HNE adducts by phase II metabolic pathways such as glutathione-*S*-transferases. Depending on its relative concentration, HNE can induce a range of hormetic effects in vascular endothelial and smooth muscle cells, including kinase activation, proliferation, induction of phase II enzymes and in high doses inactivation of enzymatic processes and apoptosis. HNE also plays an important role in the pathogenesis of vascular diseases such as atherosclerosis, diabetes, neurodegenerative disorders and *in utero* diseases such as pre-eclampsia. In recent studies, we have examined the effects of electrophilic agents on redox signaling in human and murine endothelial cells adapted to physiological oxygen concentrations encountered *in vivo*, noting that Nrf2 mediated antioxidant gene expression is significantly down-regulated in cells adapted to 5% O_2. The known production, metabolism and consequences of HNE synthesis in vascular endothelial and smooth muscle cells will be reviewed, highlighting alterations in mitochondrial and endoplasmic reticulum function and their association with various vascular pathologies.

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Revealing 4-hydroxynonenal-histidine adducts as qualitative and quantitative biomarker of oxidative stress, lipid peroxidation and oxidative homeostasis

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Findings on growth regulating activities of the end-product of lipid peroxidation 4-hydroxy-2-nonenal (HNE), which acts as a "second messenger of free radicals", overlapped with the development of

antibodies specific for the aldehyde-protein adducts. These led to qualitative immunochemical determinations of the HNE presence in various pathophysiological processes and to the change of consideration of the aldehyde's bioactivities from toxicity into cell signalling

Moreover, findings of the HNE-protein adduct in various organs under physiological circumstances support the concept of "oxidative homeostasis", which implies that oxidative stress and lipid peroxidation are not only pathological but also physiological processes. Reactive aldehydes, at least HNE, could play important role in oxidative homeostasis, while complementary research approaches might reveal the relevance of the aldehydic-protein adducts as major biomarkers of oxidative stress, lipid peroxidation and oxidative homeostasis.

Aiming to join efforts in such research activities researchers interacting through the International 4-Hydroxynonenal Club acting within the SFRR-International and through networking projects of the system of the European Cooperation in Science and Technology (COST) carried validation of the methods for lipid peroxidation and further developed the genuine 4-HNE-His ELISA founding quantitative and qualitative methods for detection of 4-HNE-His adducts as valuable tool to study oxidative stress and lipid peroxidation in cell cultures, various organs and tissues and eventually for human plasma and serum analyses [1].

Reference

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Protein modification and phospholipid oxidation

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Phospholipid oxidation can generate reactive and electrophilic products that are capable of modifying proteins, especially at cysteine, lysine and histidine residues. Such lipoxidation reactions are known to alter protein structure and function, both with gain of function and loss of activity effects. As well as potential importance in the redox regulation of cell behaviour, lipoxidation products in plasma could also be useful biomarkers for stress conditions. Although studies with antibodies suggested the occurrence of lipoxidation adducts on ApoB-100, these products had not previously been characterized at a molecular level. We have developed new mass spectrometry-based approaches to detect and locate adducts of oxidized phospholipids in plasma proteins, as well as direct oxidation modifications of proteins, which avoid some of the problems typically encountered with database search engines leading to erroneous identifications of oxidative PTMs. This approach uses accurate mass extracted ion chromatograms (XICs) of fragment ions from peptides containing oxPTMs, and allows multiple modifications to be examined regardless of the protein that contains them. For example, a reporter ion at 184.074 Da/e corresponding to phosphocholine indicated the presence of oxidized phosphatidylcholine adducts, while 2 reporter ions at 100.078 and 82.025 Da/e were selective for allysine. ApoB-100-oxidized phospholipid adducts were detected even in healthy human samples, as well as LDL from patients with inflammatory disease. Lipidomic studies showed that more than 350 different species of lipid were present in LDL, and were altered in disease conditions. LDL clearly represents a very complex carrier system and one that offers a rich source of information about systemic conditions, with potential as indicators of oxidative damage in ageing or inflammatory diseases.

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High resolution mass spectrometric strategies for studying AGEs inhibitors and RAGE antagonists

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AGEs are involved in the onset and progression of different oxidative based diseases and their inhibition, together with the blockade of the AGEs-RAGE interaction, represents a promising drug target. In order to exploit AGEs and RAGE as drug targets, validated analytical methods able to screen compounds acting as AGEs inhibitors or as RAGE antagonists are required. An analytical platform based on high-resolution mass spectrometry (MS) which permits to evaluate the ability of the tested compounds to inhibit AGEs-ALEs formation and their interaction with RAGE is here reported.

Testing AGEs/ALEs inhibitors: the method we set-up offers the unique advantage of evaluating the efficacy of pure compound, mixture and raw extract on inhibiting AGEs and ALEs generated by incubating ubiquitin as protein target with reactive carbonyl species (RCS) such as glyoxal, methylglyoxal, 2-hydroxynonenal, acrolein, or reducing sugars (glucose and fructose). The method is based on automated injection and quantitative analysis of ubiquitin in native and adducted forms, using an Orbitrap mass spectrometer. The method was validated by investigating the effect of known inhibitors of AGEs and ALEs formation such as carnosine, hydralazine, aminoguanidine and pyridoxamine as well as of some natural extracts.

Testing RAGE antagonists: a MS method was firstly set up to study the non-covalent interactions between ligands and recombinant sRAGEs (V1-C1), representing the ligand binding domain of RAGE. The V1-C1 protein target was expressed in E. coli and the ligand-protein binding properties (stoichiometry and Kd values) were determined by a high-resolution mass spectrometric (orbitrap) approach carried out in not-denaturating conditions and using a static nano-ESI source. The method was then validated by using well known low molecular weight sRAGE ligands and the Kd values were in line with those previously reported by fluorescence titration experiments.

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SESSION 10

Glutathione S-transferase gene polymorphisms and susceptibility to renal cell carcinoma: Serbian case-control study

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Objective: Obesity, hypertension, smoking and professional exposure to carcinogens are the recognized risk factors for clear renal cell carcinoma (cRCC). Cytosolic glutathione transferases (GST) catalyze conjugation reaction of electrophylic compounds to glutathione. Although polymorphic expression of GST enzymes confers increased risk for various cancers, the role of GST polymorphisms in susceptibility to cRCC is still controversial. We aimed to assess whether common GST polymorphisms are associated with higher risk for cRCC, independently or in conjunction with recognized risk factors as well as to identify their value in tumor progression.

Methods: A hospital-based case-control study recruited 98 patients with cRCC and 240 healthy controls. *GSTA1*, *GSTT1, GSTP1* and *GSTO1* genotypes were determined by PCR. The associations between the genotypes and cRCC risk were examined by using logistic regression to calculate odds ratios (ORs) and 95% confidence intervals (CIs).

Results**:** Regression analysis showed that *low activity GSTA1* (*CT/TT)* and *active GSTT1* genotypes were associated with higher, but statistically non-significant effect on risk for cRCC (OR=1.4 CI:0.7-2.4 and OR=1.8 CI:0.9-3.5, respectively) after adjustment for age, gender and obesity. However, when the effect of these two genotypes was analyzed in combination, patients with combined *low activity GSTA1/active GSTT1* genotype exhibited 5–fold higher risk (CI:1.1-22.9, p=0.034) than those with *GSTA1 CC/GSTT1 null* genotype. Patients homozygous for *GSTO1 A* and *GSTP1 Ile* alleles exhibited higher, but non-significant risk compared to the patients homozygous for *GSTO1 C* and *GSTP1 Val* alleles (OR=1.6, CI:0.7-3.8, OR=1.5 CI:0.6-3.6, respectively). Concerning the association of GST genotypes with cRCC progression, patients with *active GSTT1* genotype or carrying *GSTO1 A* and *GSTP1 Ile* alleles had tumors of higher grade than those with *GSTT1 null* genotype or homozygous for *GSTO1 C* or *GSTP1 Val* alleles.

Conclusions: Patients with combined *low activity GSTA1* and *active GSTT1* genotypes are at higher risk of developing cRCC.

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Development and in vitro proof-of-concept of interstitially targeted zinc-phthalocyanine liposomes for photodynamic therapy

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Background: Photodynamic therapy (PDT) has been used to treat numerous solid cancers. However, some cancer types respond poorly to PDT, including urothelial carcinomas, nasopharyngeal carcinomas, and extrahepatic cholangiocarcinomas. The therapeutic recalcitrance is in part due to the use of photosensitizers with suboptimal optical/photochemical properties and poor pharmacokinetics.

Objective: To circumvent these drawbacks, a second-generation photosensitizer with improved optical/photochemical properties, zinc phthalocyanine (ZnPC), was encapsulated in interstitially targeted, polyethylene glycol-coated liposomes (ITLs) intended for systemic administration. The ZnPC-ITLs were examined for reactive oxygen species (ROS) generation and oxidation capacity and validated for tumoricidal efficacy in human extrahepatic cholangiocarcinoma (Sk-Cha1) cells. ZnPC-ITL uptake as well as the mechanism and mode of PDT-induced cell death were also studied.

Methods: The ITL formulation was optimized on the basis of fluorescence spectroscopy. The extent of ROS generation, protein oxidation, and membrane oxidation were determined by the 2',7'-dichlorodihydrofluorescein assay, tryptophan oxidation assay, and calcein leakage assays using cell phantoms, respectively. PDT efficacy was evaluated by measuring mitochondrial activity and apoptosis-/necrosis-specific staining in combination with flow cytometry. The uptake of fluorescently labeled ITLs was assayed by confocal microscopy, flow cytometry, and fluorescence spectroscopy.

Results: ZnPC-ITLs exhibited maximum ROS-generating and oxidation potential at a ZnPC:lipid molar ratio of 0.003. PDT of Sk-Cha1 cells incubated with ZnPC-ITLs induced cell death in a lipid concentration-dependent manner. The mode of PDT-induced cell death comprised both apoptosis and necrosis, with necrotic cell death predominating. Post-PDT cell death was attributable to pre-PDT ZnPC-ITL uptake by cancer cells, which was more efficient at smaller ITL diameters and a more positive surface charge.

Conclusions: ZnPC-ITLs constitute a nanoparticulate photosensitizer delivery system capable of inducing apoptosis and necrosis in cultured extrahepatic cholangiocarcinoma cells by PDT-mediated oxidative processes. Animal studies are underway to provide in vivo proof-of-concept regarding the utility of this formulation in PDT.

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Radical mediated degradation of cereal β-glucan

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Cereal β-glucan possesses well-established health benefits, namely the improvement of glucose metabolism and the decrease in the level of cholesterol in the case of hypercholesterolemia. These functionalities have often been related to the ability of β-glucan in forming highly viscous solutions, a feature which is directly controlled by the concentration and the molecular weight of the polysaccharide. However, β-glucan in solution undergoes a non-enzymatic degradation in presence of iron(II), which could alter its viscosity–related health benefits. This degradation process has been attributed to a hydroxyl radical-mediated oxidative cleavage, however no direct link between the formation of hydroxyl radical and β-glucan degradation has been reported, moreover the mechanism of the oxidative cleavage is not known. In the present study, we demonstrated that the presence iron(II) with a reducing agent (ascorbic acid) in β-glucan solutions causes the formation of a large amount of hydroxyl radicals, which further degrade the polysaccharide. The mere presence of iron(II) in β-glucan solutions also promoted the formation of hydroxyl radicals, hence β-glucan degradation, although to a significantly lower extent. Moreover, the radical mediated degradation of β-glucan was fully inhibited by catalase and slowed down by superoxide dismutase, which indicates that superoxide and hydrogen peroxide are intermediate species occurring during the generation of hydroxyl radical responsible β-glucan degradation. Additionally, the characterization of β-glucan oxidation products obtained

after treatment with hydroxyl radical generating system (H_2O_2/Fe^{2+}) shows that the hydroxyl radical mediated degradation is accompanied by the formation of peroxyl radicals and new oxidized functional groups, as detected by ESR and NMR, respectively. More importantly, the results indicate that the cleavage is initiated by the formation of a carbon centered radical at the anomeric carbon (C1). Thus it demonstrates that the hydroxyl radical cause the degradation of beta-glucan while changing its structural properties with the introduction of new functional group.

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Influence of dietary restriction on astrocytic response in the rat brain following traumatic brain injury

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Traumatic brain injury (TBI) is a widespread cause of death and adult disability. Besides primary loss of neurons, secondary injury, which is the following event, leads to further neuronal damage and loss. One of the hallmarks of the secondary injury is microglial activation resulting in increased cytokine production and subsequent astrocytic activation. When activated, astrocytes create physical and chemical barrier around the damaged tissue which prevents axonal regrowth. This research was aimed to examine whether dietary restriction (DR) affects astrocytic response and thus modulates processes of recovery after cortical injury. Astrocytic response was followed by measuring expression of delta isoform of glial fibrillary acidic protein (GFAPσ), galectin-1 and neurocan; molecules that are mainly expressed by astrocytes and have important roles in neuronal regeneration after TBI. In this study we used male Wistar rats (3 months old) which were subjected to DR (3 months on 50% of the daily food intake) prior to stab injury in the somatosensory cortex. Tissue was collected in several time points (2, 7, 14 and 28th day post-injury) and using Western blot and immunohistochemical analyses (IHC), the level and localization of GFAPσ, galectin-1 and neurocan were examined. Our results have shown that levels of GFAPσ, galectin-1 and neurocan were downregulated in DR animals compared to animals that had unlimited approach to the food. These effects were most notably 2 and 7 days after the injury. Additionally, IHC revealed cell type specific pattern of expression of those molecules in the injured area, which was also modulated by DR. Notwithstanding that our study demonstrated that DR prior to an acute brain injury affects astrocytic response, the exact mechanism remains unclear. Revealing it might be of interest for potential therapeutic application.

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Application of fluorous chemistry for the identification of carbonylation sites from oxidised proteins

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Protein carbonylation is an irreversible oxidative modification, affecting both peptide backbone and amino acid side chains. It triggers structural and functional changes to proteins often leading to their proteasomal degradation. Protein carbonylation is a well-known hallmark of oxidative stress-related diseases such as *diabetes mellitus* and Alzheimer's disease. Therefore understanding mechanism of action and characterisation of carbonylated proteins might facilitate treatment of such diseases in the future. Analytically, carbonylation is one of the most challenging post-translational modifications to study. This is due to the high reactivity, low stoichiometry and reduced ionisation efficiency of carbonylated species. To overcome these limitations enrichment of carbonylated peptides is crucial prior to mass spectrometry-based identification of carbonylation sites. We have chosen a strategy based on fluorous chemistry, known as a highly selective and efficient method for enrichment and identification of various post-translational modifications. We have adapted and optimized it for analysis of carbonylome, carbonyl content of proteins. We have synthesized a fluorous tag containing carbonyl-reactive hydrazide moiety. Utilising this tag we are able to label and enrich aldehyde-containing model peptides by fluorous solid phase extraction with a recovery of above 60%. Application of our strategy to analysis of carbonylated BSA provided information about 51 carbonylation sites, 30% of which has been reported previously. Final validation was analysis of rat liver mitochondrial proteome under oxidative stress. We have identified numerous proteins carrying at least one carbonylation site. Amongst these, proteins involved in oxidative stress-response, i.e. Proteasome activator complex subunit 1 and mitochondrial respiratory complex, i.e. ATP synthase subunit beta. Interestingly, carbonylation sites were also associated with proteins of mitochondrial DNA replication machinery. We believe that our methodology will bring insights into the role of oxidative stress in cellular homeostasis and will facilitate treatment of oxidative stress-related diseases in the future.

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The mechanisms behind the inhibition of cytokine-induced inflammatory response by Cyanidin 3-Glucoside and Resveratrol in human intestinal cells: comparison with 5-ASA

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Polyphenols are naturally occurring compounds widely spread in human diet. The advantage of polyphenols in the prevention and treatment of chronic inflammatory diseases has been described by several studies; however the precise mechanisms and targets involved in cellular signaling are still not fully understood. The aim of this study was to assess the protection afforded by two dietary polyphenols, Cyanidin 3-Glucoside (C3G), a typical anthocyanin that belongs to the flavonoid group of polyphenols, and Resveratrol, a non-flavonoid polyphenol, against cytokine-induced inflammatory response in the human intestinal HT-29 cell line, in comparison with 5-aminosalicylic acid (5-ASA), a well-known anti-inflammatory drug, commonly used in inflammatory bowel disease. For this purpose, some key inflammatory mediators and pro-inflammatory enzymes were evaluated. HT-29 cells were pretreated with 25 μM C3G or 25 μM Resveratrol

and/or 500 μM 5-ASA and then exposed to a combination of cytokines (IL-1α, TNF-α, IFN-γ) for a certain period of time. Nitric oxide (NO) was measured by a fluorimetric assay whereas prostaglandin E_2 (PGE_2) was evaluated by using a competitive immunoassay. The protein levels of iNOS, COX-2 and IkB-α were analyzed by Western blotting. Our data showed that both C3G and Resveratrol were able to inhibit cytokine-induced inflammation in intestinal cells, in terms of NO, PGE_2 and IL-8 production and of iNOS and COX-2 expressions, at a much lower concentration than 5-ASA, suggesting a higher anti-inflammatory efficiency of C3G and Resveratrol. Interestingly, neither of the above mentioned compounds prevents IκB-α degradation, suggesting the involvement of another cell signaling pathway. Therefore, the ability of these compounds to suppress cytokine-induced STAT1 phosphorylation has been studied. As a matter of fact, polyphenols can reach high concentrations in the gastrointestinal tract which make Cyanidin-3-glucoside and Resveratrol promising nutraceuticals, able to give complementary benefits in the context of inflammatory bowel disease.

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Involvement of Reactive Oxygen Species (ROS) in skeletal muscle function during ageing: Study in a model of isolated single skeletal muscle fibre

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Reactive oxygen species (ROS) are constantly produced in skeletal muscle and the hypothesis that ROS are involved in the process of ageing is extensively accepted. Several studies indicate that ROS might be responsible of different adaptive responses that lead to maintain muscle mass and function. However, during ageing these adaptive responses are attenuated or disrupted and ROS might be the key regulators of this patho-physiological process.

We study the effect of ROS in skeletal muscle using an *ex-vivo* physiological model, the single skeletal muscle fibre isolated from the *Flexor Digitorum Brevis* muscle. This model is suitable for the use of specific detectors for different ROS to monitor, in real time, intracellular ROS production in single skeletal muscle fibres.

We have demonstrated that contractile activity generates a net intracellular increase of ROS in single muscle fibres and the effect was abolished when glutathione was replenished. In addition, contractile activity produces an intracellular increase of nitric oxide in fibres and this effect was attenuated by treatment with nitric oxide synthase inhibitors. Moreover, contractile activity induces an intracellular increase of superoxide in muscle fibres and this effect was abolished by treatment with superoxide scavengers. In another study we have demonstrated that passive elongation of skeletal muscle fibres from young mice induced an increase in intracellular superoxide with no increase in intracellular nitric oxide. In contrast, in fibres from old mice passive elongation induced an increase in intracellular nitric oxide with no change in superoxide production. Recently, we have proved that ROS are increased in fibres from old mice at rest and, surprisingly, no further increase in ROS generation during contractile activity. The defect in short-term adaptations to contractions reported in old mice may be related to a diminished or absent increase in the muscle generation of ROS that accompanies contractile activity.

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Evidence of an interplay between ER stress/UPR and mitochondria in human hepatic cells treated with the antiretroviral drug Efavirenz

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Altered function of the endoplasmic reticulum (ER) manifested as accumulation of misfolded/unfolded proteins and/or depletion of $[Ca^{2+}]_{er}$, giving rise to a state of "ER-stress", has been related to various hepatic diseases including drug-induced hepatotoxicity. Efavirenz, a non-nucleoside analog reverse transcriptase inhibitor, is a cornerstone of the current combined anti-HIV1 therapy. Despite being generally safe, it has been associated with several adverse events including liver damage. We have recently reported involvement of mitochondrial dysfunction in this event, using human hepatic cells exposed to clinically relevant concentrations of EFV. In parallel, these cells display markers of ER-stress and UPR activation. Here, we analyzed the relation between EFV-induced ER stress and mitochondrial (dys)function in the same model. Primary human hepatocytes, the human hepatoma cell line Hep3B and rho^o cells generated in Hep3B background (phenotype lacking functional mitochondria) were exposed to EFV (10 and 25μM) EFV for 24h. The concentration-dependent increase in both mRNA and protein expression of GADD153/CHOP (CCAAT/enhancer binding protein) and GRP78 (Glucose-regulated protein 78) was largely diminished in rho^o cells. Similarly, unlike WT cells, rho^o cells displayed no increase in the ER-signal (fluorescence microscopy). The specific interconnection between ER-stress and mitochondria was also shown by studying calcium levels. EFV exposure resulted in a decrease in $[Ca^{2+}]_m$ and an increase in $[Ca^{2+}]_c$, which differs from the action of a classic-stressor such as thapsigargin. Moreover TEM experiments revealed that EFV-treated cells exhibited a higher level of contact (closer location) between mitochondria and ER (both displaying altered morphology). In conclusion, human hepatic cells treated with clinically relevant concentrations of Efavirenz present markers of ER-stress with a specific involvement of mitochondria in this effect. These findings expand our knowledge of the mechanisms that trigger ER-stress and throw light on the mitochondria/ER interplay in drug-induced hepatic challenge with specific relevance for the patients undergoing EFV-containing therapy.

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SESSION 11

Beneficial role of nitric oxide in cholesterol-induced steatohepatitis and LPS-induced endotoxemia

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The study was designed to elucidate the role of nitric oxide generated by inducible nitric oxide synthase (iNOS) during high cholesterol diet (HCD)-induced steatohepatitis with and without LPS treatment. Herein, wild type (WT) and iNOS-knockout (iNOS-/-) mice were fed with a HCD for 6 weeks. Following diet period, some of the mice were injected with LPS (5 mg/Kg). Results: Chronic consumption of HCD led to steatohepatitis WT and iNOS-/- mice. LPS administration caused marked liver damage only in cholesterol-fed mice, which was further exacerbated in the absence of iNOS. Metabolic effect: Enhanced liver injury by HCD and LPS in iNOS-/- mice was associated with a fatal hypoglycemia. Glycogen contents were significantly retained in iNOS-/- mice while hypoxia inducible factor 1 (HIF1) signaling was markedly attenuated compared to control WT. Results also demonstrated increased oxidative stress and reduced heme oxygenase-1 (HO-1) mRNA in the livers of iNOS-/- mice. Furthermore, the amounts of plasma tumor necrosis factor-α (TNFα) and intrahepatic TNFα mRNA) were significantly elevated in the absence of iNOS. These data highlight the essential role of iNOS axis in the glycemic response to LPS in NASH and argues for beneficial effects of acute NO production under chronic and acute liver stress.

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Metabolic aging in diabetes: The glucoliptoxic connection

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'Metabolic aging' describes the premature and accelerated end-organ dysfunction due to severe metabolic ab'normalities. Obesity and type-2 diabetes are major risk factor in this process. An altered crosstalk between fat depots and peripheral organs, low grade inflammation, insulin resistance and altered mitochondrial function contribute to this phenomenon. The latter is of great interest due to its central role in the generation of reactive free radicals causing cellular damage. We have hypothesized that enhanced radical-induced lipid peroxidation of polyunsaturated fatty acids and the subsequent generation of 4-hydroxynonenal (4-HNE), underlie some aspects of 'metabolic aging' in insulin-secreting pancreatic β-cells. We have studied the impact of the combination of high levels of glucose and palmitic acid (glucolipotoxic condition) on β-cell function and survival and observed a biphasic function of 4-HNE in these cells. In the early stages of exposure when of 4-HNE is present at low non-toxic levels, it acted as a hormetic agent, enhancing glucose-stimulated insulin secretion from the INS-1E β-cell line and from freshly isolated rodent islets. This may enable a short-term defense against the developing peripheral insulin resistance in obese and/or diabetic subjects. However, at higher concentrations, typical of long-term obesity and hyperglycemia, 4-HNE induced β-cell death in both preparations. This was due to 4-HNE induced apoptosis and accompanied with a substantial increase in the abundance of 4-HNE-protein adducts. Co-treatment of cells with the scavenger L-carnosine, protected the cells against this damage. Moreover, we used *Psammomys obesus*, an animal model of diabetes, and found a marked time-dependent increase in 4-HNE levels in the plasma of high energy-fed hyperglycemic animals, in comparison with healthy controls fed a low energy diet. These results suggest that lipid peroxidation and the resulting formation of 4-HNE are related to end-organ metabolic aging and its consequences.

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The *in vivo* antioxidant capacity of fruits, barks and leaves of cornelian cherry (*Cornus mas L., Cornaceae*) and myrtle oil (*Myrtii Oleum*; MO) related with their antidiabetic effects from Turkish folk medicine

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In Turkey, during a field survey we observed that cornelian cherry (*Cornus mas L., Cornaceae*), fruits, leaves and bark of branches have been used against diabetes among the country people. Besides *Myrtus communis L.* (Myrtaceae) leaves as well as the volatile oil (*Myrtii Oleum*; MO) obtained from the leaves are also used to lower the blood glucose level in type-2 diabetic patients in Turkish folk medicine. However, little attention has been paid to the therapeutic use of those plants. When we investigated the literature for *Cornus mas*, we were not able to find enough number of scientific studies however there are some studies especially focusing on the effect of antioxidant effects, but most of them were not done *in vivo*. In addition, its effect on diabetes has not been investigated in detail. In our studies for both remedies, we performed diabetes groups that had been taken all different forms of extracts at various doses and as well as creating another set of study groups, performed the oral glucose tolerance test. As a result we evaluated the both antidiabetic and antioxidant affects of cornelian cherry and volatile oil (*Myrtii Oleum*; MO) by measuring antioxidant enzyme activities, index of lipid peroxidation, free radicals and diabetes research panel from serum and tissue samples. In this lecture, I will mainly focus on our results of antioxidants and radicals.

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Explosive-type moderate resistance training affects cellular and biochemical parameters of exercise-induced oxidative stress in the elderly

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Regular physical activity has been hypothesized to provide protection against oxidative damage at all ages. Several studies have incorporated, as an attractive exercise modality for older adults, a low frequency explosive-type moderate resistance training (EMRT). The purpose of this work was to investigate the effect of EMRT on both biochemical and cellular markers of oxidative

stress evaluated in trained elderly subjects in response to a maximal exercise stress test (MEST).

Sixteen elderly persons (aged 72.5 years), were randomly assigned to two different groups: a training group (EMRT for 12 weeks) and a control group. Plasma redox homeostasis (total antioxidant status, (TAS) and glutathione (GSH)), oxidative damage (malondialdheyde (MDA) and protein carbonylation levels), and repair system (Hsp70 and Hsp27 expression in leukocytes) were evaluated before and after the maximal exercise stress test.

MEST lead to an increase of all the parameters related to oxidative stress. However, after the EMRT, the training group showed a less pronounced increase in oxidative stress parameters following the maximal exercise stress test compared to control group. A 25% reduction ($p < 0.05$) were found in GSSG levels and GSH/GSSG ratio was increased by 20% ($p < 0.05$) compared to control group. MDA and protein carbonylation levels were 25 % reduced respect those found in control group. Hsp70 and Hsp27 expression showed a lower protein basal levels in the trained group, however, for both groups any significant induction of HSPs was not recognized within 24 hours after MEST.

Our data show that, in trained elderly subjects, the EMRT protocol is able to induce a cellular adaptation allowing them to respond more effectively to an acute oxidative stress than the aged-matched sedentary subjects. Hence, the EMRT protocol may be considered an effective workout that can improve the general adaptive response to oxidative stress contributing to the overall health of the older people.

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Heat Shock Protein 90 role in oxidized protein aggregates

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Protein carbonylation, the most frequent type of protein oxidation, which often results from oxidative stress conditions, leads to an irreversible non-enzymatic protein modification and dysfunction. Cells recycle these proteins and prevent their accumulation, using the proteasome. However, if protein oxidation formation rate exceeds proteasome capacity, oxidized proteins form insoluble, undegradable, high molecular weight, protein aggregates that may cause senescence or apoptosis.

We have recently shown oxidized protein aggregates formation in a T cell line model after several hours of oxidative stress leading to proteasome inhibition and proliferation arrest. However, the underlying mechanism that follows these disturbances remains elusive.

We hypothesized that molecular chaperones as hsp90 and hsp70 would have a role in this process as they are known to be involved in cellular proteostasis by forwarding proteins to refolding or degradation depending on triage decision; in fact, their precise role on oxidized proteins (or oxidized aggregates) in this setting is largely unexplored. Therefore to provide insights on that model, we decided to investigate hsp90/70 involvement.

After exposing cells to oxidative stress for 24h, followed by recovery up to 216h, preliminary results indicate a role of hsp90, but not hsp70, involvement. In fact, there was a co-localization of hsp90 and protein aggregates shown by immunocytochemistry. In addition, in Western blots against hsp90 (constitutive form around 84 kDa), there was a positive band in the stacking zone suggesting high molecular weight aggregates binding. Interestingly, further analysis revealed that it contained an unexpected hsp90 band around 73 kDa, suggesting an isoform with a specific role in protein aggregates establishment.

This band may be an inducible form of hsp90, which will be clarify by mass spectrometry after purifying aggregates from insoluble fractions, a work that is currently in progress. Also, we hope to verify its role in vivo as well, as we hypothesize that this may also take place. Studies with organisms models should follow.

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SESSION 12

How to Write a Great Research Paper, and Get it Accepted by a Good Journal

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Background: Knowing the best way of structuring your paper when writing it, and the most appropriate journal to send it to, really helps in getting your paper accepted. Also understanding how editors and publishers think and what they expect, and knowing how the peer review process works, is invaluable insight into the publishing process.

Results: After attending this workshop, one in the Elsevier Publishing Connect Workshop series, participants will have a clear idea of the steps needed to be taken before starting to write a paper. They will also be able to plan writing manuscripts using the logical step sequence – not the sequence in which the paper will be read. Authors are also made aware of what aspects of their papers Editors and Publishers look at critically, and to ensure that in taking care of these areas, their papers are much more likely to be accepted. Dealing with referees' comments and the art of polite rebuttal are also described such that these can be used to improve the submitted paper suitably. Sensitive areas such as publishing ethics, plagiarism, duplicate publishing, etc are also clearly explained such that participants have a clear understanding of what is allowed, and what is not allowed.

Conclusions: These insights into the publishing and peer-review process will enable the participants to be more confident as an author in the world of science publishing, and will help them get their papers published more easily, and help them to progress their career.

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TRAINING SCHOOL 1

Combinatorial approaches and models in the study of human ageing *in vitro* and *in vivo: emphasis on Caenorhabditis elegans as a model organism*

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Ageing is a natural biological process determined by both genetic and environmental/stochastic factors. The ageing process results in the gradual decline of physiological function and the eventual failure of organismal homeostasis. Several models can be used in the study of human ageing *in vitro* or *in vivo*. Given that longitudinal (follow individuals throughout their lives) and cross-sectional studies (comparison of young and old individuals) in human are laborious, animal models including *Saccharomyces cerevisiae*, *Caenorhabditis elegans, Drosophila melanogaster, Mus musculus* and *Rattus norvegicus* along with *in vitro* cellular models and mathematical/computer models have been also used in the study of human ageing. Many studies have revealed several conserved pathways governing the ageing phenomenon across species while other studies have also shown pitfalls in the use of these models. The basic characteristics of the complex biological process of ageing will be delivered while the advantages and disadvantages of the used combinatorial models will be discussed. Special emphasis will be given on the use of *C. elegans* as a model to study human ageing.

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TRAINING SCHOOL 2

Yeast as a model to study aging

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The budding yeast *Saccharomyces cerevisiae*, which can proliferate in both haploid and diploid states, has been used extensively in aging research. Despite the fact that ageing necessarily displays unique aspects in a single-cell organism, yeast, in particular *Saccharomyces cerevisiae*, are useful as model organisms to study ageing. The budding yeast divides asymmetrically to form a 'mother' cell and a bud. Two major approaches, 'budding life span' and 'stationary phase' have been used to determine 'senescence' and 'life span' in yeast. Discrepancies observed in metabolic behavior and longevity between cells studied by these two systems raise questions of how 'life span' in yeast is defined and measured. Added to this variability in experimental approach and results is the variety of yeast strains with different genetic background used as 'wild type' and experimental organisms. We discuss the inherent, advantageous attributes that make the yeast an attractive choice for modern biological research as well as certain pitfalls in the choice of this model for the study of aging. Discrepancies between the yeast and mammalian systems with regard to aging are pointed out. Here we review mitochondrial characteristics involved in yeast longevity, including biogenesis, autophagy, respiration and oxidative phosphorylation, nutrient sensing, mitochondria-nuclear signaling, redox state and mitochondrial DNA integrity. In *S. cerevisiae* oxidatively damaged proteins accumulate in the mother cell via a Sir2p-dependent mechanism, which allows the newly formed bud to be born nearly damage-free. We will discuss rejuvenation in yeast.

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The mouse as a model organism in aging research: Usefulness, pitfalls and possibilities

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The mouse has become the favorite mammalian model. Among the many reasons for this privileged position of mice is their genetic proximity to humans, the possibilities of genetically manipulating their genomes and the availability of many tools, mutants and inbred strains. Also in the field of aging, mice have become very robust and reliable research tools. Since laboratory mice have a life expectancy of only a few years, genetic approaches and other strategies for intervening in aging can be tested by examining their effects on life span and aging parameters during the relatively short period of, for example, a PhD project. Moreover, experiments on mice with an extended life span as well as on mice demonstrating signs of (segmental) premature aging, together with genetic mapping strategies, have provided novel insights into the fundamental processes that drive aging. Finally, the results of studies on caloric restriction and pharmacological anti-aging treatments in mice have a high degree of relevance to humans. We will review a number of recent genetic mapping studies that have yielded novel insights into the aging process. We will discuss the value of the mouse as a model for testing interventions in aging, such as caloric restriction, and we will critically discuss mouse strains with an extended or a shortened life span as models of aging.

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TRAINING SCHOOL 3

Proteostasis assurance mechanisms as key determinants of longevity in *Drosophila*

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The ubiquitin-proteasome system (UPS) and the NFE2-related factor 2 (Nrf2)-mediated antioxidant responses are central modules of the proteostasis (homeostasis of the proteome) assurance network in higher metazoans. We have been studying the alterations in the functionality of the proteasome during *in vivo* ageing of the model organism *Drosophila melanogaster* and found that proteasome peptidase activities and expression decline in the soma but not in the gonads of the aged flies. Moreover, we have found that sustained partial proteasome loss of function in young flies promoted several “old-age” phenotypes and a reduction of flies’ lifespan. Loss of proteasome activity induced in the young somatic tissues, and independently of age in the gonads, higher expression levels and assembly rates of proteasome subunits. Proteasome dysfunction was signaled to the proteostasis network of the responsive tissues by reactive oxygen species that originated from malfunctioning mitochondria and triggered an Nrf2-dependent upregulation of the proteasome subunits. RNAi-mediated Nrf2 knock down reduced proteasome activities and longevity, while activation of Nrf2 in transgenic flies upregulated basal proteasome expression and

peptidase activities independently of flies' age. Interestingly, prolonged overactivation of Nrf2 decreased longevity. Our observations add further experimental evidence to the *trade-off* theories of aging evolution, where aging is considered a consequence of increased energetic investment in maintenance of the germ line (preserving viability across generations) over maintenance of the soma (only needed to support survival of a single generation) and exemplify the UPS and Nrf2 cross-talk towards the maintenance of organismal homeostasis.

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Rabbit as a model system used to study age related diseases

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To overcome some of the limitations in human studies, several animal model systems are utilized to highlight the mechanisms of age related diseases. Rabbits are among these animal models, mostly used in the studies including hypercholesterolemia induced atherosclerosis and age-related macular degeneration (AMD). White New Zealand rabbit (Oryctolagus cuniculus) is frequently used as a model for *in vivo* studies. Rabbits are the first and classical model for atherosclerosis studies. While other species, such as mice and rat, need additional chemicals and knock-out processing, cholesterol induced atherosclerosis is established in rabbits by only high cholesterol diet. Therefore rabbit model is accepted to be the most popular model in cholesterol induced atherosclerosis. Four weeks have found to be sufficient to provide high serum cholesterol levels. Additionally increase in foam cell formation is easily observed suggesting this model to be useful for the enough material to investigate the changes in the aortic tissue. Cross-linking of lens proteins are widely investigated in rabbit eye model. Lipofuscin as insoluble material in drusen biogenesis and pathogenesis of AMD is determined in rabbit models. Glycoxidation induced AMD takes also a wide place in the literature for the investigations in rabbit models. The wide usage of rabbits in above mentioned studies and in several other experimental models confirms the involvement of rabbits as an accepted animal model for the age related diseases.

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