## 5) SFRR-E Symposia Abstracts in Sequence of Presentation

### SFRR-E Symposium 1 – Bench to bedside transition for pharmacological regulation of NRF2 in non-communicable diseases

#### THE ROLE OF NRF2 FOR MITOCHONDRIAL ADAPTATION IN INFLAMMATORY MACROPHAGES

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The transcription factor Nrf2 nuclear factor erythroid 2 p45-related factor 2 (Nrf2) and its negative regulator, Kelch-like ECH associated protein 1 (Keap1), regulate the expression of complex networks of genes encoding cytoprotective proteins that provide adaptation to oxidative, electrophilic, inflammatory, and metabolic stress. Using quantitative high-resolution mass-spectrometry approaches, we characterized the proteomes of bone-marrow derived macrophages with graded Nrf2 transcriptional activity at resting and activated (with lipopolysaccharide, LPS) states. We found significant differences among the genotypes in the abundance of proteins that participate in numerous cellular processes, including redox, amino acid, carbohydrate and lipid metabolism, and innate immunity. Complementary metabolomics and respirometry studies supported these findings. Analysis of oxygen consumption rates (OCR) in resting macrophages confirmed a role for Nrf2 in regulating mitochondrial respiration: Nrf2 activation increased the basal respiration rates associated with ATP production, whereas Nrf2 disruption had the opposite effect. Analysis of extracellular acidification rates (ECAR) identified a role for Nrf2 in promoting glycolysis in resting and activated macrophages. In addition to changes in metabolism, we also observed an enrichment in mitochondrial fusion with Nrf2 activation, including the mitochondrial fusion proteins, Opa1, Mfn1 and Mfn2, and a significant increase in the mitochondrial fission factors, Mff and Mief2, with Nrf2 disruption in activated macrophages, suggesting that Nrf2 may play a role in mitochondrial adaptation during inflammation. Confocal microscopy analysis of mitochondrial morphology following immunofluorescence staining of the outer mitochondrial membrane protein Tom20 further showed that prolonged stimulation with LPS caused a switch in mitochondrial morphology, from intermediate to fused/elongated, which was enhanced by Nrf2 activation and suppressed by Nrf2 disruption. Together, these findings show that Nrf2 is a critical factor governing redox and intermediary metabolism and facilitating mitochondrial adaptation in macrophages encountering pro-inflammatory stimuli.

#### IDENTIFYING THERAPEUTIC VULNERABILITIES IN NRF2 DEPENDENT CANCERS

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The NRF2 pathway is often constitutively activated in various cancer types, particularly in non-small cell lung cancer (NSCLC), contributing to the malignant phenotype and drug resistance. Unfortunately, direct targeting of NRF2 has remained a challenge, as it is a transcription factor with many structural homologs and functions. Therefore, indirect means to inhibit NRF2 activity by for example targeting factors affecting transcriptional regulation of NRF2 or downstream pathways generating cancer-specific vulnerabilities may provide alternative ways to treat NRF2 overactive cancers. Herein, various multi-omics approaches are used to discover targetable co-dependencies in cancer with constitutive NRF2 activation. It is demonstrated how publicly available genetic and drug repurposing screens can reveal specific vulnerabilities, and how NRF2 activation affects the efficacy of immune therapies. Also, systems proteomics approaches are used to study proteins interacting with the KEAP1-NRF2 system, potentially identifying novel coregulators that can be targeted for therapy.

#### TARGETING THE NRF2/BETATRCP AXIS IN LIVER DISEASE

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Inflammation plays an important role in the pathology of most diseases, including non-alcoholic steatohepatitis (NASH) for which there is no currently approved drug to stop disease progression. Transcription factor NRF2 has been proposed recently as a promising target to stop NASH, but the most widely analyzed compounds are electrophiles that, in addition to inhibiting its main repressor KEAP1, display many off-target effects and also elicit a very strong supra-physiological NRF2 activation. As an alternative, we screened a chemical library of ~ 1 M small molecules to identify disrupters of the interaction between NRF2 and the E3 ligase adapter beta-TrCP. In vitro ubiquitination and cell culture experiments demonstrated that our hit compound is a beta-TrCP/NRF2 interaction inhibitor that activates NRF2 within physiological levels. This compound is specific for NRF2, as it does not disrupt the interaction between beta-TrCP and other substrates, such as beta-catenin. Pharmacodynamics studies demonstrated selective exposure and NRF2 activation in the liver. In mice submitted to LPS-induced acute liver inflammation, the compound greatly attenuated Kupfer cells' activation and the NFkB-mediated inflammatory response. We further analyzed the effect of this compound in the STAM model of progressive liver damage by NMR, measuring the fat/water ratio, and histochemistry of oil red (fat) and Sirius red (fibrosis), and correlated inflammatory and metabolic parameters with NRF2 activation. We found that this PPI inhibitor prevented NASH onset. Importantly, mice which were allowed to develop NASH and were then submitted to chronic treatment with this compound for 4 weeks exhibited a significant protection against the development of fibrosis. Our results report an innovative mechanism to activate NRF2 and protect the liver from NASH and fibrosis.

### SFRR-E Symposium 2 – Post-translational modification of proteins in health and disease

#### CHARACTERIZATION AND QUANTIFICATION OF POST-TRANSLATIONAL MODIFICATIONS ON PROTEINS IN CARDIOVASCULAR DISEASE

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Cardiovascular disease (CVD) is a leading cause of morbidity and mortality worldwide. Atherosclerosis, which is characterized by cholesterol and lipid accumulation in the artery wall, is the major underlying cause of CVD and is often asymptomatic for decades. Unfortunately, destabilization and rupture of atherosclerotic lesions can be sudden and give rise to an acute myocardial infarction or stroke. Despite the importance of lesion stability in CVD, the mechanisms underlying lesion rupture are poorly understood, though there is considerable evidence for extracellular matrix (ECM) alterations and a weakening of lesion structure. Compared to stable lesions, rupture-prone lesions typically contain higher levels of activated inflammatory cells that generate potent oxidants, such as hypochlorous acid and nitrating species which can both damage ECM proteins directly, or activate proteases that degrade ECM components.

In this presentation, data consistent with alterations to the nature and type of materials in lesion ECM will be presented, together with modifications to these materials, as determined by immunocytochemistry, immunoblotting and liquid-chromatography (LC-MS) studies. Analysis of materials present in, or extracted from carotid lesions, has allowed identification of 890 differentially-abundant proteins between soft (rupture-prone) and hard (stable) lesions. Many of the overabundant proteins in soft lesions are involved in inflammatory responses and ECM remodeling. Detailed LC-MS analyses have shown the presence of chlorinated, nitrated and oxidized species on multiple ECM components, together with a marked increase in cleaved proteins, as judged by the N-terminal proteomics that has allowed detection of 837 cleaved peptides. The detection of these species is consistent with marked protein damage. The protein identities and the sites of cleavage have been characterized in some cases. These species are present at significantly higher abundance in unstable compared to stable lesions. Together, these data offer a unique insight into the inflammatory and proteolytic mechanisms of lesion destabilization in CVD.

#### IDENTIFICATION OF COMPOUNDS DERIVED FROM THE GREEK FLORA WITH ANTI-AGGREGATION PROPERTIES

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Protein homeostasis (proteostasis) refers to the molecular mechanisms that are responsible for the maintenance of the cellular protein network. Proteostatic

mechanisms tend to decline with age and this often leads to accumulation of toxic protein aggregates. The Aβ peptide that has been causally related to Alzheimer's disease (AD) onset and progression represents one of these aggregation-prone proteins. Plant secondary metabolites have been shown to be beneficial for proteostasis maintenance and/or restoration. Here, we have searched for natural products with anti-aggregating properties from the Greek flora, using various C. elegans AD models for screening. We have identified a mountain tea extract with anti-aggregation properties derived from the Greek endemic Sideritis clandestina subsp. Peloponnesiaca (SCP). We have further fractionated the extract to identify the specific bioactive compounds that are responsible for these properties. We show that the identified compounds may decelerate: (1) the progression of the AD phenotype in CL4176 nematode strain, a strain expressing the human Aβ1-42 in its body wall muscle cells that undergoes paralysis upon temperature upshift due to Aβ aggregation, as well as, (2) the accumulation of Aβ aggregates in CL2331 nematode strain, a strain expressing the human Aβ3-42 peptide fused to green fluorescent protein (GFP) in its body wall muscle cells where Aβ aggregates can be visualized in vivo. Our study uncovers the need to identify bioactive compounds (that ideally are part of our diet) with anti-aggregation properties.

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## MACROMOLECULAR CROWDING AND MICRO-DOMAINS AS MODULATORS OF PROTEIN OXIDATION

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Biological systems are characterized by being highly-packed and heterogeneous environments. Such crowded milieus have been shown to modulate different aspects of protein structure and function including protein folding, protein diffusion, protein aggregation and protein-protein interactions. Therefore, we hypothesized that these highly crowded and heterogeneous systems, which contain micro-domains, may also modulate protein oxidation and modification reactions both inside and outside cells.

We have recently reported the occurrence of short-chain reactions that lead to propagation of oxidative damage in concentrated solutions of intrinsically disordered proteins (casein proteins) and in solutions containing high concentrations of inert crowding agents such as dextran. These short chain reactions involve the formation and propagation of tryptophan-derived species (i.e. tryptophanyl radicals or tryptophan-derived peroxyl radicals). In addition, enhanced damage to other susceptible residues such as methionine and tyrosine residues has also been detected. The latter is supported by kinetic studies that demonstrated that 60 mg mL-1 dextran enhanced the rate of oxidation of free Trp, and peptide Trp, elicited by AAPH-derived peroxyl radicals. For free Trp, the rates of oxidation were $15.0 \pm 2.1$ and $30.5 \pm 3.4$ μM min−1 without and with dextran, respectively. LC-MS studies also confirmed enhanced amino acid loss in casein proteins, and altered formation of protein cross-links as observed by SDS-PAGE analysis. Moreover, recent studies on the glycation of human serum albumin and human transferrin induced by methylglyoxal and glyoxal under crowded conditions has shown that crowding modulates the formation of protein carbonyls and the formation of protein oligomers. Overall, these data indicate that molecular crowding, as commonly encountered in biological systems affect the rates, and extents of oxidation, and particularly of Trp residues, illustrating the importance of appropriate choice of in vitro systems to investigate oxidative processes.

### SFRR-E Symposium 3 – Redox mechanisms in neurodegenerative disorders - From genes to proteins quality and propagation

## EFFECT OF APOE ε4 ALLELE AND REDOX SIGNATURE IN CIRCULATING EXTRACELLULAR VESICLES IN COGNITIVELY IMPAIRED PATIENTS CONVERTED TO ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is an age-related brain disorder and the leading cause of dementia. Oxidative stress is a unifying paradigm in the pathophysiology of AD and the presence of the apolipoprotein E4 variant (APOE ε4) is assumed to stimulate oxidative damage and enhance AD risk.

Exosomes or extracellular vesicles (EVs) (50-150 nm) are released by all cell types in the body. We have determined the impact of APOE ε4 on the level of apolipoproteins with antioxidant activities (apoE, apoJ, and apoD) along with oxidative markers in circulating extracellular vesicles (cEVs) and plasma from cognitively impaired-not demented (CIND) individuals converted to AD (CIND-AD).

Methods: EVs were isolated using the Total Exosome Isolation reagent and characterized according to the ISEV guidelines. Apolipoproteins E, J, and D and antioxidant response markers were determined in cEVs and plasma using immunoblotting, electrochemical examination, and spectrofluorimetry.

Results: We observed a significant decrease in the total antioxidant capacity (TAC) in the CIND-AD group. Levels of apoD in plasma and cEVs were higher in CIND-AD participants. Interestingly, protein carbonyls content and apoJ/D ratio were statistically different in cEVs but not in plasma from CIND-AD. Our data also indicate that TAC, cEVs protein carbonyls, cEVs apoJ/D levels were correlated with the neurocognitive Mini-Mental State Exam (MMSE) scores and are APOE ε4-dependant.

Discussion: Our results demonstrate that cEVs redox signature is more relevant than plasma for reflecting specific brain and systemic changes in early AD onset and particularly in APOE ε4 carriers.

Conclusion: Our findings support the pathological redox linkage between APOE ε4 and AD onset and suggest the use of cEVs oxidative signature in early AD diagnosis.

## TRISOMY 21 AND ABERRANT REDOX HOMEOSTASIS: A SYNERGISTIC PATH TO ALZHEIMER DISEASE

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Down syndrome (DS) is the most common genetic form of intellectual disability that results from the triplication of the entire portion or part of chromosome 21 (Trisomy21). DS represents a unique population for studying changes of brain aging across the lifespan; individuals with DS are the largest population under 60 years of age characterized by the early appearance of AD neuropathological features and are currently classified as early onset AD (EOAD).

The complexity of DS neurodegeneration involves multiple molecular mechanisms, similar to what observed in AD brain, including the deposition of beta-amyloid (Aβ) into senile plaques and tau hyperphosphorylation in neurofibrillary tangles. Intriguingly, several trisomic genes in addition to being primarily linked to Aβ pathology, are responsible for increased oxidative stress (OS) conditions, including SOD1, BACH-1, CBS among others. Indeed, our studies support the hypothesis that OS contributes to neurodevelopmental defects, neuronal dysfunction as well as the accelerated aging phenotype of DS population. We have demonstrated that oxidative damage to proteins occurs in young DS individuals, before the onset of AD-like neurodegeneration, and is associated to dysfunction of several cellular processes such as energy production, protein quality control, stress response, cytoskeleton network and synaptic function. Further, the OS phenotype is closely associated with mitochondrial defects, that likely sustains a vicious cycle further exacerbating ROS production and mitochondrial dysfunction with aging. Redox proteomics studies contributed to highlight that the impairment of energy metabolism is a key pathological feature of DS brain, showing aberrant activity of several enzymes involved in glycolysis, Krebs cycle and oxidative phosphorylation.

Collected results support the idea that mitochondrial defects, increased OS levels and impaired glucose metabolism lead to reduced ATP production, thus catalysing a synergistic path to accelerated aging and dementia.

## DECIPHERING THE NOXIOUS RELATIONSHIP BETWEEN OXIDATIVE STRESS AND THE UNFOLDED PROTEIN RESPONSE IN ALZHEIMER-LIKE NEUROPATHOLOGY

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Increasing evidence demonstrated that increased oxidative stress (OS), associated with mitochondrial dysfunction and antioxidant responses failure, is an early signature of alzheimer-like pathology, promoting protein oxidation and the