EUROPEAN SCHOOL OF MOLECULAR MEDICINE

SEDE DI NAPOLI

UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II

Ph.D. in Molecular Medicine – Ciclo VIII/XXVI

Human Genetics

Calorie Restriction and Anorexia Nervosa: Molecular markers and pathogenetic mechanisms

Supervisor:
Prof. Francesco Salvatore

Internal co-supervisor:
Prof. Giuseppe Castaldo

External Supervisor:
Prof. Luigi Fontana

Coordinator:
Prof. Andrea Ballabio

Ph.D. student:
Dr. Daniela Omodei

Academic Year: 2014-2015

TABLE OF CONTENTS

List of Abbreviations

CR: calorie restriction

ApoE: apolipoprotein E

WD: western diet

BMI: body mass index

IGF-1: insulin-like growth factor 1

IGFBP-3: insulin-like growth factor-binding protein 3

mTOR: mammalian target of rapamycin

AN: anorexia nervosa

DSM-V: diagnostic and statistical manual of mental disorders, fifth edition

NK: natural killer

PBMCs: peripheral blood mononuclear cells

sICAM1: soluble intracellular adhesion molecule 1

MCP-1: monocyte chemoattractant protein 1

MPO: myeloperoxidase

sTNFR: soluble tumour necrosis factor receptor

SOD2: superoxide dismutase 2

GPX1: glutathione peroxidase 1

IDH2: isocitrate dehydrogenase 2

GSTK1: glutathione S-transferase kappa 1

GSTT1: glutathione S-transferase theta 1

AS: autologous serum

HS: heterologous serum

ECAR: extracellular acidification rate

OCR: oxygen consumption rate

Figure Index

Abstract

Calorie restriction (CR) without malnutrition is the most robust intervention to slow aging and extend healthy lifespan in experimental model organisms. Several metabolic and molecular adaptations have been hypothesized to play a role in mediating the anti- aging effects of CR, including enhanced stress resistance, reduced oxidative stress and several neuroendocrine modifications. However, little is known about the independent effect of circulating factors in modulating key molecular pathways. In this study, we used sera collected from individuals practicing long- term CR and from age- and sex- matched individuals on a typical US diet to culture human primary fibroblasts and assess the effects on gene expression and stress resistance. We show that treatment of cultured cells with CR sera caused increased expression of stress- response genes and enhanced tolerance to oxidants. These cellular and molecular adaptations mirror some of the key effects of CR in animals, and further suggest that circulating factors contribute to the CR- mediated protection against oxidative stress and stress- response in humans as well.

Anorexia nervosa (AN) is an excessive form of calorie restriction associated with pathological weight loss and alterations of the immune system. However, AN patients seem to be protected from common viral infections. In this study, we investigated the metabolic and molecular adaptations induced by sustained extreme CR in the peripheral blood mononuclear cells (PBMCs) of patients with restrictive alimentary AN. Compared with controls, AN patients had significantly fewer leucocytes, lymphocytes and NK cells, lower

serum concentrations of leptin, IGF-1 and sTNFR1, and higher levels of adiponectin, sCD40L and sICAM-1 ($p < 0.05$). IL-1β, TNFα, and IL-6 produced by PBMC cultured with autologous serum for 48 h were significantly lower in AN patients than in controls ($p < 0.01$). Moreover, glycolysis and mitochondrial respiration were lower, and the antioxidant transcriptional profile was higher in the PBMCs of AN patients. Fibroblasts cultured in serum from AN patients showed a 24% increase in resistance to H_2O_2 damage.

In conclusion, extreme CR in AN patients is associated with a reduction in several immune cell populations, but with higher antioxidant potential, stress resistance and an anti-inflammatory status.

1. INTRODUCTION

1.1 Calorie Restriction

Calorie Restriction (CR) with adequate nutrition improves multiple parameters of health and extends lifespan. CR is defined as a dietary intervention where calorie intake is reduced below the usual ad libitum intake while adequate intake of proteins and micronutrients are maintained at sufficient levels to avoid malnutrition. The effects of CR on disease risk and life expectancy is widely studied in model organisms and humans.

1.1.1 Calorie Restriction in model organisms

The first evidence that CR can extend the mean and maximum lifespan of rats was published in 1935 by McCay et al. (1935). Subsequent data have shown that CR slows aging and increases maximum lifespan in different species, including yeast, fruit flies, worms, spiders, fish, mice and dogs (Weindruch and Walford 1988, Masoro 2005). Invertebrate model organisms (i.e., yeast, Caenorhabditis elegans, and Drosophila) are well-suited for the analysis of the molecular anti-aging mechanisms of CR due to their relative simplicity and shorter time needed to complete longevity studies, as discussed in more detail elsewhere (Fontana et al 2010 a, Kenyon 2005). However, the metabolic, anatomical, physiological and lifespan differences between these invertebrate model organisms and the mammalian systems are enormous. Rodents provide an extremely valuable and flexible animal model in which to determine the ability of CR to extend maximum lifespan and healthspan in a mammalian system. In rodents, a 30–60% reduction in calorie intake below usual ad libitum intake initiated early in life caused a

proportionate 30–60% increase in maximum life span (Weindruch and Walford 1988, Masoro 2005). CR started in adulthood (age 12 months) extended maximum life span by only 10–20% (Weindruch and Walford 1982). To date, mice and rats are the only mammals in which CR has clearly been shown to increase both average and maximal lifespan, and to decelerate many age-dependent physiological and structural changes in multiple organs and tissues. In addition, data from studies with laboratory rodents found that CR without malnutrition increases healthspan by preventing or delaying the occurrence of a wide range of chronic diseases (Figure 1).

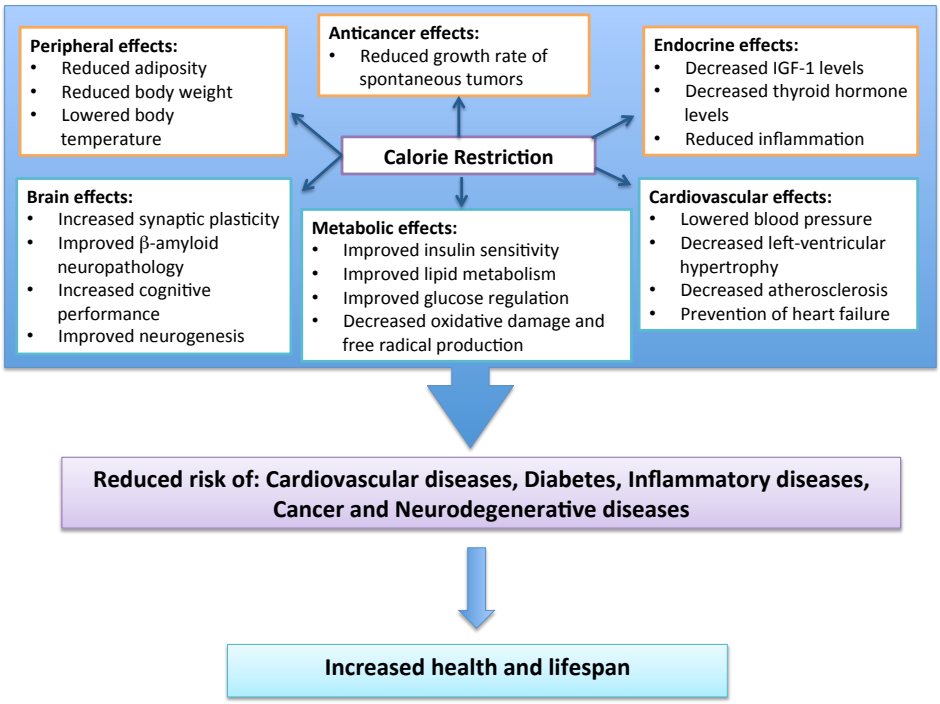

Figure 1: *Some of the physiological changes associated with calorie restriction in mammals.*

In rodents, cancer is the leading cause of death, accounting for 70–80% of all deaths, and CR has been shown to inhibit spontaneous, chemically-

induced and radiation-induced tumors in several murine models of cancer (Longo and Fontana 2010). CR without malnutrition has also been shown to prevent or delay the occurrence of chronic nephropathies and cardiomyopathies, the second leading causes of death in rodents (Weindruch and Walford 1988, Shimokawa et al 1993). Moreover, in ApoE knockout mice CR reduced the size and progression of atherosclerotic lesions when compared to ApoE-/- mice fed ad libitum, which developed more advanced and fibrotic lesions (Guo et al 2002). Diabetes, autoimmune and respiratory diseases are also prevented by CR (Weindruch and Walford 1988, Masoro 2005). Finally, CR in mice decreases neurodegeneration, β-amyloid deposition in the brain and enhances neurogenesis in animal models of Alzheimer disease, Parkinson disease, Huntington disease, and stroke (Cohen et al 2009, Mattson 2005). However, under certain conditions chronic CR may also potentially impair some key functions, such as immunity and wound healing. For example, the healing of skin wounds is reduced in long-term CR mice, but is greatly accelerated by a short period of ad libitum feeding before the wound is inflicted in CR animals (Reed et al 1996). In addition, accumulating data suggest that in mice housed in pathogen free facilities severe CR increases susceptibility to infections by bacteria, virus and worms, even though CR has been shown to delay the age-dependent decline in certain immune functions (Kristan 2008). Considering the breadth of organisms that respond positively to CR, should it be expected that non-human primates would likewise show similar results? There are two active randomized, non-human primates studies testing the benefits of long-term CR on disease prevention and longevity in rhesus monkeys, one at the

University of Wisconsin at Madison and another at the National Institute on Aging (NIA) (Ingram et al 1990, Kemnitz et al 1993). Both trials have shown that long-term, moderate (~30%) CR can be safely initiated and maintained in a primate species. The recent results reported by the Wisconsin group are the first to show a significant CR-induced benefit in reducing age-related mortality and disease in rhesus monkeys (Colman et al 2009). However, when also deaths due to acute conditions (e.g., complications of anesthesia, gastric bloat, endometriosis, and injury) were included, overall mortality was not significantly different between the CR and control monkeys, even if the trend was still in the anticipated direction ($P = 0.16$). Although the demonstrated health and lifespan benefits are significant findings, a number of previous studies have suggested the plausibility of this outcome. Similar to rodents, CR in rhesus monkeys results in lower total and abdominal adiposity, improved insulin sensitivity and lipid/lipoprotein profile, decreased body temperature, decreased serum triiodothyronine concentration and reduced inflammation (Kemnitz 1994, Lane 1996, Roth 2002, Kim 1997). However, as rhesus monkeys have a maximum life span of 40 years, it may be another 10 years before maximal life span data become available on these primates.

1.1.2 Calorie Restriction in humans

It is difficult to determine whether CR has beneficial effects on intrinsic aging and maximal lifespan in humans, because there are no validated biomarkers of aging and because it is impractical to conduct randomized diet-controlled, long-term survival studies in normal-weight humans. Data from epidemiological studies suggest that CR has beneficial effects on

human longevity. These studies include natural experiments, such as a study on the inhabitants of Okinawa (Japan) who were known to consume fewer calories than residents of the main Japanese islands (Willcox et al 2007). In this cohort of Okinawans (aged 65+) mortality from coronary heart disease and cancer was markedly lower than in the average mainland Japanese and US population (Kagawa 1978). Another category of studies in humans includes more controlled demonstrations of the effects of CR in normal-weight individuals, such as occurred with Biosphere 2 which took place in a closed ecosystem in Arizona from 1991 to 1993, involved four men and four women who experienced a forced decrease in calorie intake for 18 months, because of an unanticipated decrease in food availability (Walford et al 1992). This reduced energy intake resulted in a reduction of many anthropometric and physiological parameters, including reductions in body weight, blood pressure, fasting blood glucose, insulin, cholesterol, triiodothyronine and white blood cells (Walford et al 1992).

Another series of metabolic and physiological studies have been conducted in members of the Calorie Restriction Society, which is a group that practices self-imposed CR in the belief that CR will extend their healthspan and lifespan. The CR group consists of lean volunteers, who had been eating about 1800 kcal/day for an average of 6.5 years, which is ~30% less calories than age-matched and sex-matched volunteers consuming a typical Western diet (WD) (Holloszy and Fontana 2007). The CR society members eat a diet rich in nutrient-dense foods, including a wide variety of vegetables, fruits, whole grains, nuts, egg whites, fish, low-fat dairy products and lean meat, which supplies more than 100% of the recommended daily intake (RDI) for

all essential nutrients. The decrease in energy intake resulted in a decrease in BMI from 23.7 kg/m^2 at the beginning of CR to a currently steady BMI of 19.6 kg/m^2 (Holloszy and Fontana 2007); total body fat averaged 6.7% in the CR men and 22.4% in the comparison group men. The metabolic and physiological data from members of the calorie restriction society show that CR provides powerful protective effects against overweight/obesity, type 2 diabetes, inflammation, and left ventricular diastolic dysfunction that are similar to those that occur in CR rodents and monkeys (Meyer et al 2006, Fontana et al 2004). Serum total cholesterol, low density lipoprotein cholesterol, triglycerides, fasting glucose, fasting insulin were all significantly lower, whereas HDL-C was higher, in the CR group than in the US diet control group (Fontana et al 2004). In particular, the CR society members appear to have much lower levels of blood pressure (both systolic and diastolic blood pressure) and inflammatory markers (i.e., C-reactive protein, tumor necrosis factor-α, and interleukin-6) than healthy, age- and sex-matched controls eating typical Western diets (Meyer et al 2006, Fontana et al 2004, Fontana et al 2010 b).

Although research on CR in humans is still at an early stage, available information suggests that CR induces a number of the same adaptive response that occurs in laboratory animals. For example, CR results in some of the same hormonal adaptations related to longevity in CR rodents, including lower circulating concentrations of triiodothyronine, testosterone, and estradiol, and increased adiponectin and steroid hormone binding protein concentrations (Fontana et al 2010 b, Fontana et al 2006, Cangemi et al 2010). However, key differences in the metabolic effects of CR exist

between mice and humans. In rodents, CR without protein restriction induces a 20–40% reduction in the level of insulin-like growth factor-1 (IGF-1), an important growth factor that mediates proliferation and inhibits apoptosis (Sonntag 1999). In contrast, in humans, severe CR does not reduce serum IGF-1 and IGF-1/IGFBP-3 concentrations, unless protein intake is also reduced (Fontana et al 2008).

1.1.3 Metabolic and molecular mechanisms of Calorie restriction

Since 1935, many mechanisms have been proposed as the biological basis of the life-prolonging and anti-aging actions of CR; none is strongly supported by available evidence, but it is entirely possible that the actions of CR involve a combination of metabolic, physiological and cellular adaptations to CR itself (McCay et al 1935, Brown-Borg 2007). It is well established that nutrient-sensing pathways are key modulators of the aging process; different nutrients can activate different pathways directly or indirectly (Fontana et al 2010 a). For example in mice, CR down-regulates the insulin/IGF-1/mTOR pathways, which, in turn, activates other anti-aging pathways in various mammalian cells (Salmon et al 2005, Kennedy et al 2003) (Figure 2). Mutations that cause a down-regulation of the insulin/Igf-1/mTOR signaling pathways can substantially increase healthspan and lifespan in mice (Fontana et al 2010 a, Bartke 2005). For example, decreased IGF-1 signaling is involved in the delayed aging phenotype of IGF-1 receptor-deficient mice, klotho transgenic mice, and pregnancy-associated plasma protein A (PAPP-A) knock-out mice (Holzenberger et al 2003, Kurosu et al 1998, Conover and Bale 2007). In contrast, mice overexpressing the GH receptor have very high concentrations of IGF-1, larger body size, shorter lifespan, and an

increased incidence of cancer, kidney and neurodegenerative disease (Bartke et al 2002). In addition to alteration in IGF-1 signaling, alterations of the insulin and mTOR pathways appear to contribute to the effect of CR on longevity.

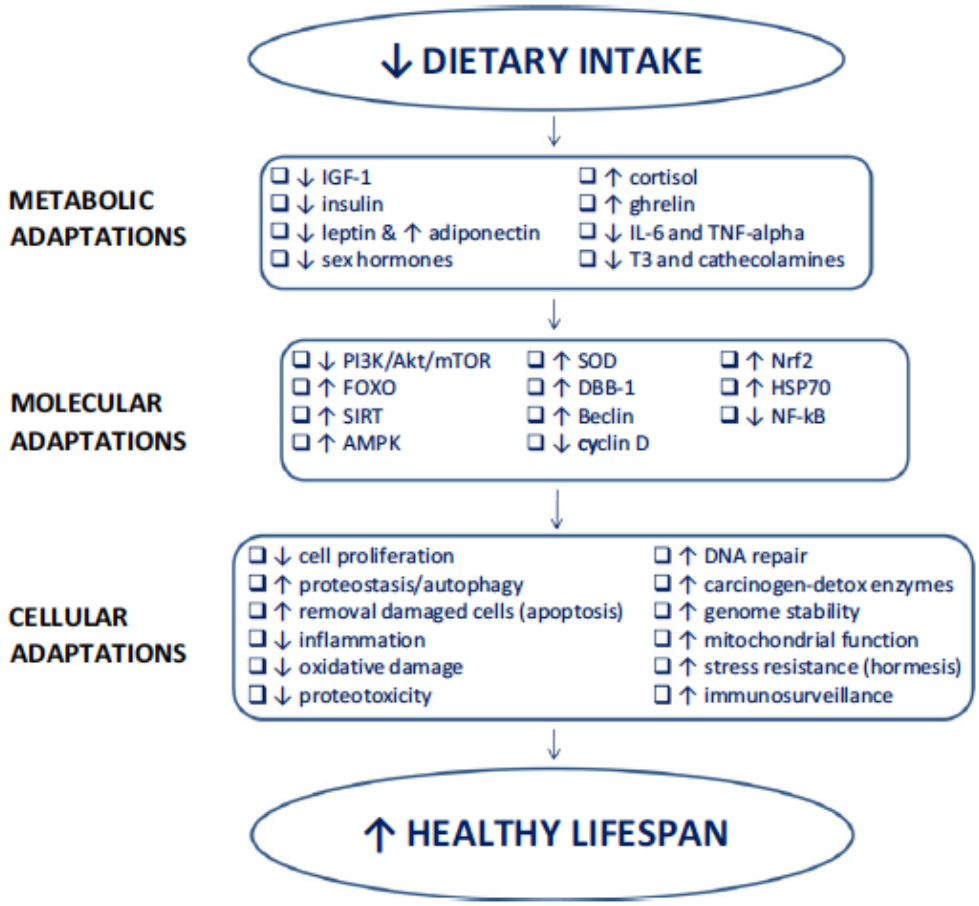

Figure 2: *Calorie restriction (CR)-induced metabolic, molecular, and cellular adaptations. A chronic negative energy balance induced by dietary restriction triggers multiple metabolic adaptations including a reduction in circulating levels of growth factors (i.e., IGF-1), anabolic hormones (e.g., insulin, testosterone, estradiol, leptin), inflammatory cytokines (e.g., IL-6 and TNF-alpha), and key hormones that regulate thermogenesis and cellular metabolism (e.g., triiodothyronine (T3), cathecolamines), and an increase in hormones that suppress inflammation (e.g., cortisol, ghrelin, and*

adiponectin). Concomitantly, several key molecular adaptations take place, including a down-regulation of the insulin/IGF pathway (i.e., PI3K/Akt/mTOR), and an upregulation of two energy-sensing pathways (i.e., SIRT and AMPK which activates FOXO. The activation of FOXO in turn modifies several 'longevity genes,' including up-regulation of DNA repair genes (e.g., DDB1), autophagy genes (e.g., beclin), and a down-regulation of genes that control cell proliferation (e.g., cyclin D). DR induces also a down-regulation of inflammatory pathways (e.g., NFkB), and an up-regulation of genes that enhance protection against molecular damage (e.g., Nrf2, HSP70). These DR-induced metabolic and molecular modifications are responsible for several important cellular adaptations, that antagonize the accumulation of macromolecular damage, abnormal cell proliferation, and cellular senescence. (Fontana et al 2014).

Other important CR-mediated neuroendocrine adaptations, that have been hypothesized to play an important role in mediating the anti-aging effects of CR, are: (1) reduced levels of hormones that regulate thermogenesis and cellular metabolism (e.g., thyroid hormones, cathecolamines), (2) reduced levels of anabolic hormones (e.g., testosterone, estradiol, insulin, leptin), and (3) increased levels of hormones that suppress inflammation (e.g., glucocorticoids, adiponectin, ghrelin) (Fontana and Klein 2007) (Figure 2).

Accretion of oxidative damage with time has been hypothesized to play a central role in the biology of aging and age-associated diseases (Harman theory of aging) (Sohal et al 2002). Oxidative damage to macromolecules

(i.e., DNA/RNA, proteins and lipids) in cells and tissues exponentially increases with aging. Long-term CR reduces the age-associated accumulation of oxidative damage to proteins, lipids and DNA (Sohal and Weindruch 1996). This attenuation of the accumulation of oxidative damage can be due to either a decreased rate of generation of reactive oxygen molecules, or to increased efficiency of protective processes, or to an increase in repair activity, or to a combination of these processes (Figure 2). However, most of the evidence in support of the Harman theory of aging is just associative, and accumulating data do not support a key and independent role of oxidative stress in modulating aging in mammals (Muller et al 2007).

1.2 Anorexia Nervosa

Anorexia Nervosa (AN) is a complex multi-factorial disease in which genetic, biological and psychological factors, interacting each other, contribute significantly to susceptibility (Kaye et al 2009). AN primarily affects adolescent girls and young women; it is characterized by distorted body image and excessive dieting that leads to severe weight loss with a pathological fear of becoming fat.

1.2.1 Definition of Anorexia Nervosa according to the DSM-V

The American Psychiatric Association published the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) 2013, classifying anorexia nervosa as:

- Persistent restriction of energy intake leading to significantly low body weight (in context of what is minimally expected for age, sex, developmental trajectory, and physical health).
- Intense fear of gaining weight or becoming fat, even though underweight.
- Disturbance in the perception of body weight or shape.

Furthermore DSM-V allows us to specify two different types of anorexia nervosa:

- Restricting Type: in which individuals limit food intake;
- Binge-Eating / Purging Type: in which a person engages in binge eating followed by subsequent purging behavior (self-induced vomiting / laxative abuse).

1.2.2 Prevalence and Incidence of Anorexia Nervosa

The average prevalence rate for strictly defined AN young women is about 0.3%, whereas the incidence of anorexia nervosa is 8 cases per 100,000 population per year (Hoek et al 2003). Studies based on long study periods have revealed an upward trend in the incidence of anorexia nervosa since the 1950s. The increase is most substantial among 15–24-year old females with an estimated rate of increase of 1.03 per 100,000 persons from 1935 to 1989 (Hoek et al 2003).

1.2.3 Anorexia Nervosa and Immune System

Extreme CR and low body fat stores in AN patients are associated with alterations of the immune system (Marcos et al 2003). In particular, AN patients frequently have leukopenia and relative lymphocytosis, but interestingly they seem to be protected against some infectious disease (e.g. common viral infections and other nosocomial infective disease) (Nova and Marcos 2006).

Lymphocytes and natural killer (NK) cells play a central role in regulating the immune response against pathogens. Several adipokines (i.e. leptin and adiponectin) produced by the adipose tissue and some nutrients (e.g. vitamins and trace elements) play a key role in preserving the homeostasis of T-cells and NK cells (Muñoz and Costa 2013, Gostner et al 2013, Kowalska et al 2011, Gustafsson et al 2013, Caspar-Bauguil et al 2006, La Cava and Matarese 2004, Galgani et al 2010). However, little is known on the metabolic, molecular and functional adaptations induced by extreme and sustained CR in young AN patients on lymphocytes and NK cells. To the best of our knowledge, the only few studies conducted so far on lymphocyte populations in AN subjects were inconclusive, probably due to heterogeneity of the study subjects (Saito et al 2007, Pászthy et al 2007).

1.3 First Aim of the study

Calorie restriction without malnutrition is the most robust intervention to slow aging and extend healthy lifespan in experimental model organisms.

Several metabolic and molecular adaptations have been hypothesized to play a role in mediating the anti- aging effects of CR, including enhanced stress resistance, reduced oxidative stress and several neuroendocrine modifications. However, little is known about the independent effect of circulating factors in modulating key molecular pathways. The first aim of this study was to use sera collected from individuals practicing long- term CR and from age- and sex- matched individuals on a typical US diet to culture human primary fibroblasts and assess the effects on gene expression and stress resistance. These cellular and molecular adaptations could mirror some of the key effects of CR in animals.

1.4 Second Aim of the study

Anorexia nervosa is an excessive form of calorie restriction associated with pathological weight loss and alterations of the immune system. However, AN patients seem to be protected from common viral infections. The second aim of the present study was to investigate the metabolic, molecular and immunological adaptations induced by sustained extreme CR in the peripheral blood mononuclear cells (PBMCs) of patients with restrictive alimentary AN.

2. MATERIALS AND METHODS

2.1 Human subjects

Fifteen women who met DSM-V criteria for AN, twelwe individuals belonging to the Calorie Restriction society who had been practicing severe CR with adequate nutrition for an average of 7 years (range 3-20 years) and 33 normal weight controls were enrolled in this study (American Psychiatric Association 2013). Control subjects were matched with the CR and AN groups in terms of age and sex. The characteristics of the study subjects are shown in Tables 1 and 2. No patient had been affected by infectious diseases or required medical treatment or hospitalization during the previous 5 years. All had undergone standard vaccination programs during childhood. None of their parents had experienced a major eating disorder. The research was performed according to the Helsinki II declaration and all enrolled subjects gave informed consent to the study. Height was measured without shoes to the nearest 0.1 cm. Body weight was obtained on a balance scale in the morning. Body mass index (BMI) was calculated by dividing body weight by the square of height (kg/m^2). A venous blood sample was obtained from each participant the morning after a 12-hour fast and analyzed by routine assays for complete blood count as well as a comprehensive metabolic panel to assess current metabolic, kidney and liver function and electrolyte balance. Additional blood samples were stored at −80 °C, and later assayed in batch to measure levels of inflammatory and anti-inflammatory cytokines, adipokines and hormones. Specifically, soluble CD40L (sCD40L), soluble

intracellular adhesion molecule (sICAM-1), monocyte chemoattractant protein-1 (MCP-1), myeloperoxidase (MPO), resistin and soluble tumour necrosis factor receptor (sTNFR) were analyzed using the bead-based analyte detection system Human Obesity 9plex kit (Bender MedSystem Inc., Burlingame, CA) in duplicate serum samples. Leptin, adiponectin, IGF-1 and IGFBP-3 were measured with a highly sensitive ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol. Total catecholamines were determined by HPLC (Hewlett Packard 1100, BioRad, Herts, UK) in 24h urine.

2.2 Human fibroblast cell culture

The BJ human fibroblast cell line was obtained from ATCC and used in these experiments. BJ cells were cultured in 75 cm^2 culture flasks containing basic culture medium: 86% of E-MEM medium supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 1% Sodium Pyruvate, 1% non-essential amino acids, 1% GlutaMAX and 1% antibiotics (GIBCO, Carlsbad, CA, USA) until the time of treatment. Cells were cultured in a humidified incubator at 37°C and 5% CO_2. For the experiments, FBS was replaced with human serum from CR, AN and control participants. Both FBS and human serum were heat inactivated for 30 minutes at 55°C.

2.3 Microarray analysis

BJ cells were seeded at a concentration of 4.000 cells/cm^2 in 25 T-25 flasks; when they reached 60% of confluence, the cells were starved for 24h without FBS. After starvation, 10% of human serum from 12 CR and 13 control individuals was added to the cultures for 48h. Whole cells were detached

from the flask with trypsin/EDTA (Sigma-Aldrich, St. Louis, MO, USA) and Total RNA was extracted using the mirVANA (Ambion, Inc, Austin, TX, USA) according to manufacturer's protocol. Integrity of total RNA was evaluated using capillary electrophoresis (Bioanalyzer 2100, Agilent technologies, Santa Clara, CA, USA) and quantified using a Nanodrop 1000 (Nanodrop, Wilmington, DE). Aliquots of RNA (400 ng) samples were amplified according to the specifications of the Illumina® TotalPrep™ RNA Amplification Kit (Ambion, Austin, TX, USA) to produce a pool of biotin-labeled RNA corresponding to the polyadenylated (mRNA) fraction. The cRNA samples were applied to the arrays of Illumina whole-genome HumanHT-12 v 4.0 (Illumina, San Diego, CA, USA) and hybridized according to the manufacturer's specification. Each array on the BeadChip targets over 25.000 annotated genes using 3-micron beads bearing covalently attached 50-base oligonucleotide probes. Each probe interrogates a single gene, and each bead type is represented with an average 15-fold redundancy on every array. The BeadChips were scanned with the Illumina's Beadarray system scanner (Illumina, San Diego, CA, USA). The hybridization images signal intensity was extracted and background subtracted using Illumina Inc. BeadStudio software version 3.3.7. The produced data were checked for the Illumina internal quality control and loaded into Bioconductor software (Gentleman et al 2004, Ritchie et al 2006) for statistical analysis using lumi package (Du et al 2008) (see below). The data discussed in this thesis have been deposited in NCBI's Gene Expression Omnibus (Edgar et al 2002) and are accessible through GEO Series accession number GSE41790.

(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=nnonbsicqouiilw&acc=GSE41790).

2.4 Real Time PCR

Real-time PCR was used to verify microarray data and to evaluate the antioxidant status in PBMCs from AN patients and BJ cells cultured with sera from AN and control subjects. Total RNA was DNase I treated and reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA, USA). The cDNA was then used to evaluate the relative expression of selected genes from the microarray results including SOD2, GPX1, IDH2, GSTK1 and GSTT1 using specific TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA), which are pre-designed and pre-optimized gene-specific probe sets. To evaluate the antioxidant status in PBMCs from AN patients and BJ cells cultured with sera from AN and control subjects 2.9 μg of reverse transcribed RNA were added to 540 μL of TaqMan Fast Universal PCR Master Mix (Applied Biosystems), and dispensed 10 μL per well into a TaqMan Human Antioxidant Mechanisms 96-well Fast Plate (Applied Biosystems) that evaluates 92 antioxidant-associated genes and 4 endogenous control genes. DNA was amplified using the Applied Biosystems 7900 Fast Real-Time PCR system according to the manufacturer's protocol. The relative amount was calculated from the threshold cycles with the instrument's software (SDS 2.0, Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. We used the comparative cycle threshold (C_t) method ($2^{-\Delta\Delta CT}$) to calculate relative gene expression under experimental

and control conditions normalized to GAPDH. The results are expressed either as the abundance of the gene of interest relative to the house keeping gene ($2^{-\Delta CT}$), or as fold change over control values ($2^{-\Delta\Delta CT}$).

2.5 Differential stress resistance assays in response to hydrogen peroxide

To determine the differential stress resistance to hydrogen peroxide of serum from CR, AN and control individuals, BJ cells were seeded in 96-well plates at a concentration of 3000 cells/well in 86% D-MEM low glucose without phenol red supplemented with 1% sodium pyruvate, 1% non-essential amino acids, 1% GlutaMAX and 1% antibiotics (GIBCO, Carlsbad, CA, USA). After cells had seeded, media were removed and replaced with media containing 10% of human serum from 11 CR, 10 AN or from 10 control individuals for 48h. Each assay was performed in triplicate. After incubation, freshly prepared hydrogen peroxide was added to the plates at concentrations of 200, 400, 600, 800 and 1000 μM, and cells were challenged for 24h. Cell viability was determined by the addition of 20 μl of the ready-to-use WST1 reagent (Roche diagnostics, Mannheim, Germany) to each well; the cells were incubated for 3h at 37°C and 5% CO_2 in a humidified incubator. The absorbance of the formazan dye formed was measured at a wavelength of 450 nm using a microplate spectrometer. The measured absorbance directly correlated to the number of viable cells.

2.6 Immunophenotyping

Immunophenotyping was performed on a whole blood sample from 10 AN and 10 controls with an EPICS XL flow cytometer (Beckman Coulter, Milan, Italy) using the Beckman Coulter XL System II software program. Cell

populations were identified using triple combinations of human monoclonal antibodies, namely, fluorescein isothiocyanate [FITC]- and phycoerythrin [PE]-anti-CD3, PE- and PC-5-anti-CD4, PC5-anti-CD8, PE-anti-CD16, PC5-anti-CD19, PE-anti-CD25, FITC-anti-CD45, and PE-anti-CD56, all from Coulter Immunotech (Marseille, France).

2.7 Cell cultures, proliferation assays and cytokine measurement

Human peripheral blood mononuclear cells (PBMCs) were isolated from AN patients and controls by stratifying 15 mL of whole blood on 5 mL of Ficoll-Paque PREMIUM (GE Healthcare) and centrifuging the solution at 1.2×g for 20 min. PBMCs (2×10^5 per well) were cultured, in triplicate, in 96-well round-bottomed plates, in medium supplemented with 5% (vol/vol) autologous (AS) subject serum or 5% (vol/vol) heterologous (HS) commercial human serum (Sigma-Aldrich, St. Louis, MO, USA) and were stimulated or not in parallel with 0.1 μg/mL anti-CD3 mAb (OKT3) (Orthoclone, Janssen-Cilag, Cologno Monzese, Italy). Stimulated PBMCs were maintained in culture for 48h, and H3 thymidine (Amersham-Pharmacia Biotech, Cologno Monzese, Italy) was added (0.5 μCi per well) during the last 12h. Cells were harvested on fiberglass filters using a 96-well cell harvester (Tomtec Inc., Hamden, CT, USA) and were counted in a Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD). Supernatants were collected from 48h TCR-stimulated T cells from AN patients and control subjects, and stored at -80°C before analysis. The human Th1/Th2/Th9/Th17/Th22 13plex kit FlowCytomix (Bender Medsystems GmbH, Vienna, Austria) was used to detect human TNFα, IFNγ, IL-1β, IL-

2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17α, IL-22 by flow cytometry (Becton-Dickinson, San Diego, CA) according to the manufacturer's instructions.

2.8 Bioenergetics and metabolism of T lymphocytes

The mitochondrial respiratory rate was measured in PBMCs from a random subset of AN (n=4) and control (n=3) subjects, in basal condition and upon TCR-mediated stimulation (anti-CD3). Real-time measurements of the extracellular acidification rate (ECAR) and of the oxygen consumption rate (OCR) were made using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA). Cells were cultured ($2x10^5$ cells/well in XF-96-well culture plate) in triplicate in 100 µl DMEM complete medium, leaving the temperature control wells empty, and incubated at 37°C for 12h. ECAR was measured in XF media in basal condition and in response to 10 mM glucose (basal glycolysis), 5 µM oligomycin (maximal glycolysis) and 100 mM 2-DG (glycolytic capacity). OCR was measured in XF media (non-buffered DMEM medium, containing 10 mM glucose, 2 mM L-glutamin, and 1 mM sodium pyruvate), under basal conditions (basal respiration) and in response to 5 µM oligomycin (ATP-linked respiration), 1.5 µM of carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) and 1 µM antimycin and rotenone (maximal respiration) (Sigma Aldrich).

2.9 Statistical analysis

Microarray data produced were quality checked with arrayQualityMetrics (Ritchie et al 2006) package under R version 2.15.1 Bioconductor software

(Gentleman et al 2004) and undetectable and low detection value probes were filtered out from further analysis accordingly to the lumi package standards (Du et al 2008). To identify differentially expressed genes, based on a moderate t-test, the Limma package (Wettenhall et al 2006) was used and genes were selected based on a p-value cut-off <0.05 (after FDR adjustment) and absolute fold change >1 following the Macroarrays Quality Control Program criteria (Shi et al 2006). IPA software was then used (www.ingenuity.com) to detect the enrichment of biofunctions and networks in the resulting list. Core analysis was performed using the following settings: Reference set: HumanHT-12 v 4.0; Relationship to include: Direct and Indirect; Includes Endogenous Chemicals; Filter Summary: Consider only molecules and/or relationships where (species = Uncategorized (e.g. chemicals) OR Human) AND (confidence = Experimentally Observed). Canonical pathways analysis has been performed using as scoring method BH Multiple Testing Correction p-values set to a threshold value of 0.05. Meta-analysis was performed using the CAMERA function implemented in the Bioconductor Limma package for a 'competitive gene set test' (Wu and Smyth 2012). The resulting top Molecular signatures have been further analyzed using a ‘focused’ gene set test ROAST (Wu et al 2010). All the list of genes organized as Signature and present in The Molecular Signature Database C2 collection (MSigDB) (www.broadinstitute.org/gsea/msigdb/) were updated to official symbols using the illumina annotation file HumanHT-12_V3_0_R2_11283641_A. Representation of differentially expressed genes in their Genomic localization and visual representation of their relationships with the different pathways were produced using CIRCOS

software (Krzywinski et al 2009). The Mann Whitney U test was used to compare group variables in all the other tests. Statistical significance was set at $P<0.05$. All data were analyzed by using the PASW package for Windows (v18; SPSS Inc Headquarters Chicago IL, USA) and Graphpad InStat3 version 4.0.

3. RESULTS

Table 1 and 2 provide demographic, anthropometric and biochemical characteristics of the enrolled subjects. Mean values for body weight and BMI were significantly different between the selected groups. Total body weight and BMI were significantly lower in the CR and AN group than in the Control group (Table 1 and 2).

Table 1: Characteristics of the calorie restricted (CR) and subjects eating Western Diets (WD)

	CR group (n=12)	WD group (n=13)	P value
Age (yrs)	57.8±8.4	55.7±8.7	ns
Sex (M/F)	10/2	11/2	
Height (m)	1.73±0.07	1.76±0.12	ns
Weight (Kg)	57.1±6.6	79.8±14.0	0.0002
BMI (kg/m^2)	19.1±1.2	25.7±2.3	P<0.0001
Total body fat (%)	9.8±4.8	25.9±5.6	P<0.0001

Values are means ± SD

Plasma glucose, insulin, leptin, IGF-1, prolactin, DHEA-s, and sTNFR1 concentrations in the AN group were significantly lower than the CTR group (Table 2). However, serum concentrations of adiponectin, sCD40L, sICAM-1, LDH, ALP and liver enzymes (i.e. ALT, GGT) were significantly higher in the AN group than in the CTR group (Table 2 and 3). Serum MPO and resistin levels were higher in the AN group than in CTR, but the difference was not significant (Table 2). Both the leptin/adiponectin (L/A) and the IGF-

1/IGFBP-3 ratio were significantly lower in the AN than in the control group (Table 2). Despite undernutrition, AN patients did not show selective malnutrition in terms of vitamins and minerals (Table 3).

Table 2: Main clinical and biochemical characteristics of the enrolled Anorectic (AN) and control (CTR) subjects

	AN (n=15)		CTR (n=20)		P value
	Mean	**SEM**	**Mean**	**SEM**	
Age (yrs)	19.70	0.97	22.57	0.83	ns
Height (m)	1.65	1.2	1.66	2.0	ns
Weight (Kg)	43.3	1.2	56.6	2.6	<0.001
BMI (kg/m^2)	15.95	0.40	20.40	0.70	<0.001
Glucose (mmol/L)	3.76	0.11	4.43	0.12	<0.001
Total cholesterol (mmol/L)	4.44	0.14	4.41	0.24	ns
HDL-c (mmol/L)	1.66	0.12	1.86	0.14	ns
Leptin (ng/mL)	1.00	0.34	15.08	1.88	<0.001
Adiponectin (µg/mL)	16.43	1.51	11.09	1.41	0.02
Leptin/adiponectin ratio	0.06	0.01	1.60	0.28	<0.001
Insulin (µU/mL)	1.03	0.04	9.05	1.57	<0.001
IGF-1 (ng/mL)	158.8	13.41	191.6	11.13	0.03
IGFBP-3 (µg/mL)	3.33	0.18	2.99	0.15	ns
IGF-1/IGFBP-3 ratio	47.94	3.32	64.90	3.69	0.004
DHEA-s (µg/dL)	87.20	9.17	218.50	34.97	<0.001
Prolactin (ng/mL)	5.95	0.71	19.84	3.29	<0.001
sTNFR1 (ng/mL)	1.14	0.06	1.45	0.12	0.04
sCD40L (ng/mL)	865.6	102.8	608.2	108.8	0.04

sICAM-1 (ng/mL)	430.5	75.50	302.9	20.52	0.04
MPO (ng/mL)	268.7	28.98	216.50	25.00	ns
MCP-1 (ng/mL)	377.0	39.67	365.3	35.32	ns
Resistin (µg/mL)	9.51	1.60	7.54	0.67	ns
Catecholamines (µg/24h)	24.56	3.80	76.80	9.04	<0.001

*DHEA-s: Dehydroepiandrosterone-sulphate; IGFBP-3: IGF-1 binding protein 3; MCP-1: monocyte chemotactic protein 1; MPO: myeloperoxidase; sCD40L: soluble CD40 ligand; sICAM-1: soluble intracellular adhesion molecule; sTNFR1: soluble tumor necrosis factor receptor 1. All biochemical parameters were measured in serum except catecholamines in 24h urine sample. *p value at Mann Whitney U test.*

Table 3: Main biochemical characteristics of the enrolled AN and control subjects

	AN (n=15)		**CTR (n=20)**		**P value**
	Mean	**SEM**	**Mean**	**SEM**	
Na^+ (mg/dL)	141.40	0.76	141.64	0.79	ns
K^+ (mg/dL)	4.32	0.14	4.47	0.07	ns
Ca^{++} (mg/dL)	9.72	0.08	9.63	0.08	ns
Phosphorus (mg/dL)	3.86	0.04	3.77	0.18	ns
Iron (mg/dL)	85.50	6.34	84.00	9.74	ns
Total Protein (g/dL)	7.45	0.11	7.34	0.14	ns
Albumin (g/dL)	5.02	0.09	4.84	0.07	ns
Triglycerides (mmol/L)	0.79	0.09	0.55	0.04	0.03
AST (U/L)	30.10	4.64	19.09	2.96	ns

ALT (U/L)	49.00	10.90	19.82	7.75	0.04
GGT (U/L)	25.20	6.00	11.67	0.89	0.05
ALP (U/L)	60.10	2.61	49.50	1.51	0.01
CHE (U/L)	6336.43	826.84	7952.83	486.64	ns
CK (U/L)	59.50	6.90	81.25	7.03	ns
AMS (U/L)	82.67	8.81	74.11	8.58	ns
LDH (U/L)	387.40	21.63	301.83	13.83	0.01
Uric acid (mmol/L)	0.21	0.01	0.23	0.01	ns
Urea (mmol/L)	4.98	0.50	4.71	0.25	ns
Creatinine (mmol/L)	70.72	3.23	66.70	4.21	ns
RBC (x10^6/mL)	4.35	0.13	4.39	0.08	ns
HB (g/dL)	12.91	0.26	12.84	0.20	ns
HCT (%)	39.43	1.03	37.86	0.45	ns
MCV (fl)	90.57	1.45	86.45	1.17	0.04
Estradiol (pg/mL)	76.67	37.74	153.25	36.19	ns
Progesterone (ng/mL)	2.26	1.41	5.17	1.62	ns
LH (mU/mL)	3.23	1.22	4.69	0.77	ns
FSH (mU/mL)	5.22	0.98	5.36	0.65	ns
TSH (µU/mL)	2.44	0.45	1.76	0.22	ns
FT3 (pg/mL)	2.59	0.17	2.69	0.07	ns
FT4 (ng/dL)	1.04	0.07	1.05	0.03	ns
Vitamin B12 (pg/mL)	608.75	93.39	413.10	35.66	ns
Folate (ng/mL)	6.73	1.26	5.78	0.96	ns
Vitamin A (µg/dL)	43.34	4.63	48.05	4.24	ns
Vitamin E (µg/dL)	1260.03	81.63	1217.86	60.29	ns

: alanine aminotransferase; ALP: alkaline phosphatase; AMS: amylase; AST: aspartate aminotransferase; CHE: cholinesterase; CK: creatine kinase; FSH: follicle-stimulating hormone; GGT: γ-glutamyl transferase; HB: hemoglobin; HCT: hematocrit; LDH: lactate dehydrogenase; LH: luteinizing hormone; MCV: mean cell volume; RBC: red blood cells; TSH:

*thyrotropin; FT4: free thyroxine; FT3: free 3,5,3'-triiodothyronine. All biochemical parameters were measured in serum. *p value at Mann Whitney U test.*

3.1 Gene expression analysis shows up-regulation of genes involved in NRF2-mediated oxidative stress response in fibroblasts cultured with CR serum

Mammalian fibroblasts have been extensively used to assess global transcriptional changes induced by circulating factors (i.e. serum) on cell function (Iyer et al 1999). To test the hypothesis that the CR-induced changes in circulating factors can influence cell function, we compared the transcriptional profiles of human primary fibroblasts treated with CR or WD serum. Differentially expressed genes were selected using a moderate t-test approach (Wettenhall et al 2006) and multiple test correction (Shi et al 2006), and these genes were used to identify transcriptionally altered pathways. Ingenuity Pathways Analysis software (IPA) showed a significant association of a subgroup of 83 genes with many oxidative response-related pathways (Figure 3).

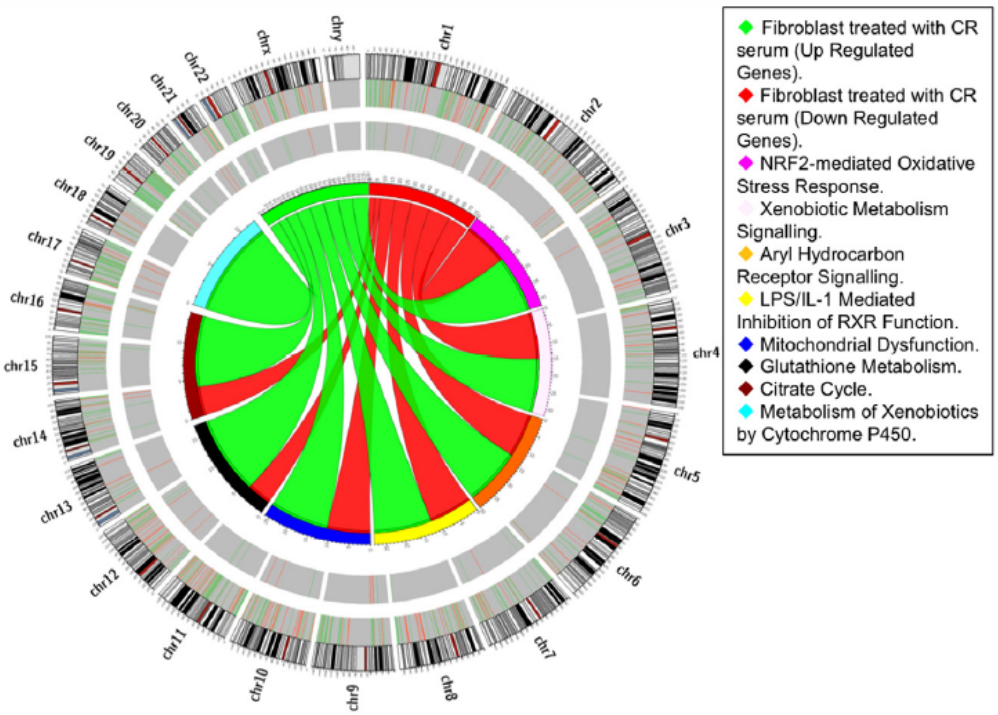

Figure 3: *Pathway-based analysis. Circos diagram shows the relationship between CR differentially expressed genes and oxidative-related pathways. Ribbon ends represent links between genes and pathways while the width of the ribbon correlates with the number of genes involved. Segments in the outer ring indicate the total number of genes involved in the corresponding pathways while thin internal segments indicate the number of up-regulated (light green) and down-regulated (orange) genes.*

In particular, NF-E2-related factor 2 (Nrf2)-mediated oxidative stress response contained 30 genes that were dysregulated in the treated fibroblasts (BH p-value $3.33E^{-02}$), and many of them were direct expression effectors of Nrf2 activity (Figure 4). Meta-analysis performed using a competitive gene set test (Wu and Smyth 2012) pinpoints 11 molecular signatures significantly downregulated in a 'self-contained' gene test (Table 4 red Highlights).

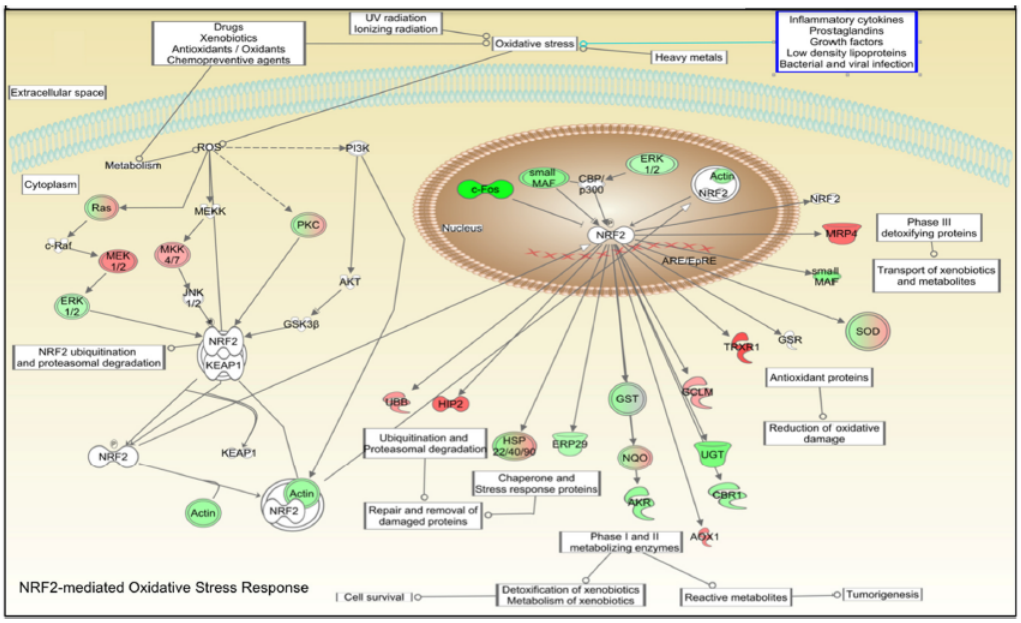

Figure 4: *Connectivity map of NRF2-mediated oxidative stress response adapted from IPA software. Pathways analysis identified the NRF2-mediated oxidative stress response pathway with a statistically significant value (BH p-value 3.33E^{-02}). Green colored shapes represent up-regulated genes while red ones represent down-regulated genes of fibroblast cell lines treated 48 hours with CR serum.*

Table 4: Gene Set Enrichment CAMERA and ROAST results

Gene Set Enrichment results.		Correlation Adjusted Mean Rank Gene				Rotation gene set testing for linear models					
Molecular Signature	NGenes	Cor	Down	Up	TwoSided	Adj.P.Value .Down	Adj.P.Value. Up	Adj.P.Value .Mixed	Active.Proportion .Down	Active.Proportion. Up	Active.Proportion .Mixed
PROTEIN_MATURATION	17	0.027	0.9976	0.0024	0.00	1.00	0.10	0.00	0.18	0.59	0.76
DNA_FRAGMENTATION_DURING_APOPTOSIS	31	-0.002	0.9951	0.0049	0.01	1.00	0.07	0.00	0.13	0.42	0.55
AMYLOID_PRECURSOR_PROTEIN_METABOLIC_PROCESS	24	0.005	0.9948	0.0052	0.01	1.00	0.07	0.00	0.08	0.38	0.46
HYDROLASE_ACTIVITY_ACTING_ON_CARBON_NITROGEN_BUT_NOT_PEPTIDE_BONDS_IN_LINEAR_AMIDES	24	0.005	0.9925	0.0075	0.02	1.00	0.10	0.00	0.13	0.29	0.42
MITOTIC_CELL_CYCLE_CHECKPOINT	45	0.012	0.0076	0.9924	0.02	0.06	1.00	0.01	0.31	0.09	0.40
RAS_GTPASE_BINDING	35	-0.010	0.0074	0.9926	0.01	0.04	1.00	0.01	0.34	0.14	0.49
HOMEOSTASIS_OF_NUMBER_OF_CELLS	36	-0.009	0.0069	0.9931	0.01	0.03	1.00	0.00	0.31	0.08	0.39
U12_DEPENDENT_SPLICEOSOME	10	-0.069	0.0063	0.9937	0.01	0.03	1.00	0.01	0.40	0.20	0.60
APICAL_PLASMA_MEMBRANE	25	-0.004	0.0056	0.9944	0.01	0.04	1.00	0.04	0.24	0.04	0.28
Stress_response_CR	11	0.002	0.0052	0.9948	0.01	0.03	1.00	0.00	0.55	0.09	0.64
INSOLUBLE_FRACTION	28	-0.003	0.0046	0.9954	0.01	0.03	1.00	0.01	0.29	0.04	0.32
DNA_DAMAGE_RESPONSE_SIGNAL_TRANSDUCTION_BY_P53_CLASS_MEDIATOR	31	-0.002	0.0043	0.9957	0.01	0.03	1.00	0.01	0.32	0.10	0.42
APICAL_PART_OF_CELL	29	-0.004	0.0042	0.9958	0.01	0.03	1.00	0.06	0.24	0.03	0.28
SMALL_CONJUGATING_PROTEIN_BINDING	21	-0.015	0.0040	0.9960	0.01	0.03	1.00	0.02	0.33	0.05	0.38
AMINOPEPTIDASE_ACTIVITY	19	0.005	0.0015	0.9985	>0.01	0.03	1.00	0.00	0.47	0.00	0.47
UBIQUITIN_BINDING	14	-0.016	0.0008	0.9992	>0.01	0.03	1.00	0.01	0.50	0.07	0.57

To further investigate the result of microarray data and confirm the association with oxidative response-related pathways, we performed quantitative real-time PCR for the following genes which were shown by microarray to be detectable and significantly dysregulated: GSTK1, GPX1, SOD2, GSTT1, and IDH2. As shown in Figure 5, relative abundance of message for these genes was significantly higher in the CR group than the control group ($p<0.0005$) with a fold change difference $\geq$ 2.2-fold.

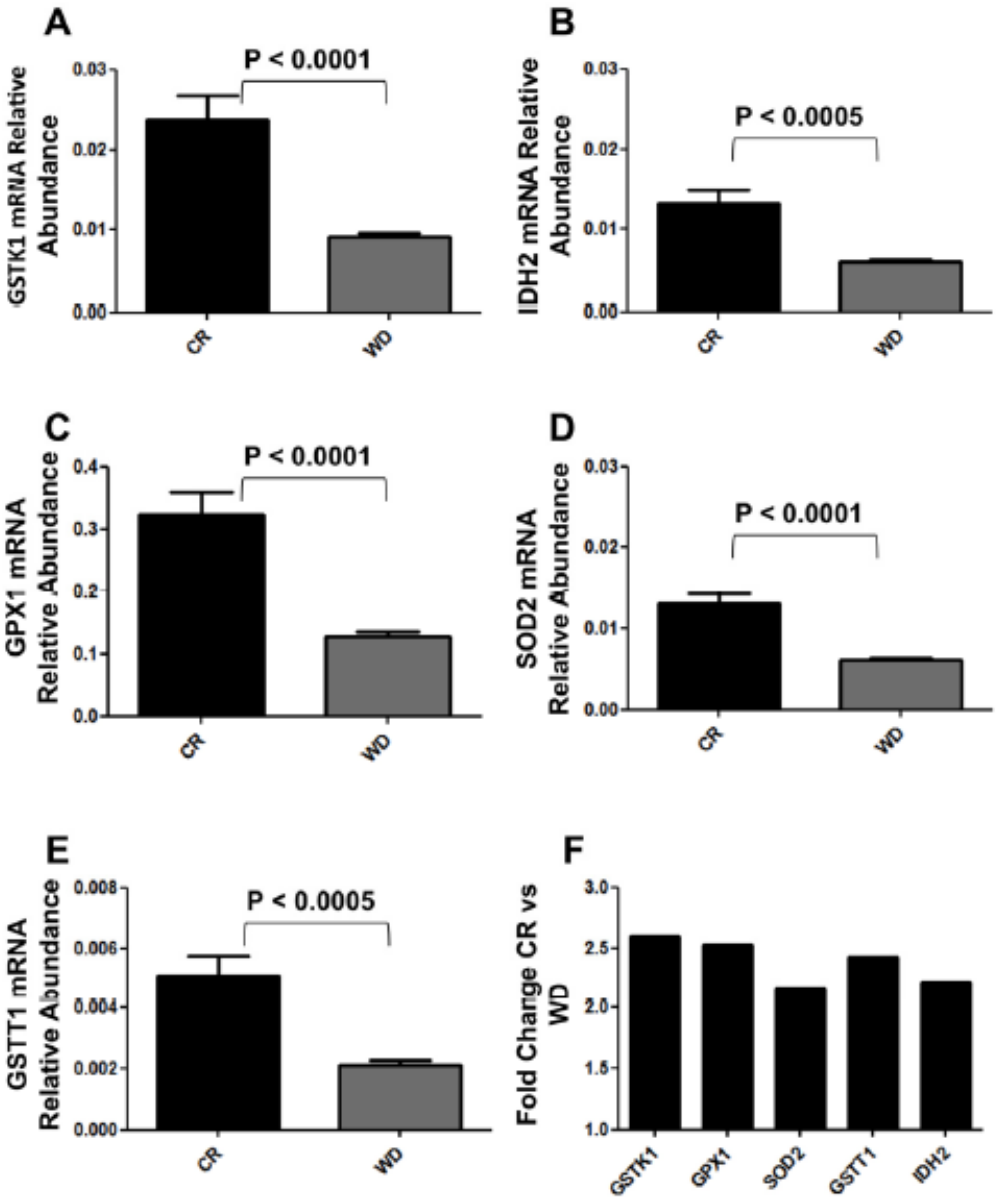

Figure 5: *Real Time qPCR analysis of GSTK1, GSTT1, IDH2, GPX1 and SOD2 expression in BJ cells cultured 48hs with CR and WD sera. (A-E) Data are expressed as the gene of interest abundance relative to the GAPDH (Mean ± SEM); n = 12 CR, n = 13 WD. Student's t test was used to determine P-values. (F) Data are expressed as fold changes relative to CR cultures.*

3.2 Incubation with human CR serum increases stress resistance to hydrogen peroxide in vitro

To confirm the gene expression findings with a functional test, human primary fibroblasts cultured for 48 hours with CR or WD sera were exposed to increasing concentrations of the powerful oxidizing agent, hydrogen peroxide (H_2O_2) for 24 hours. As shown in Figure 6, treatment with H_2O_2 produced a dose-dependent decline in survival, but fibroblasts cultured with CR serum were significantly protected from the cytotoxic effect of the oxidant.

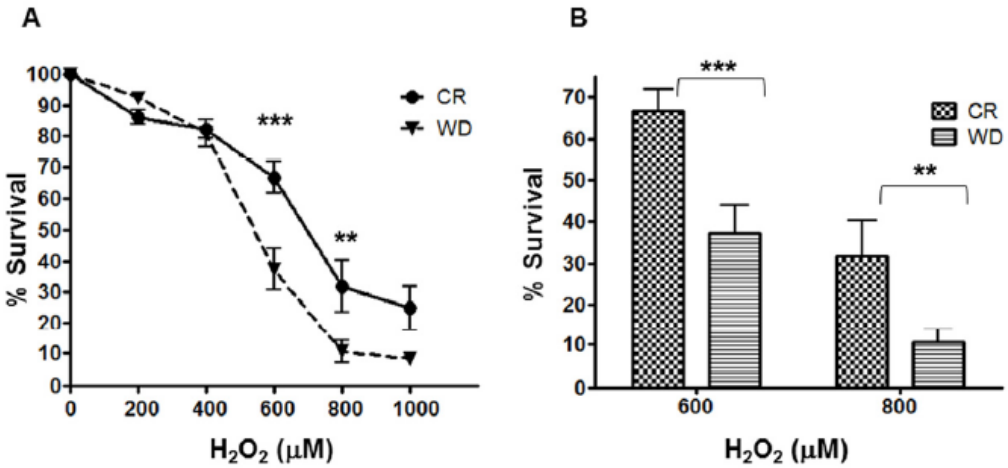

Figure 6: *Differential Stress Resistance to hydrogen peroxide. A human fibroblast cell line (BJ) was cultured 48-hrs with sera from CR and WD individuals. Cell viability (WST1 assay) was determined after a 24 h*

*treatment with H_2O_2 (0 – 1000 μM). Data are represented as means ± SEM. P- values were determined with 2way ANOVA with Bonferroni post test. ***P < 0.001; **P < 0.01.*

At 600 μM about 67% of cells pretreated with CR serum were viable, while only 37% of cells pretreated with WD serum survived ($p< 0.001$), and even at higher H_2O_2 concentrations (800 μM) 32% of the cells pretreated with CR serum survived, while only 10% of the cells exposed to WD serum were still alive ($p< 0.01$).

3.3 Immunophenotyping of AN patients and controls

As shown in Table 5, AN patients had significantly fewer total leucocytes (p=0.033), lymphocytes (p=0.009), $CD3^+$ cells (p=0.040), and NK ($CD3^-CD16^+CD56^+$) cells (p=0.033) than controls. Furthermore, AN patients had significantly fewer $CD3^+$ T cells with a naive phenotype ($CD3^+CD45^+RA^+$) (p=0.022); the same, albeit not significant, trend was observed for total $CD4^+$ T cells and $CD4^+$ T cells with a naive phenotype ($CD4^+CD45^+RA^+$) (p=0.068). The absolute number of circulating $CD4^+CD28^+$, $CD8^+CD11b^+$ and $CD3^+CD16^+CD56^+$ T cells was also significantly lower in AN patients (p=0.012, p=0.015, p=0.012 respectively).

Table 5: Immunophenotype of AN and CTR subjects

Cell type	AN (n=10) Cells/mm³ (%)	CTR (n=10) Cells/mm³ (%)	*p* value
Leucocytes	3712.67±1132.88	5642.73±1957.73	0.033
Lymphocytes	1596.89 ± 445.23 (44.12 ± 8.53)	2068.64 ± 475.65 (39.29 ± 10.77)	0.009
$CD3^+$	1196.11 ± 320.40 (75.22 ± 4.10)	1502 ± 341.60 (72.91 ± 5.58)	0.040
$CD4^+$	666.33 ± 120.13 (43.11 ± 6.61)	847.55 ± 197.60 (41.36 ± 6.19)	ns
$CD8^+$	426.56 ± 194.29 (25.89 ± 4.41)	533.18 ± 147.76 (25.82 ± 4.77)	ns
$CD3^-CD16^+CD56^+$	123.22 ± 83.97 (7.67 ± 5.12)	268.82 ± 162.89 (12.63 ± 6.13)	0.033
$CD19^+$	251.00 ± 125.73 (15.33 ± 4.55)	259.09 ± 104.54 (12.27 ± 3.93)	ns
$CD3^+CD45^+RA^+$	593.06 ± 171.16 (37.78 ± 7.24)	815.24 ± 198.81 (39.91 ± 6.61)	0.022
$CD3^+CD45^+RO^+$	603.16 ± 250.39 (37.44 ± 8.25)	686.85 ± 196.52 (33.00 ± 5.92)	ns
$CD4^+CD45^+RA^+$	310.03 ± 64.08 (20.78 ± 6.30)	428.80 ± 166.87 (21.09 ± 7.53)	ns
$CD4^+CD45^+RO^+$	356.30 ± 121.55 (22.33 ± 4.82)	418.75 ± 140.84 (20.27 ± 5.08)	ns
$CD8^+CD45^+RA^+$	283.02 ± 169.30 (17.00 ± 7.87)	386.44 ± 121.03 (18.82 ± 4.89)	ns
$CD8^+CD45^+RO^+$	143.53 ± 144.74	146.74 ± 69.88	ns

	(15.11 ± 8.71)	(12.73 ± 4.38)	
$CD4^+CD28^+$	601.83 ± 113.86	766.90 ± 168.33	0.012
	(38.89 ± 6.26)	(37.64 ± 6.31)	
$CD4^+CD25^+$	15.75 ± 9.60	22.31 ± 6.14	ns
	(1.00 ± 0.47)	(1.09 ± 0.30)	
$CD4^+CD8^+$	15.27 ± 11.69	34.07 ± 44.52	ns
	(0.89 ± 0.57)	(1.55 ± 1.57)	
$CD4^+DR^+$	17.31 ± 9.89	19.75 ± 8.82	ns
	(1.11 ± 0.57)	(1.00 ± 0.45)	
$CD8^+DR^+$	23.00 ± 10.77	25.47 ± 15.95	ns
	(1.44 ± 0.50)	(1.36 ± 1.12)	
$CD8^+CD11b^+$	41.93 ± 37.89	117.58 ± 95.55	0.015
	(2.44 ± 1.57)	(5.45 ± 3.62)	
$CD3^+CD16^+CD56^+$	29.11 ± 24.81	119.91 ± 176.83	0.012
	(1.67 ± 1.05)	(5.27 ± 6.28)	
$CD3^-CD8^+$	71.11 ± 50.04	118.94 ± 94.33	ns
	(4.22 ± 2.39)	(5.55 ± 3.93)	
$CD4^+/CD8^+$ (ratio)	26.31 ± 6.52	34.55 ± 11.70	ns
	(1.74 ± 0.51)	(1.70 ± 1.66)	

3.4 T-cell proliferative response and cytokine profiling in supernatants of isolated T cells

We next evaluated the PBMC proliferative profile in AN and control subjects in medium supplemented with 5% pooled HS or 5% AS after stimulation with anti CD3 (OKT3 mAb). T-cell proliferation did not differ between AN patients and controls in either PBMC cultured with AS or HS serum (Figure 7A and 7B). The cytokine profile evaluated in the supernatant of TCR-

stimulated PBMCs (Figure 7C-O) revealed significantly higher IL-6 levels in AN than in control supernatants from cells cultured in HS medium ($p<0.01$) (Figure 7G). Interestingly, this effect was abolished when AN cells were cultured in AS medium (Figure 7G). TNFα levels were significantly decreased in stimulated-AS cultured AN and control cells compared with HS cultured cells ($p<0.01$). Similarly, IL-1β levels were decreased in supernatant from AS-cultured AN cells versus HS cultured AN cells ($p<0.01$). Treatment with AS resulted in a reduction of all the other tested inflammatory cytokines in anti CD3-stimulated AN cells compared with HS AN cultured cells although the trend was not statistically significant.

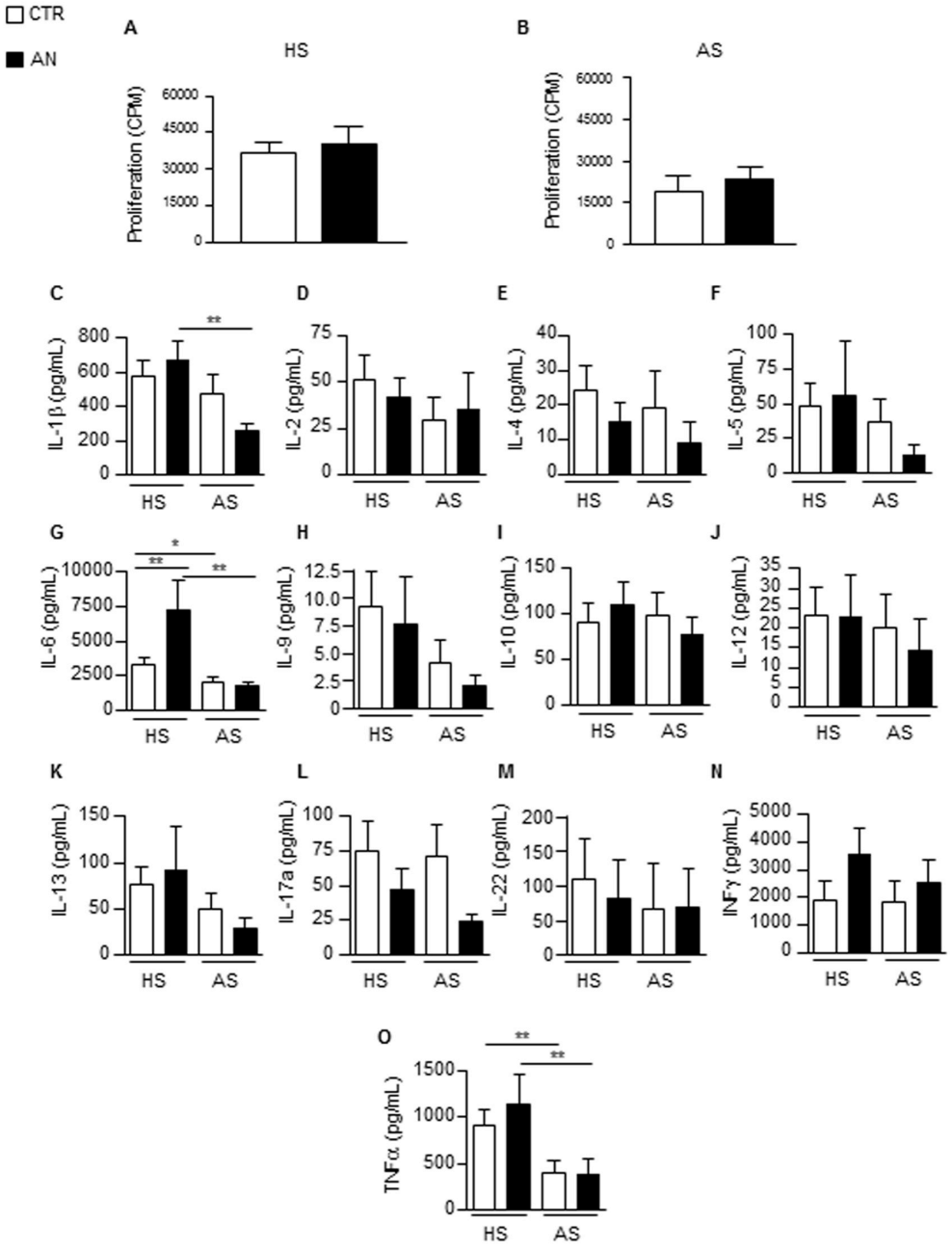

Figure 7: *TCR-mediated PBMC proliferation in heterologous (HS) or autologous (AS) serum (A, B) and cytokine secretion (C-O) in supernatants from 48h anti-CD3 (OKT3 mAb) stimulated PBMCs from AN (n=10) and CTR subjects (n=10). Data are expressed as mean ± SEM and statistical analysis was performed with the Mann Whitney non parametric U test. (** p≤0.01, *p<0.05).*

3.5 Impaired glycolysis and mitochondrial respiration in T cells from AN patients

To elucidate the impact of chronic caloric deprivation on the metabolic profile of immune cells we measured the ECAR and OCR, which are indicators of glycolysis and mitochondrial respiration, respectively, in PBMCs from AN and control subjects in unstimulated conditions (Figure 8) and upon TCR-mediated stimulation (anti-CD3) (Figure 9). After TCR-mediated stimulation, activation of the glycolytic pathway was impaired in AN patients (Figure 9A), as testified by the reduced basal glycolysis (p=0.0126) (Figure 9B) and maximal glycolysis (p=0.0071) (Figure 9C), and also in terms of glycolytic capacity (p=0.0031) (Figure 9D). The same trend was observed in unstimulated cells (Figure 8A), but it was not significant (Figure 8B-D). Oxidative phosphorylation was also lower in PBMCs from AN patients (Figure 9E and Figure 8E) than in controls, as indicated by the decreased basal OCR in both unstimulated cells (Figure 8F) (p<0.0001) and anti-CD3 stimulated cells (Figure 9F) (p<0001). The same result was obtained after the addition of oligomycin (an ATP synthase inhibitor). Indeed, the amount of basal OCR required for ATP production, calculated as the difference between basal and oligomycin-induced OCR ("ATP-coupled respiration"), was significantly reduced only in AN after TCR-mediated stimulation (p=0.0173) (Figure 9G and Figure 8G). Finally, as expected, the addition of the mitochondrial uncoupler (FCCP) resulted in a dramatic increase in the OCR thereby showing that mitochondria had maximal respiratory capacity. Interestingly, OCR did not differ significantly between

AN and control subjects in either unstimulated or TCR-stimulated cells (Figure 8H and Figure 9H).

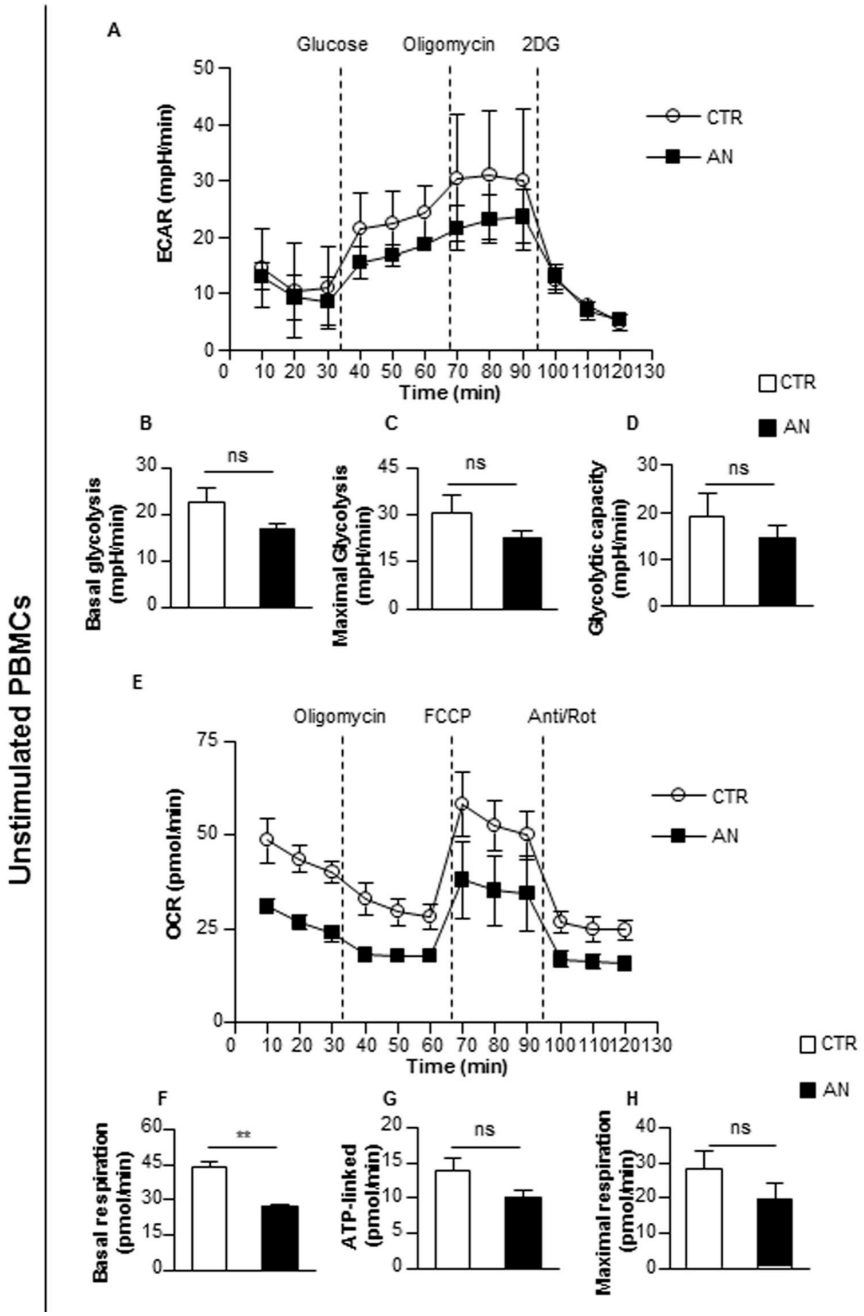

Figure 8: *Impaired glycolysis and mitochondrial respiration in unstimulated PBMCs from AN subjects. (A) Extracellular acidification rate (ECAR) bioenergetic profile. ECAR was measured in real time under basal conditions and in response to glucose, oligomycin and 2-Deoxy-D-glucose*

*(2DG) in unstimulated PBMCs from AN and CTR subjects. Indices of glycolytic pathway activation, calculated from the PBMCs bioenergetic profiles: (B) Basal (after the addition of glucose), (C) maximal (after the addition of oligomycin), and (D) glycolytic capacity (calculated as the difference of oligomycin rate and 2-DG rate) ECAR in AN and CTR. (E) Oxygen consumption rate (OCR) bioenergetic profile in unstimulated PBMCs from AN and CTR subjects. OCR was measured in real time under basal conditions and in response to indicated mitochondrial inhibitors: oligomycin, carbonylcyanide-4- (trifluoromethoxy) -phenylhydrazone (FCCP), Antimycin A and Rotenone (Ant+Rot), using an XF-96 Extracellular Flux Analyzer. Indices of mitochondrial respiratory function calculated from the AN and CTR anti-CD3 stimulated PBMC bioenergetic profiles: (F) Basal (before addition of oligomycin), (G) ATP-linked (calculated as the difference of basal rate and oligomycin rate) and (H) maximal (calculated as the difference of FCCP rate and Ant+Rot rate) OCR in AN and CTR. Cumulative data from 4 AN patients and 3 CTR subjects expressed as mean ± SEM, statistical analysis by Mann Whitney test. (**p≤0.01; ns=not significant).*

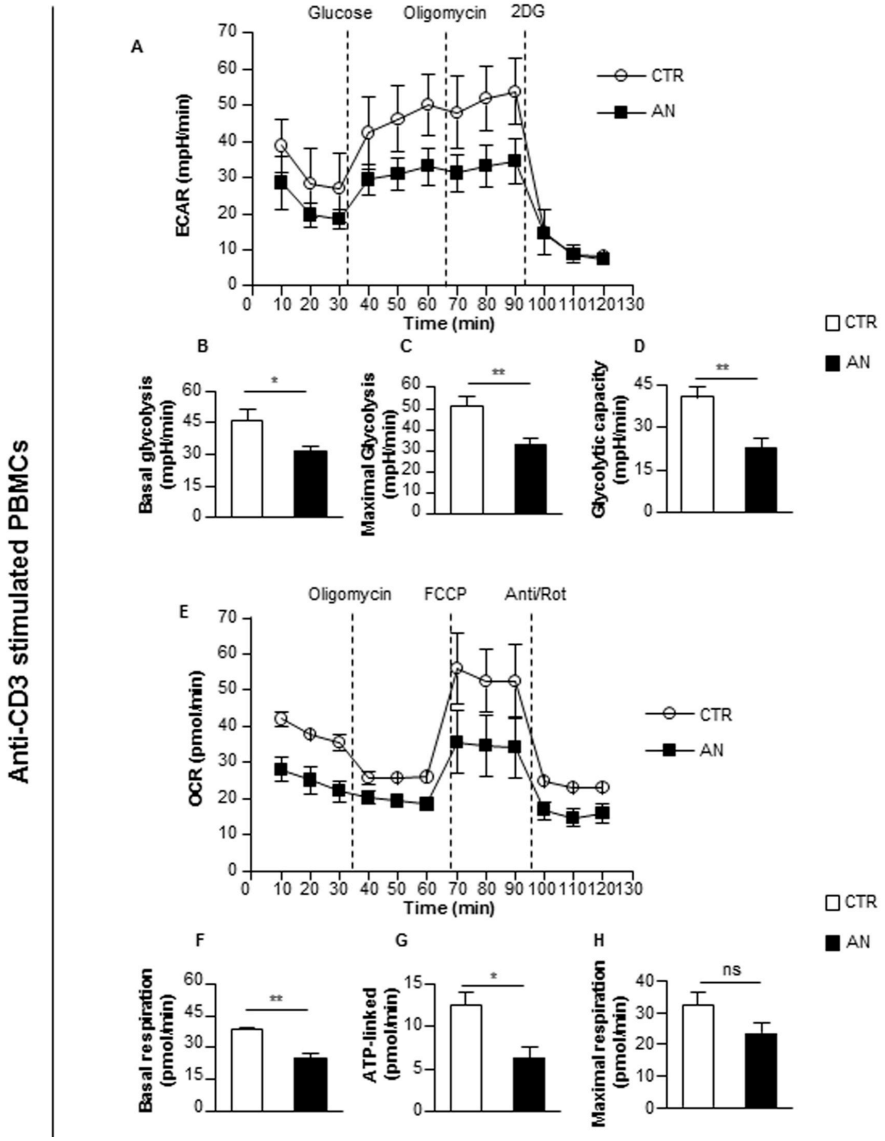

Figure 9: *Impaired glycolysis and mitochondrial respiration in AN PBMCs after TCR-mediated stimulation. (A) Extracellular acidification rate (ECAR) bioenergetic profile. ECAR was measured in real time under basal conditions and in response to glucose, oligomycin and 2-Deoxy-D-glucose (2DG) in anti-CD3-stimulated PBMCs from AN and CTR subjects. Indices of the glycolytic pathway activation, calculated from the PBMC bioenergetic profiles: (B) Basal (after the addition of glucose), (C) maximal (after the addition of oligomycin), and (D) glycolytic capacity (calculated as the difference of oligomycin rate and 2DG rate) ECAR in AN and CTR. (E)*

*Oxygen consumption rate (OCR) bioenergetic profile in anti-CD3-stimulated PBMCs from AN and CTR subjects. OCR was measured in real time under basal conditions and in response to indicated mitochondrial inhibitors: oligomycin, carbonylcyanide-4- (trifluoromethoxy) -phenylhydrazone (FCCP), Antimycin A and Rotenone (Ant+Rot), using an XF-96 Extracellular Flux Analyzer. Indices of mitochondrial respiratory function, calculated from PBMC bioenergetic profiles: (F) Basal (before addition of oligomycin), (G) ATP-linked (calculated as the difference of basal rate and oligomycin rate) and (H) maximal (calculated as the difference of FCCP rate and Ant+Rot rate) OCR in AN and CTR. Cumulative data from 4 AN patients and 3 CTR subjects expressed as mean ± SEM, statistical analysis by Mann Whitney test. (**p≤0.01; * p≤0.03; ns=not significant).*

3.6 Evaluation of antioxidant status in PBMCs and in human fibroblasts cultured with AN or control serum

As shown in Figure 10, 14 genes (RPLP0, ALOX12, ApoE, BNIP3, CCL5, DUOX2, GPX1, GPX3, MT3, PRDX2, PRDX6, PRNP, PTGS2 and RNF7) were significantly up-regulated ($p<0.05$) in PBMCs isolated from AN patients compared with those from control subjects (fold change range: 2-9).

To determine whether serum from AN patients affected the cell antioxidant potential, we analysed the gene expression of mRNA isolated from the BJ human fibroblasts cultured with AN or control serum. Eighty-three of the 92 genes were expressed in BJ fibroblasts; NOX5 and PTGS2 were significantly down-regulated ($p=0.02$ and $p=0.004$, respectively), while GPX3 was

significantly up-regulated (p=0.002) in the fibroblasts cultured with AN serum versus those treated with control serum (Figure 11).

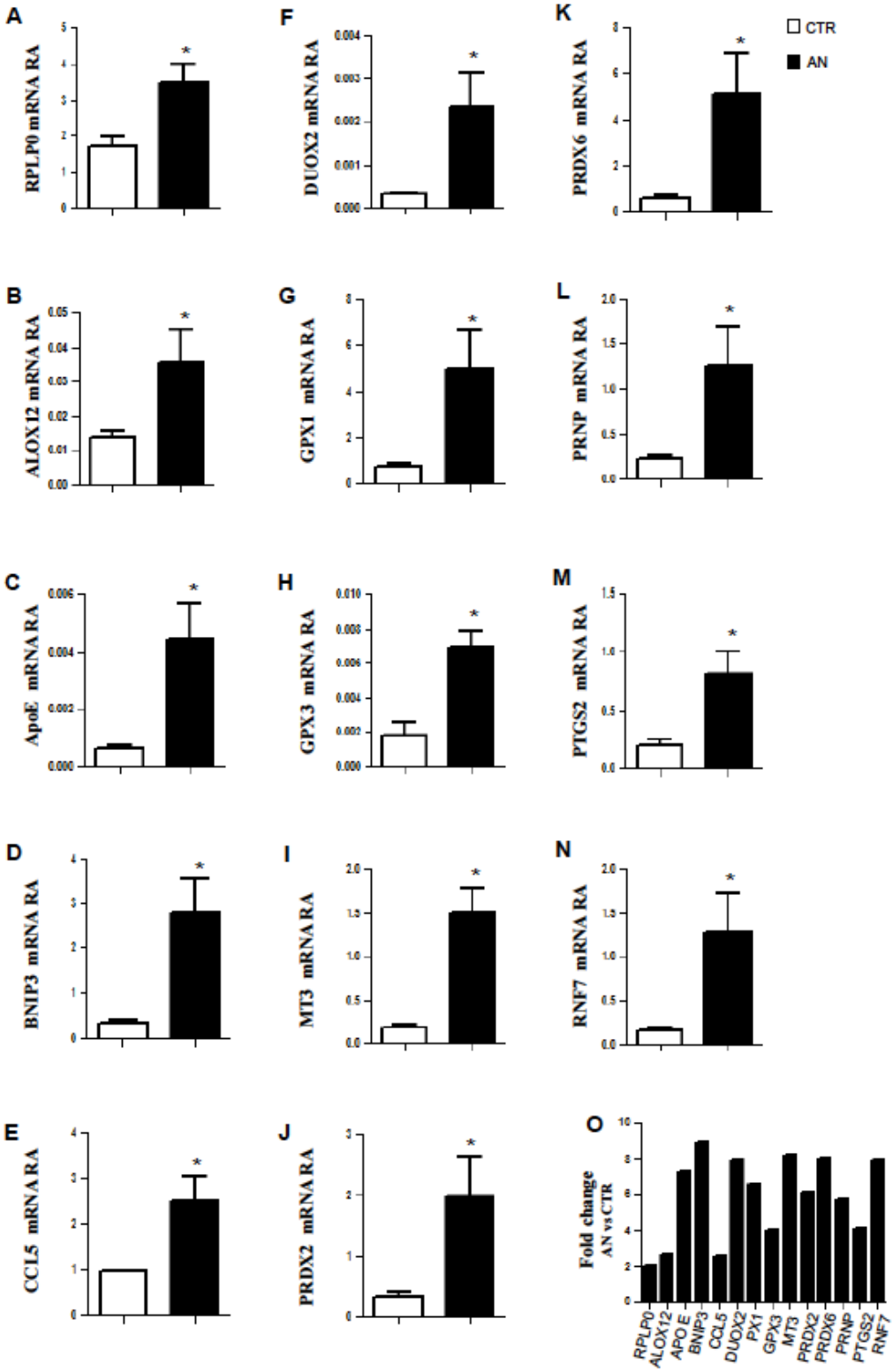

Figure 10: *Significantly higher levels of antioxidant genes in PBMCs fromAN with respect to control subjects. (A-N) Relative amount of the*

*indicated mRNA using GAPDH as endogenous reference genein both AN and CTR subjects. (O) Fold change of mRNA from AN versusmRNA in CTR subjects.RPLP0:60S Ribosomal Protein L10; ALOX12:Arachidonate 12-Lipoxygenase; ApoE: Apolipoprotein E; BNIP3: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; CCL5: chemokine (C-C motif) ligand 5; DUOX2: dual oxidase 2; GPX1 and GPX3: Glutathione peroxidase 1 and 3; MT3: Metallothionein-3; PRDX2 and PRDX6: Peroxiredoxin-2; PRNP: prion protein; PTGS2: prostaglandin-endoperoxide synthase 2; RNF7: ring finger protein 7. *: p< 0.05.*

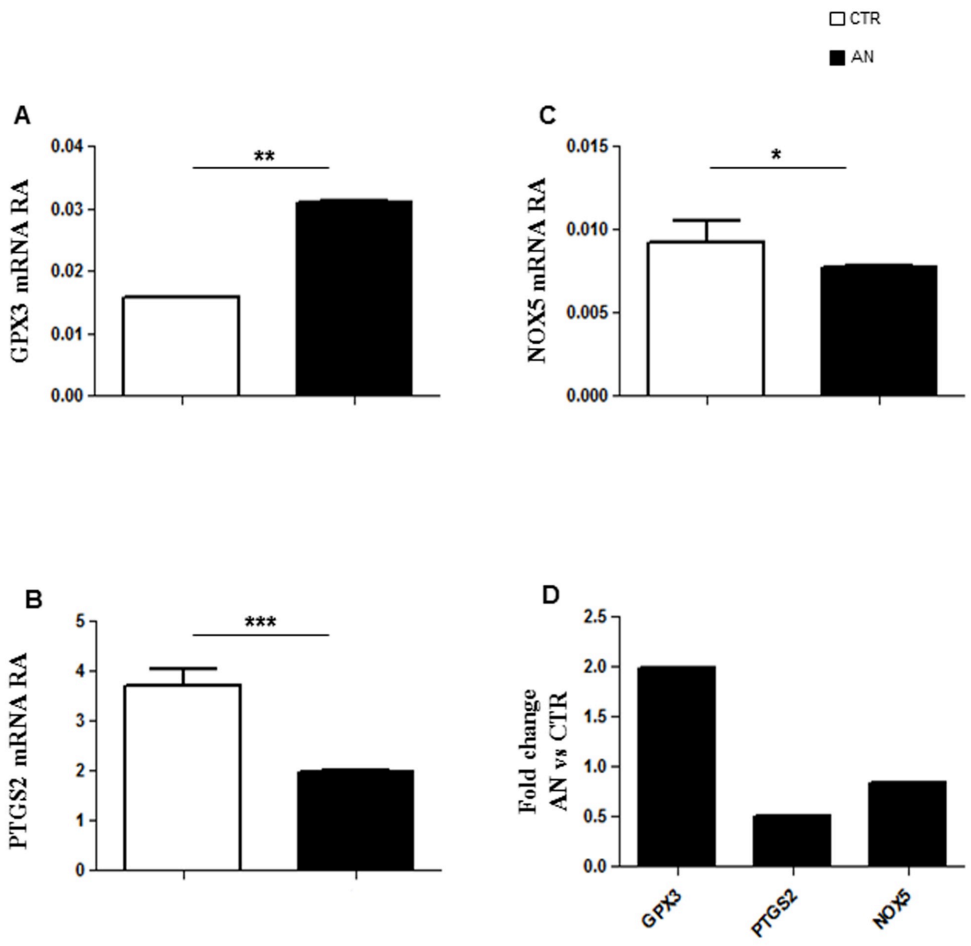

Figure 11: *Antioxidant gene expression in BJ cells cultured with AN or CTR sera. (A-C) Relative amount of the indicated mRNA using GAPDH as*

*endogenous reference gene in both AN and CTR sera cultured cells. (D) Fold change of mRNA from AN serum cultured cells versus mRNA measured in CTR serum cultured cells. GPX3: Glutathione peroxidase 3; NOX5: NADPH oxidase, EF-hand calcium binding domain 5; PTGS2: prostaglandin-endoperoxide synthase 2. *:p= 0.02; **: p=0.002; ***: p=0.004.*

3.7 Sera from AN patients enhanced stress resistance to hydrogen peroxide in human fibroblasts

To determine the effect induced by AN and control sera on resistance to oxidative stress, we cultured BJ cells for 48 hours with AN or control sera and then exposed them to increasing concentrations of the powerful oxidizing agent, H_2O_2 for 24 hours. H_2O_2 induced a dose-dependent decline in survival, but fibroblasts cultured with AN serum were significantly protected from the cytotoxic effect of the oxidant (Figure 12). In fact, at 200 μM H_2O_2, 73% of cells pretreated with AN sera versus 49% of cells pretreated with control sera were viable ($p<0.01$). The higher stress resistance induced by AN sera occurred also at higher H_2O_2 concentrations. In fact, the survival rates of cells pretreated with AN or control sera were 63% vs 40% at 400μM, and 56% versus 29% at 600 μM H_2O_2, respectively ($p<0.05$).

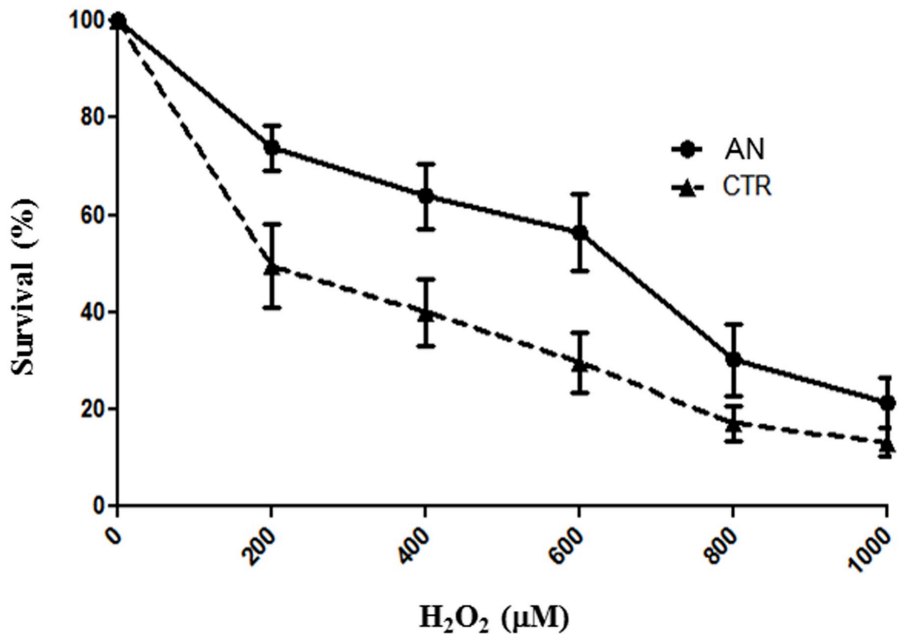

Figure 12: *Sera from AN patients enhances stress resistance to hydrogen peroxide in human fibroblasts. AN serum cultured cells were more resistant to oxidative stress than CTR serum cultured cells. The solid and the dotted lines represent the percentage of survival of BJ cells cultured with AN or CTR serum, respectively, after treatment with increasing doses of H_2O_2. *:$p<0.05$; **: $p<0.01$.*

4. DISCUSSION

4.1 First Aim

We report here that some molecular and functional adaptations of CR, such enhanced stress resistance, can be mimicked in vitro by culturing cells with serum from individuals practicing long-term CR without malnutrition. Our findings show that the incubation of human fibroblasts with CR sera induces a transcriptional activation of several anti-oxidant genes along the Nrf2 pathway. In addition, we found that the incubation of fibroblasts with human CR serum is sufficient to increase stress resistance to hydrogen peroxide in vitro. These molecular and cellular adaptations are consistent with findings in CR rodents and monkeys, and may contribute at least in part to the protective anti-aging effects of CR (Anderson et al 2009, Lee et al 1999). In particular, accumulating data indicate that aging is associated with a down-regulation of stress-response genes and pathways (Lee et al 1999). To test the hypothesis that CR can counteract some of these age-associated detrimental effects in vitro, we first sought to determine if circulating factors of CR sera modify gene expression profiles of fibroblasts, which are the most abundant cell type in connective tissues and form the structural framework of tissues through their secretion of extracellular matrix components. To this end, we cultured human primary fibroblasts with sera from 12 middle-aged (57.8±8.4 yrs), weight-stable very lean (BMI=19.1±1.2 kg/m^2) individuals who have been practicing ~30% CR with adequate nutrition (at least 100% of RDI for each nutrient), for 3-20 years, and a control group of 13 non-obese (BMI=25.7±2.3 kg/m^2) age-matched sedentary controls eating a typical Western diet. Remarkably, we found that culturing cells with human CR sera

for 48-hrs was sufficient to trigger a rapid transcriptional up-regulation of important genes and pathways that control stress resistance. In particular, we found that a highly significant number of transcripts along the Nrf2 pathway were altered by CR, and genes encoding important antioxidant enzymes, such as GSTK1, GSTT1, IDH2, GPX1 and SOD2, were up-regulated at least two fold in the CR group. Nrf2 is a key transcription factor that binds to the antioxidant response element (ARE) in response to environmental carcinogens or low levels of insulin, and increases the transcription of a variety of antioxidative and carcinogen-detoxification enzymes that boost protection against cellular and molecular damage and cancer (Kannan and Jaiswal 2006, Kim and Novak 2007, Kwak et al 2002). It might be also possible that CR causes oxidative stress and therefore induce a NRF2-dependent response, supporting the hypothesis that oxidative damage is not a cause of aging (Gems and Partridge 2013, Gems and de la Guardia 2013, Blagosklonny 2012).

In order to further investigate the role of CR sera in modifying the resistance of cells to oxidative stress, we next performed a series of experiments in which we exposed human primary fibroblasts to increasing concentrations of H_2O_2, a powerful oxidizing agent. Our data indicate that incubating fibroblasts with CR sera exerts a rapid and profound protection against hydrogen peroxide-induced cytotoxicity. These findings are consistent with data from studies in mice, suggesting that these selected cellular adaptations induced by circulating factors are conserved among species (de Cabo et al 2003). Indeed, most of the metabolic and hormonal parameters examined in

CR humans are similar to those reported in CR rodents (Cangemi et al 2010, Fontana and Klein 2007, Fontana et al 2010 b).

In conclusion, our findings reported here show that enhanced stress responsiveness known to occur in animals on CR following oxidative stress can be reproduced in vitro by culturing cells using serum from CR patients. Our findings provide support for a possible neuroendocrine based mechanism of CR and offer a model for the screening and investigation of CR mimetic agents. We anticipate that this novel in vitro model will be instrumental in the elucidation of biochemical, molecular and cellular mechanisms underlying the anti-aging effects of CR in humans.

4.2 Second Aim

The data of the present study show that young AN women with pathologically low body weight (BMI, 15.9±0.4 kg/m^2) have significantly lower circulating levels of leptin, IGF-1, leukocytes and lymphocytes than age-matched lean control subjects. In particular, we found that the T lymphocyte $CD3^+$, $CD3^+CD45^+RA^+$, $CD4^+CD28^+$, $CD8^+CD11b^+$, $CD3^+CD16^+CD56^+$, and NK cell $CD3^-CD16^+CD56^+$ subpopulations were significantly lower in the AN patients than in control subjects. Moreover, the antioxidant potential and the anti-inflammatory status were more efficient in the PBMCs of AN patients than in controls.

Leptin is one of the key adipokines secreted by adipocytes (Engineer and Garcia 2012) that affects both innate and adaptive immunity (Galgani et al 2010) and exerts differential effects on several T-cell subpopulations by activating the mammalian target of the rapamycin (mTOR) pathway (De

Rosa et al 2007, Procaccini et al 2012, Procaccini et al 2010). In particular, leptin inhibits Treg proliferation (De Rosa et al 2007), whereas it enhances conventional T-cells (Tconv) proliferation (Procaccini et al 2012). The enhanced proliferation of $CD4^+CD25^-$ Tconv is associated with inflammatory cytokine secretion, whereas leptin neutralization has the opposite effect, suggesting that this adipokine plays a key role in T-cell homeostasis and function (Conde et al 2010, Matarese and La Cava 2007). Accordingly, it has been reported that women with hypothalamic amenorrhea and concurrent chronic-acquired relative leptin deficiency have a reduced number and function of T cells, which are restored after leptin treatment (Matarese et al 2013). In animal models, leptin reduced the number of natural killer cells in isolated fat pads (La cava and Matarese 2004). In line with these data, the number of circulating natural killer ($CD3^-CD16^+CD56^+$) cells was reduced in our AN patients.

Leptin levels were severely lower in our AN patients than in controls (~1 vs 15 ng/mL), which coincides with previous reports (Terra et al 2013, Dostálová et al 2007, Modan-Moses et al 2007, Janas-Kozik 2011, Föcker et al 2011). In physiological conditions, low leptin levels trigger a compensatory increase in food intake and a decrease in energy expenditure, but this mechanism is deranged in AN subjects possibly because of decreased transport of leptin through the blood-to-brain barrier induced by food deprivation (Kastin et al 2000).

Adiponectin was significantly higher in our AN patients than in control subjects, in accordance with previous studies (Pannacciulli et al 2003, Delporte et al 2003, Terra et al 2013, Dostálová et al 2007). The enhanced

adiponectin levels in AN patients could result from loss of the negative feedback physiologically exerted by fat depots on their own adiponectin production, or from the altered sympathetic nervous tone (Delporte et al 2003). In line with the latter hypothesis, 24h urinary catecholamine was reduced in our AN patients, and could have induced the increase of serum adiponectin levels.

Parallel to hyperadiponectinemia, insulin levels were very low in our AN patients in the presence of normoglycemia, which coincides with increased insulin sensitivity in AN subjects (Terra et al 2013, Dostálová et al 2007, Modan-Moses et al 2007). Accordingly, the leptin:adiponectin ratio that we previously identified as a marker of insulin resistance (Labruna et al 2011), was significantly lower in AN than in control subjects.

Adiponectin exerts anti-inflammatory effects by down-regulating the expression of pro-inflammatory molecules such as TNF-α and IL-6, and stimulating the secretion of anti-inflammatory cytokines like IL-10 (Ouchi et al 2011, Holland et al 2013). Its secretion is modulated by IL-6, which in turn inhibits adiponectin synthesis. Accordingly, levels of IL-6 and TNF-α were higher in HS-cultured AN PBMCs than in controls. This secretion was abolished in AS-cultured PBMCs, which suggests that serum adiponectin indirectly affects IL-6 and TNF-α secretion. Moreover, despite lower pro-inflammatory cytokine secretion (IL-1β, TNF-α, IL-6) in supernatants of stimulated AS-cultured AN PBMCs, T-cell proliferation did not differ between HS and AS-cultured PBMCs in our AN patients and control subjects. Interestingly rAN patients had also lower levels of sTNF-R1 than

control subjects. Since the soluble forms of TNF receptors (sTNF-Rs) can be considered a marker of activation of immune system (Spoettl et al 2007), our data suggest that decreased levels of sTNF-R1 in rAN patients, is associated with an anti-inflammatory immune response.

Recent findings have expanded our knowledge of how aspects of cellular metabolism can modulate several T cell responses thereby controlling the fine balance between inflammation and immune tolerance (Pearce et al 2013). Consequently, we investigated the bioenergetic profile of AN and control PBMCs in unstimulated and stimulated conditions by studying ECAR and OCR, the two major energy-producing pathways essential for the functions of all living cells, including immune cells (Pearce et al 2013). Interestingly, we found, for the first time, lower glycolysis and mitochondrial respiration in AN PBMCs compared with healthy controls both in basal condition and after anti-CD3 stimulation, which suggests impaired bioenergetic metabolism in AN immune cells. However, maximal respiration did not differ between AN and control T cells, suggesting that the former are able to compensate the mitochondrial functional deficit in response to increased metabolic demand. The afore-mentioned data indicate that T cells from AN patients are bioenergetically different from those of control subjects. In fact, they are metabolically less active, which indicates that caloric deprivation could play a role in modulation of the cellular metabolic machinery. These results are in line with experimental data showing that activated T cells have high metabolic demands, associated with increased glycolysis activation, mitochondrial activity, ATP synthesis and ROS production (Pearce et al 2013). Therefore, the low metabolic profile observed

in AN patients could justify the anti-inflammatory status characterizing AN patients. According to the latter hypothesis, the antioxidant status of PBMC of our AN patients was enhanced. In particular, genes encoding the antioxidant enzymes GPX1, GPX3, PRDX2, PRDX6 and DUOX2 were significantly up-regulated (between 4-8 fold). To determine whether circulating factors of AN sera could be responsible for this enhanced antioxidant status, we evaluated the antioxidant profile of fibroblasts, which are the most abundant cell type in connective tissues, cultured with AN or control sera. We found that 48h of culture with AN serum was sufficient to induce the expression of GPX3 and to down-regulate NOX5, an enzyme that generates superoxide. These data are consistent with data obtained in individuals on long-term calorie restriction that have also increased levels of serum adiponectin (Omodei et al 2013, Fontana et al 2010 b).

To probe further the effect exerted by AN sera on the antioxidant response, we challenged fibroblasts cultured with AN and control sera with increasing doses of H_2O_2. Our data indicate that AN serum confers protection against the cytotoxic effect of H_2O_2. This effect could be explained by the high plasma adiponectin concentrations in our patients and by the high assumption of vitamin and antioxidant-rich foods. Interestingly, adiponectin ensured normal oxidative stress levels and glutathione levels in cells exposed to oxidized low-density lipoprotein (Plant et al 2008). Moreover, a positive association between plasma adiponectin levels and glutathione was also described in elderly individuals (Gustafsson et al 2013). However, further studies are required to determine whether or not adiponectin is responsible for the enhanced antioxidant and anti-inflammatory status of AN patients.

In summary, here we demonstrated that AN patients have lower leptin and IGF-1, and higher adiponectin serum levels; the analysis of lymphocyte subpopulations also showed, although in the normal range, fewer leucocytes, lymphocytes, $CD3^+$ and NK cells. Moreover, AN patients showed an impaired bioenergetic metabolism, as evidenced by lower glycolysis and mitochondrial respiration than in controls. Furthermore, an increased antioxidant potential was described in AN patients, either in terms of increased antioxidant gene expression in PBMCs or as augmented resistance to oxidative stress of human fibroblasts cultured with AN sera.

In conclusion, our data suggest that excessive CR in AN patients is associated with a reduction in several key immune cell populations, impaired metabolic activity, but preserved immune function. Moreover, our findings suggest that chronic severe CR in young AN patients results in an enhanced anti-oxidant and anti-inflammatory status, which may protect cells from biochemical stress.

5. REFERENCES

American Psychiatric Association. 2013. Diagnostic and statistical manual of mental disorders (5th ed.). Arlington, VA: American Psychiatric Publishing.

Anderson RM, Shanmuganayagam D, Weindruch R. Caloric restriction and aging: studies in mice and monkeys. Toxicol Pathol. 2009; 37:47-51.

Bartke, A., Chandrashekar, V., Bailey, B., Zaczek, D. and Turyn, D. (2002) Consequences of growth hormone (GH) overexpression and GH resistance. Neuropeptides 36, 201–208.

Bartke, A. (2005) Minireview: role of the growth hormone/insulin-like system in mammalian aging. Endocrinology 146, 3718–3723.

Blagosklonny MV. Answering the ultimate question "what is the proximal cause of aging?". Aging (Albany NY). 2012; 4:861-277.

Brown-Borg, H.M. (2007) Hormonal regulation of longevity in mammals. Ageing Res. Rev. 6, 28–45.

Cangemi, R., Friedmann, A.J., Holloszy, J.O. and Fontana, L. (2010) Effects of long-term calorie restriction on serum sex hormones concentration in men. Aging Cell 9, 236–242.

Caspar-Bauguil S, Cousin B, André M, Nibbelink M, Galinier A, Periquet B, Casteilla L, Pénicaud L. Weight-dependent changes of immune system in adipose tissue: importance of leptin. Exp Cell Res 2006; 312:2195-2202.

Cohen, E., Paulsson, J.F., Blinder, P., Burstyn-Cohen, T., Du, D., Estepa, G., Adame, A., Pham, H.M., Holzenberger, M., Kelly, J.W., Masliah, E. and

Dillin, A. (2009) Reduced IGF-1 signaling delays age-associated proteotoxicity in mice. Cell 139, 1157–1169.

Colman, R.J., Anderson, R.M., Johnson, S.C., Kastman, E.K., Kosmatka, K.J., Beasley, T.M., Allison, D.B., Cruzen, C., Simmons, H.A., Kemnitz, J.W. and Weindruch, R. (2009) Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 325, 201–204.

Conde J, Scotece M, Gómez R, Gómez-Reino JJ, Lago F, Gualillo O. At the crossroad between immunity and metabolism: focus on leptin. Expert Rev Clin Immunol 2010; 6:801-808.

Conover, C.A. and Bale, L.K. (2007) Loss of pregnancy-associated plasma protein A extends lifespan in mice. Aging Cell 6, 727–729.

De Cabo R, Furer-Galban S, Anson RM, Gilman C, Gorospe M, Lane MA. An in vitro model of caloric restriction. Exp Gerontol. 2003; 38:631-639.

Delporte ML, Brichard SM, Hermans MP, Beguin C, Lambert M. Hyperadiponectinaemia in anorexia nervosa. Clin Endocrinol (Oxf) 2003; 58:22-29.

Dostálová I, Smitka K, Papezová H, Kvasnicková H, Nedvídková J. Increased insulin sensitivity in patients with anorexia nervosa: the role of adipocytokines. Physiol Res 2007; 56:587-594.

Du P, Kibbe WA, Lin SM. lumi: a pipeline for processing Illumina microarray. Bioinformatics. 2008; 24:1547-1548.

De Rosa V, Procaccini C, Calì G, Pirozzi G, Fontana S, Zappacosta S, La Cava A, Matarese G. Key role of leptin in the control of regulatory T cells proliferation. Immunity 2007; 26:241-255.

Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002; 30:207-210.

Engineer DR, Garcia JM. Leptin in anorexia and cachexia syndrome. Int J Pept 2012; 2012:287457.

Föcker M, Timmesfeld N, Scherag S, Bühren K, Langkamp M, DempfleA, Sheridan EM, de Zwaan M, Fleischhaker C, Herzog W, Egberts K, Zipfel S, Herpertz-Dahlmann B, Hebebrand J. Screening for anorexia nervosa via measurement of serum leptin levels. J Neural Transm 2011; 118:571-578.

Fontana, L., Meyer, T.E., Klein, S. and Holloszy, J.O. (2004) Long-term calorie restriction is highly effective in reducing the risk of atherosclerosis in humans. Proc. Natl. Acad. Sci. USA 101, 6659–6663.

Fontana, L., Klein, S., Holloszy, J.O. and Premachandra, B.N. (2006) Effect of long-term calorie restriction with adequate protein and micronutrients on thyroid hormones. J. Clin. Endocrinol. Metab. 91, 3232–3235.

Fontana, L. and Klein, S. (2007) Aging, adiposity and calorie restriction. JAMA 297, 986–994.

Fontana, L., Weiss, E.P., Villareal, D., Klein, S. and Holloszy, J.O. (2008) Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. Aging Cell 7, 681–687.

Fontana, L., Partridge, L. and Longo, V.D. (2010 a) Extending healthy lifespan—from yeast to humans. Science 328, 321–326.

Fontana, L., Klein, S. and Holloszy, J.O. (2010 b) Effects of long-term calorie restriction and endurance exercise on glucose tolerance, insulin action and adipokine production. Age 32, 97–108.

Fontana L, Hu FB. (2014) Optimal body weight for health and longevity: bridging basic, clinical, and population research. Aging Cell 13, 391-400.

Galgani M, Procaccini C, De Rosa V, Carbone F, Chieffi P, La Cava A, Matarese G. Leptin modulates the survival of autoreactive CD4+ T cells through the nutrient/energy-sensing mammalian target of rapamycin signaling pathway. J Immunol 2010; 185:7474-7479.

Gems D, Partridge L. Genetics of longevity in model organisms: debates and paradigm shifts. Annu Rev Physiol. 2013;75:621-644.

Gems D, de la Guardia Y. Alternative Perspectives on Aging in Caenorhabditis elegans: Reactive Oxygen Species or Hyperfunction? Antioxid Redox Signal. 2013; 19:321-329.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004; 5: R80.

Gostner JM, Becker K, Fuchs D, Sucher R. Redox regulation of the immune response. Redox Rep 2013; 18:88-94.

Guo, Z., Mitchell-Raymundo, F., Yang, H., Ikeno, Y., Nelson, J., Diaz, V., Richardson, A. and Reddick, R. (2002) Dietary restriction reduces atherosclerosis and oxidative stress in the aorta of apolipoprotein E deficient mice. Mech. Ageing Dev. 123, 1121–1131.

Gustafsson S, Lind L, Söderberg S, Zilmer M, Hulthe J, Ingelsson E. Oxidative stress and inflammatory markers in relation to circulating levels of adiponectin. Obesity 2013; 21:1467-1473.

Hoek HW, van Hoeken D. Review of the prevalence and incidence of eating disorders. Int J Eat Disord. 2003 Dec;34(4):383-96.

Holland WL, Scherer PE. Cell Biology. Ronning after the adiponectin receptors. Science 2013; 342:1460-1461.

Holloszy, J.O. and Fontana, L. (2007) Calorie restriction in humans: an update. Exp. Gerontol. 42, 709–712.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Géloën, A., Even, P.C., Cervera, P. and Le Bouc, Y. (2003) IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 421, 182–187.

Ingram, D.K., Cutler, R.G., Weindruch, R., Renquist, D.M., Knapka, J.J., April, M., Belcher, C.T., Clark, M.A., Hatcherson, C.D., Marriott, B.M. and Roth, G.S. (1990) Dietary restriction and aging: the initiation of a primate study. J. Gerontol. 45, 148–163.

Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JC, Trent JM, Staudt LM, Hudson J Jr, Boguski MS, Lashkari D, Shalon D, Botstein D, Brown

PO. The transcriptional program in the response of human fibroblasts to serum. Science. 1999; 283:83-87.

Janas-Kozik M, Stachowicz M, Krupka-Matuszczyk I, Szymszal J, Krysta K, Janas A, Rybakowski JK. Plasma levels of leptin and orexin A in the restrictive type of anorexia nervosa. Regul Pept 2011; 168:5-9.

Kagawa, Y. (1978) Impact of westernization on the nutrition of Japanese: changes in physique, cancer, longevity and centenarians. Prev. Med. 7, 205–217.

Kannan S, Jaiswal AK. Low and high dose UVB regulation of transcription factor NF-E2-related factor 2. Cancer Res. 2006; 66:8421–8429.

Kastin AJ, Akerstrom V, Pan W. Activation of urocortin transport into brain by leptin. Peptides 2000; 21:1811-1817.

Kaye WH, Fudge JL, Paulus M. New insights into symptoms and neurocircuit function of anorexia nervosa. Nat Rev Neurosci. 2009 Aug; 10(8):573-84.

Kemnitz, J.W., Weindruch, R., Roecker, E.B., Crawford, K., Kaufman, P.L. and Ershler, W.B. (1993) Dietary restriction of adult male rhesus monkeys: design, methodology, and preliminary findings from the first year of study. J. Gerontol. 48, 17–26.

Kemnitz, J.W., Roecker, E.B., Weindruch, R., Elson, D.F., Baum, S.T. and Bergman, R.N. (1994) Dietary restriction increases insulin sensitivity and lowers blood glucose in rhesus monkeys. Am. J. Physiol. 266, 540–547.

Kennedy, M.A. et al. (2003) Long-living Ames dwarf mouse hepatocytes readily undergo apoptosis. Exp. Gerontol. 38, 997–1008.

Kenyon, C. (2005) The plasticity of aging: insights from long-lived mutants.

Cell 120, 449–460.

Kim, M.J., Aiken, J.M., Havighurst, T., Hollander, J., Ripple, M.O. and Weindruch, R. (1997) Adult-onset energy restriction of rhesus monkeys attenuates oxidative stress-induced cytokine expression by peripheral blood mononuclear cells. J. Nutr. 127, 2293–2301.

Kim SK, Novak RF. The role of intracellular signaling in insulin-mediated regulation of drug metabolizing enzyme gene and protein expression. Pharmacol Ther. 2007; 113:88–120.

Kowalska I, Karczewska-Kupczewska M, Strączkowski M. Adipocytokines, gut hormones and growth factors in anorexia nervosa. Clin Chim Acta 2011; 412:1702-1711.

Kristan, D.M. (2008) Caloric restriction and susceptibility to intact pathogens. Age (Dordr.) 30, 147–156.

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA. Circos: an information aesthetic for comparative genomics. Genome Res. 2009; 19:1639-1645.

Kurosu, H., Yamamoto, M., Clark, J.D., Pastor, J.V., Nandi, A., Gurnani, P., McGuinness, O.P., Chikuda, H., Yamaguchi, M., Kawaguchi, H., Shimomura, I., Takayama, S., Herz, J., Kahn, C.R., Rosenblatt, K.P. and

Kuro-o, M. (1998) Suppression of aging in mice by the hormone Klotho. Science 309, 1829–1833.

Kwak MK, Itoh K, Yamamoto M, Kensler TW. Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response elementlike sequences in the nrf2 promoter. Mol Cell Biol. 2002; 22:2883-2892.

La Cava A, Matarese G. The weight of leptin in immunity. Nat Rev 2004; 4:371-379.

Labruna G, Pasanisi F, Nardelli C, Caso R, Vitale DF, Contaldo F, Sacchetti L. High leptin/adiponectin ratio and serum triglycerides are associated with an "at-risk" phenotype in young severely obese patients. Obesity (Silver Spring) 2011; 19:1492-1496.

Lane, M.A., Baer, D.J., Rumpler, W.V., Weindruch, R., Ingram, D.K., Tilmont, E.M., Cutler, R.G. and Roth, G.S. (1996) Calorie restriction lowers body temperature in rhesus monkeys, consistent with a postulated anti-aging mechanism in rodents. Proc. Natl. Acad. Sci. USA 93, 4159–4164.

Lee CK, Klopp RG, Weindruch R, Prolla TA. Gene expression profile of aging and its retardation by caloric restriction. Science. 1999; 285:1390-1393.

Longo, V.D. and Fontana, L. (2010) Calorie restriction and cancer: metabolic and molecular mechanisms. Trends Pharmacol. Sci. 31, 89–98.

Marcos A, Nova E, Montero A. Changes in the immune system are conditioned by nutrition. Eur J Clin Nutr 2003; 57 Suppl 1:S66-69.

Masoro EJ. Overview of caloric restriction and ageing. Mec Ageing Dev 2005; 126: 913-922.

Matarese G, La Cava A. Leptin in autoimmunity: many questions, some answers. Tissue Antigens 2007; 70:87-95.

Matarese G, La Rocca C, Moon HS, Huh JY, Brinkoetter MT, Chou S, Perna F, Greco D, Kilim HP, Gao C, Arampatzi K, Wang Z, Mantzoros CS. Selective capacity of metreleptin administration to reconstitute CD4+ T-cell number in females with acquired hypoleptinemia. PNAS 2013; 110:E818-827.

Mattson, M.P. (2005) Energy intake, meal frequency, and health: a neurobiological perspective. Annu. Rev. Nutr. 25, 237–260.

McCay CM, Crowel MF, Maynard LA. The effect of retarded growth upon the length of the lifespan and upon the ultimate body size. J Nutr 1935; 10: 63-79.

Meyer, T.E., Kovács, S.J., Ehsani, A.A., Klein, S., Holloszy, J.O. and Fontana, L. (2006) Long-term caloric restriction ameliorates the decline in diastolic function in humans. J. Am. Coll. Cardiol. 47, 398–402.

Modan-Moses D, Stein D, ParienteC, Yaroslavsky A, Ram A, Faigin M, Loewenthal R, Yissachar E, Hemi R, Kanety H. Modulation of adiponectin and leptin during refeeding of female anorexia nervosa patients. J Clin Endocrinol Metab 2007; 92:1843-1847.

Muller, F.L., Lustgarten, M.S., Jang, Y., Richardson, A. and Van Remmen H. (2007) Trends in oxidative aging theories. Free Radic. Biol. Med. 43, 477–503.

Muñoz A, Costa M. Nutritionally mediated oxidative stress and inflammation. Oxid Med Cell Longev 2013; 2013:610950.

Nova E, Marcos A. Immunocompetence to assess nutritional status in eating disorders. Expert Rev Clin Immunol 2006; 2:433-444.

Omodei D, Licastro D, Salvatore F, Crosby SD, Fontana L. Serum from humans on long-term calorie restriction enhances stress resistance in cell culture. Aging (Albany NY) 2013; 5:599-606.

Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat Rev Immunol 2011; 11:85-97.

Pannacciulli N, Vettor R, Milan G, Granzotto M, Catucci A, Federspil G, De Giacomo P, Giorgino R, De Pergola G. Anorexia nervosa is characterized by increased adiponectin plasma levels and reduced nonoxidative glucose metabolism. J Clin Endocrinol Metab 2003; 88:1748-1752.

Pászthy B, Svec P, Vásárhelyi B, Túry F, Mazzag J, Tulassay T, Treszl A. Investigation of regulatory T cells in anorexia nervosa. Eur J Clin Nutr 2007; 61:1245-9.

Pearce EL, Poffenberger MC, Russell CC, Jones G. Fueling Immunity: Insights into Metabolism and Lymphocyte Function. Science 2013; 342.

Plant S, Shand B, Elder P, Scott R. Adiponectin attenuates endothelial dysfunction induced by oxidised low-density lipoproteins. Diab Vasc Dis Res 2008; 5:102-108.

Procaccini C, De Rosa V, Galgani M, Carbone F, Cassano S, Greco D, Qian K, Auvinen P, Calì G, Stallone G, Formisano L, La Cava A, Matarese G. Leptin-induced mTOR activation defines a specific molecular and transcriptional signature controlling CD4+ effector T cell responses. J Immunol 2012; 189:2941-2953.

Procaccini C, De Rosa V, Galgani M, Abanni L, Calì G, Porcellini A, Carbone F, Fontana S, Horvath TL, La Cava A, Matarese G. An oscillatory switch in mTOR Kinase activity sets regulatory T cell responsiveness. Immunity 2010; 33:1-13.

Reed, M.J., Penn, P.E., Li, Y., Birnbaum, R., Vernon, R.B., Johnson, T.S., Pendergrass, W.R., Sage, E.H., Abrass, I.B. and Wolf, N.S. (1996) Enhanced cell proliferation and biosynthesis mediate improbe wound repair in refed, caloric-restricted mice. Mech. Ageing Dev. 89, 21–43.

Ritchie ME, Diyagama D, Neilson J, van Laar R, Dobrovic A, Holloway A, Smyth GK. Empirical array quality weights in the analysis of microarray data. BMC Bioinformatics. 2006; 7:261.

Roth, G.S., Handy, A.M., Mattison, J.A., Tilmont, E.M., Ingram, D.K. and Lane, M.A. (2002) Effects of dietary caloric restriction and aging on thyroid hormones of rhesus monkeys. Horm. Metab. Res. 34, 378–382.

Saito H, Nomura K, Hotta M, Takano K. Malnutrition induces dissociated changes in lymphocyte count and subset proportion in patients with anorexia nervosa. Int J Eat Disord 2007; 40:575-579.

Salmon, A.B. et al. (2005) Fibroblast cell lines from young adult mice of longlived mutant strains are resistant to multiple forms of stress. Am. J. Physiol. Endocrinol. Metab. 289, E23–E29.

Shi L, Reid LH, Jones WD, Shippy R, Warrington JA, Baker SC, Collins PJ, de Longueville F, Kawasaki ES, Lee KY, Luo Y, Sun YA, Willey JC, Setterquist RA, Fischer GM, Tong W, et al. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. Nat Biotechnol. 2006; 24:1151-1161.

Shimokawa, I., Higami, Y., Hubbard, G.B., McMahan, C.A., Masoro, E.J. and Yu, B.P. (1993) Diet and the suitability of the male Fischer 344 rat as a model for aging research. J. Gerontol. 48, B27–B32.

Sohal, R.S. and Weindruch, R. (1996) Oxidative stress, caloric restriction and aging. Science 273, 59–63.

Sohal, R.S., Mockett, R.J. and Orr, W.C. (2002) Mechanisms of aging: an appraisal of the oxidative stress hypothesis. Free Radic. Biol. Med. 33, 575–

586.

Sonntag, W.E., Lynch, C.D., Cefalu, W.T., Ingram, R.L., Bennett, S.A., Thornton, P.L. and Khan, A.S. (1999) Pleiotropic effects of growth hormone and insulin like growth factor (IGF)-1 on biological aging: inferences from

moderate caloric-restricted animals. J. Gerontol. A: Biol. Sci. Med. Sci. 54, B521–B538.

Spoettl T, Hausmann M, Klebl F, Dirmeier A, Klump B, Hoffmann J, Herfarth H, Timmer A, Rogler G. Serum soluble TNF receptor I and II levels correlate with disease activity in IBD patients. Inflamm Bowel Dis 2007; 13:727-32.

Terra X, Auguet T, Agüera Z, Quesada IM, Orellana-Gavaldà JM, Aguilar C,Jiménez-Murcia S, Berlanga A, Guiu-Jurado E, Menchón JM, Fernández-Aranda F, Richart C. Adipocytokine levels in women with anorexia nervosa. Relationship with weight restoration and disease duration. Int J Eat Disord 2013; 46:855-861.

Walford, R.L., Harris, S.B. and Gunion, M.W. (1992) The calorically restricted low-fat nutrient-dense diet in Biosphere 2 significantly lowers blood glucose, total leukocyte count, cholesterol and blood pressure in humans. Proc. Natl. Acad. Sci. USA 89, 11533–11537.

Weindruch R, Walford RL. The retardation of aging and disease by dietary restriction. Springfield, IL, Charles C Thomas Publisher 1988.

Weindruch, R. and Walford, R.L. (1982) Dietary restriction in mice beginning at 1 year of age: effect on life span and spontaneous cancer incidence. Science 215, 1415–1418.

Wettenhall JM, Simpson KM, Satterley K, Smyth GK, affylmGUY: a graphical user interface for linear modeling of single channel microarray data. Bioinformatics. 2006; 22:897-899.

Willcox, B.J., Willcox, D.C., Todoriki, H., Fujiyoshi, A., Yano, K., He, Q., Curb, J.D. and Suzuki, M. (2007) Caloric restriction, the traditional Okinawan diet, and healthy aging: the diet of the world's longest-lived people and its potential impact on morbidity and life span. Ann. N.Y. Acad. Sci. 1114, 434–455.

Wu D, Smyth GK. Camera: a competitive gene set test accounting for inter-gene correlation. Nucleic Acids Res. 2012; 40:e133.

Wu D, Lim E, Vaillant F, Asselin-Labat ML, Visvader JE, Smyth GK. ROAST: rotation gene set tests for complex microarray experiments. Bioinformatics. 2010; 26:2176-2182.

6. APPENDIX

List of Pubblications

1. Simeone A., Di Salvio M., Di Giovannantonio L.G., Acampora D., **Omodei D.**, Tomasetti C. *The role of Otx2 in Adult Mesencephalic-Diencephalic Dopaminergic Neurons.* Molecular Neurobiology. 2011 Apr; 43(2): 107-13.

2. Simeone A., Puelles E., **Omodei D.**, Acampora D., Di Giovannantonio L.G., Di Salvio M., Mancuso P., Tomasetti C. *Otx genes in neurogenesis of mesencephalic dopaminergic neurons.* Developmental Neurobiology. 2011 Aug; 71(8): 665-79.

3. Soare A., Cangemi R., **Omodei D.**, Holloszy J.O., Fontana L. *Long-term calorie restriction, but not endurance exercise, lowers core body temperature in humans*. Aging (Albany NY). 2011 Apr; 3(4): 374-9.

4. **Omodei D.**, Fontana L. *Calorie Restriction and prevention of age-associated chronic disease*. FEBS Letters. 2011 June; 585(11): 1537-42.

5. Fabbrini E, Higgins PB, Magkos F, Bastarrachea RA, Voruganti VS, Comuzzie AG, Shade RE, Gastaldelli A, Horton JD, **Omodei D**, Patterson BW, Klein S. *Metabolic response to high-carbohydrate and low-carbohydrate meals in a nonhuman primate model.* Am J Physiol Endocrinol Metab. 2013 Feb 15;304(4):E444-51

6. **Omodei D**, Licastro D, Salvatore F, Crosby SD, Fontana L. *Serum from humans on long-term calorie restriction enhances stress resistance in cell culture.* Aging (Albany NY). 2013 Aug;5(8):599-606.

7. Di Giovannantonio LG, Di Salvio M, **Omodei D**, Prakash N, Wurst W, Pierani A, Acampora D, Simeone A. *Otx2 cell-autonomously determines dorsal mesencephalon versus cerebellum fate independently of isthmic organizing activity.* Development. 2014 Jan;141(2):377-88.

8. **Omodei D**, Pucino V, Labruna G, Procaccini C, Galgani M, Perna F, Pirozzi D, De Caprio C, Marone G, Fontana L, Contaldo F, Pasanisi F, Matarese G, Sacchetti L. *Immune-metabolic profiling of anorexic patients reveals an anti-oxidant and anti-inflammatory phenotype*. Metabolism. 2015 Mar; 64(3):396-405.