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XXXI

CHRONIC KIDNEY DISEASE AS AN AGE-RELATED DISEASE: NEW STUDY PERSPECTIVES FROM ANIMAL MODELS TO HOSPITALIZED PATIENTS

Settore Scientifico Disciplinare BIO/09 (Fisiologia)

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Abstract

Chronic kidney disease (CKD) is a major public health problem worldwide and its main consequences include the loss of renal function leading to end-stage renal disease (ESRD), an increased risk of cardiovascular disease (CVD), a significant increase in morbidity and mortality, and a decrease in health related quality of life.

The risk of CKD increases with age, though there seems to be a complex relationship between ageing and this disease: elderly patients are overrepresented in the dialysis population and geriatric complications are highly detectable in younger patients with ESRD. This has led to the hypothesis of a premature biological ageing process of different organ systems associated with CKD.

The present research work was based on translational approach to study the role of many CKD risk factors such as hypertension, oxidative stress/inflammation, obesity, and hyperuricemia with the aim of identifying new molecular mechanisms of kidney damage to prevent it by successful behaviour modifications. For this purpose, both human and animal models were used.

Human pathological models: in both ESRD and obese patients, the role of oxidative stress, inflammation and hyperuricemia in progression and complications of CKD was investigated.

Human physiological models: in a consistent healthy population, the oxidative status and its correlation with traditional cardiovascular risk factors were examined. In addition, the health history data of centenarian subjects was utilized to study the clinical and prognostic value of traditional cardiovascular risk factors in relation to mortality.

Animal models: the mechanisms renal damage, induced by hypertension (Spontaneously Hypertensive Rat) and obesity (Cafeteria diet rats), were verified. In this context, the antioxidant and cytoprotective effects of a nutraceutical (Bergamot extract) on obesity was also tested.

This multilevel approach has allowed us to individually and synergistically analyze some aspects of the complex pathogenic mechanism of CKD, in order to clarify the

role of the new amplifying risk factors for CKD and to prepare an effective personalized prevention plan by acting on both modifiable and non-modifiable risk factors.

Chapter 1. Introduction

1.1 Ageing as public health priority

With the extension of life expectancy, the percentage of elderly individuals in the general population has considerably increased, whereby understanding why ageing results in progressively higher vulnerability to chronic morbidity, disability, and frailty has become a public health priority [Bektas et al., 2018].

The population ageing is a worldwide burden, and the number of older adults is increasing at an accelerating rate: it is estimated that in the next 30 years at least 20% of the population will be aged >60 years and the most substantial increase will be observed in the oldest-age group (aged>85 years) [Kinsella and He, 2009]. Population ageing occurs at various rates in different geographic regions (Figure 1) wherein Europe currently includes the most aged population but, in the next years, it is anticipated that Asia, South America, and Africa will experience the most rapid rate of increase in population ageing [Bektas et al., 2018]. These demographic changes in age composition will affect needs and demand for health and social care in many countries worldwide and urgently require the adaptation of health policies to tackle complex chronic diseases and disabilities and to improve elders' quality of life [Christensen et al., 2009].

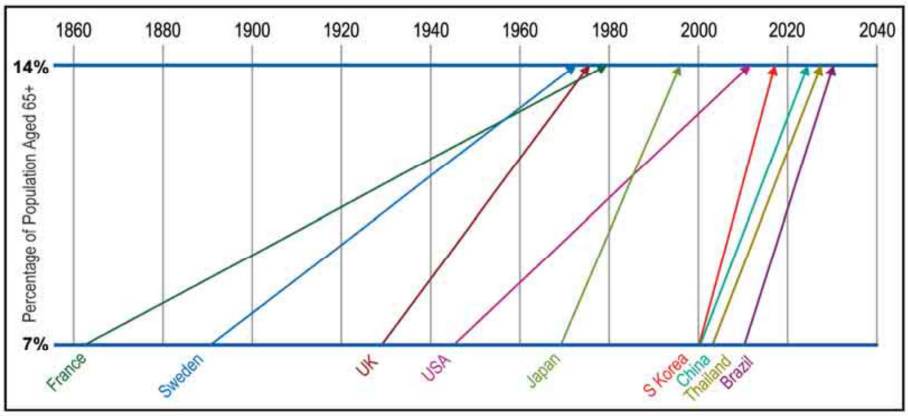


Fig. 1. The Speed of Population Ageing: time required or expected for percentage of population aged 65 and over torise from 7 to 14 % [Kinsella and He, 2009]

1.2 Inflammageing and its potential contribution to age-associated diseases

Ageing is a ubiquitous and physiological phenomenon influenced by a complex interaction between genetic and environmental factors. The intracellular and cellular processes that contribute to ageing include genomic instability, mitochondrial dysfunction, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, cellular senescence, stem cell exhaustion and altered intercellular communication [Rebelo-Marques et al., 2018]. This phenomenon can alter cell population and damage cell functionality, thereby compromising the function of physiological systems (e.g. immune system, musculoskeletal system, cardiovascular system, endocrine system, and nervous systems), increasing the risk of organism failure (Figure 2).

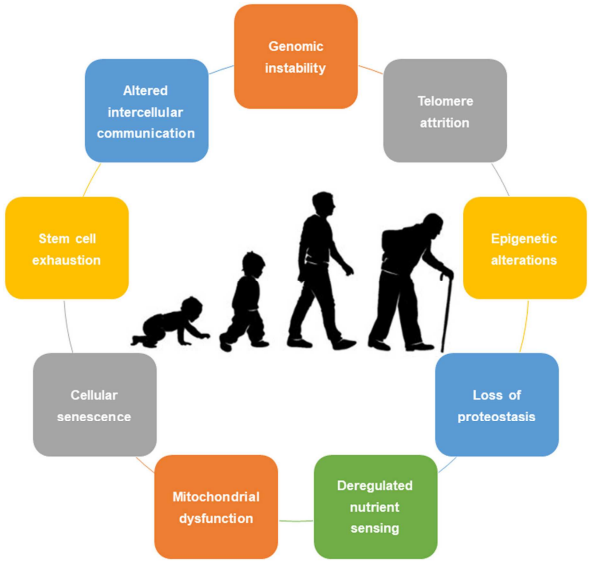


Fig. 2. Ageing hallmarks [Rebelo-Marques et al., 2018]

A salient feature of ageing tissues is chronic inflammation, characterized by high levels of pro-inflammatory markers, a condition defined “inflammageing”, a term first coined in 2000 by Claudio Franceschi [Franceschi et al., 2000]. Inflammageing describes the low-grade, chronic, systemic inflammation in ageing, in the absence of overt infection (“sterile” inflammation), and represents a highly significant risk factor

for both morbidity and mortality in elderly people [Franceschi et al., 2000]. This pro-inflammatory state is characterized by high levels of circulating pro-inflammatory mediators, including interleukines, C-reactive protein (CRP), interferon (IFNα) and IFNβ, transforming growth factor-β (TGFβ), tumour necrosis factor-α (TNF) and its soluble receptors, and serum amyloid A. Under physiological conditions, these plasmatic inflammatory mediators are involved in defense mechanisms against infections or extraneous molecules but when their expression is exacerbated and prolonged, they become detrimental. Epidemiological studies have demonstrated that inflammageing is a risk factor for many age-related diseases such as: cardiovascular diseases (CVD), cancer, chronic kidney disease (CKD), dementia, and depression as well as for global indicators of poor health status, such as multimorbidity, mobility and disability in daily activities, sarcopenia, frailty and premature death (Figure 3) [Salimi et al., 2018; Leonardi et al., 2018]. On the basis of these findings, inflammageing should be considered one of the pillars of the biology of ageing and a marker of accelerated ageing. However, it is unclear if inflammation causes the associated pathology directly or is instead a biomarker for the rate of biological ageing.

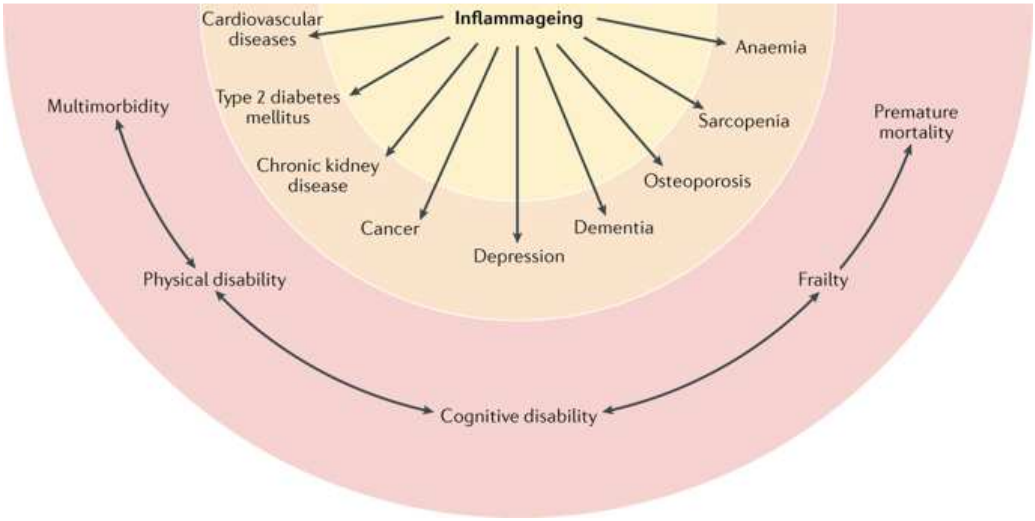


Fig. 3. Inflammageing as a risk factor for several chronic diseases [Ferrucci and Fabbri, 2018]

1.3 Oxidative stress and antioxidant defence mechanisms

Oxidative stress occurs when there is an imbalance between the production of free radical species and the antioxidant ability to neutralize their harmful effects [Salisbury et al., 2015]. Free radicals can be defined as higly reactive molecular species (atoms or molecules) that contain one or more unpaired electrons in their external shell or outer orbit and are capable of independent existence [Chandrasekaran et al., 2017]. In cells, these radicals can act as oxidants or reductants by losing or accepting a single electron and are continuously produced by the organism's normal use of oxygen [Lobo et al., 2010]. Free radicals include reactive radical and non-radical derivatives of oxygen (ROS) and nitrogen (RNS) that are collectively called reactive oxygen nitrogen species (RONS) [Powers et al., 2011]. The generation of RONS is a physiological process and, at moderate or low levels, RONS are important molecules involved in a number of cellular signaling pathways, in the extraction of energy from organic molecules, in immune defense, in mitogenic response, and in redox regulation [Genestra et al., 2007]. An excess production or a decreased scavenging of RONS has been implicated in ageing and age-related diseases [Venkataraman et al., 2013]. Both endogenous and exogenous sources of RONS have been described. The endogenous sources of RONS include different subcellular organelles such as mitochondria, peroxisomes and endoplasmic reticulum, where oxygen consumption is high [Phaniendra et al., 2015]. NADPH oxidase is the prevalent source of the superoxide radical ($\bullet O_2^-$), which is formed by the addition of one electron leak from the electron transport system during cellular respiration to the molecular oxygen [Miller et al., 1990]. Most of the superoxide is dismutated into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) [Genestra et al., 2007]. H_2O_2 is a neutral molecule because it has no unpaired electrons, but it is able to form the most reactive and dangerous radical, the hydroxyl radical (•OH), through the Fenton or Haber–Weiss reaction. Hydroxyl radicals mainly react with phospholipids in cell membranes and proteins. In activated neutrophils, in the presence of chloride and myeloperoxidase, H_2O_2 can be converted to hypochlorous acid that can react with

DNA and produce pyrimidine oxidation products and add chloride to DNA bases [Kulcharyk et al., 2001]. Another important determinant in the cellular redox equilibrium is nitric oxide (NO). In mammals, NO can be generated by three main isoforms of nitric oxide synthase (NOS): endothelial NOS, related to vasodilation and vascular regulation, neuronal NOS, linked to cellular signaling, and inducible NOS, activated in response to various endotoxin or cytokine signals [Adams et al., 2015]. All isoforms of NOS utilize arginine as the substrate, and molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates. The reaction of NO with superoxide radical ($\bullet O_2^-$) forms the potent oxidant peroxynitrite (ONOO−). This compound can cause oxidative damage, nitration, and S-nitrosylation of biomolecules including proteins, lipids, and DNA [Mikkelsen et al., 2003]. Nitrosative stress by ONOO− has been implicated in DNA single-strand breakage, followed by poly-ADP-ribose polymerase (PARP) activation [Ridnour et al., 2004].

Exogenous sources of RONS are numerous and include: air and water pollution, pesticides, tobacco, alcohol, heavy metals (Fe, Cu, Co, Cr) or transition metals (Cd, Hg, Pb, As), drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), industrial solvents, cooking (smoked meat, waste oil, fat), and radiation. Inside the body, all these substances are metabolized into free radicals [Phaniendra et al., 2015].

Endogenous or exogenous RONS are capable of damaging biologically relevant molecules with consequent cell damage and homeostatic disruption [Young et al., 2001]. Among them, lipids, carbohydrates, nucleic acids, and proteins are the major targets and their oxidative modification can also be used as markers of oxidative stress (Figure 4) [Frijhoff et al., 2015].

Free radicals can damage cells by several mechanisms:

1. lipid peroxidation and loss in membrane fluidity: double bonds in polyunsaturated membrane lipids are vulnerable to attacks by oxygen free radicals;

2. protein cross-linking: free radicals promote sulfhydryl-mediated protein cross-linking, resulting in increased degradation or loss of activity;
3. DNA fragmentation;
4. oxidative damage on carbohydrates impairs functions of some cellular receptors including those associated with hormonal and neurotransmitter responses.

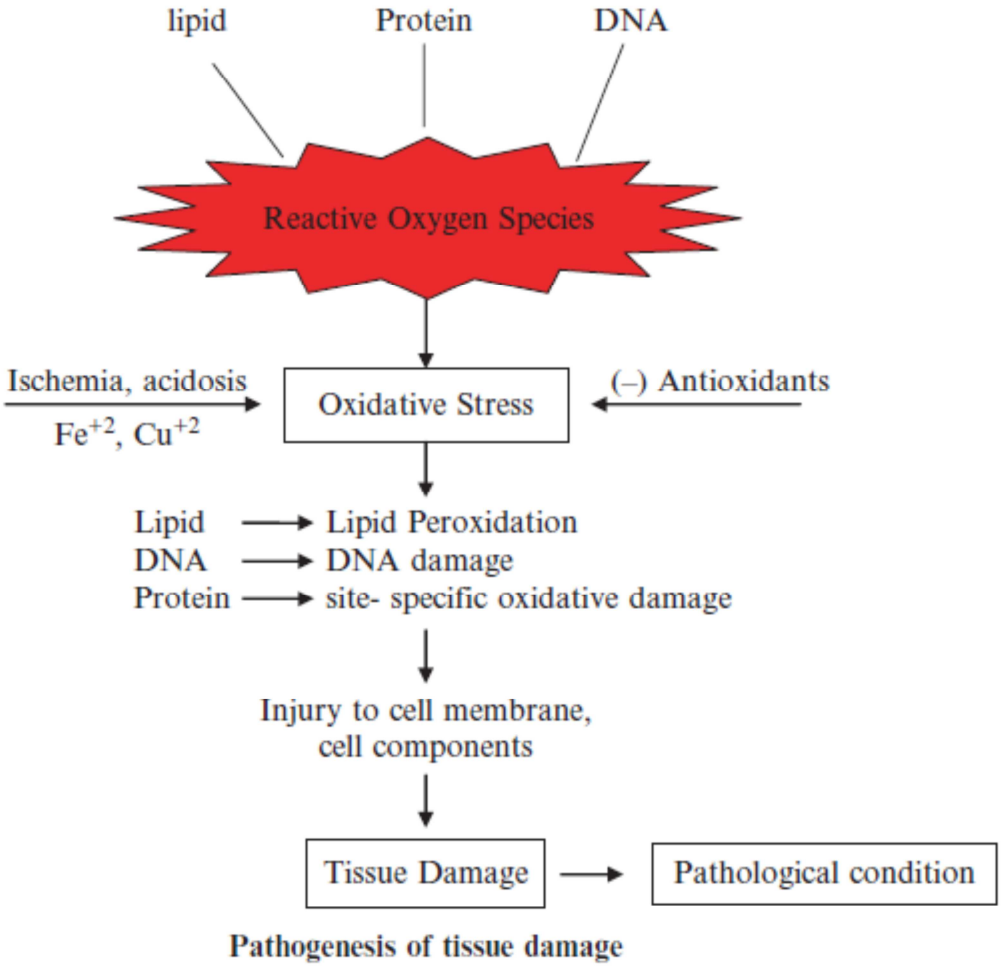


Fig. 4. Oxidative stress and tissue damage [Rao et al., 2011]

Overproduction of oxygen-derived free radicals has been implicated in the pathogenesis of over 200 clinical conditions. Tissue injury and its healing are characterized by a sequence of various events influenzed by the cause of the injury

and other factors, such as the intensity of the damageing agent, type of tissue and condition of the whole organism [Chettibi et al., 1999]. The healing process is mediated by a variety of messengers released by the immune system; for example, phagocytes produce cytotoxic agents, which not only prevent the spread of infection but also remove host cellular particles that are damaged [Juranek and Bezek, 2005].

The most important cellular defence mechanism is represented by antioxidant systems. The cells contain important antioxidant defense mechanisms that protect against free radical toxicity and include both endogenous and exogenous molecules. Endogenous antioxidants (naturally generated *in situ*) include enzymatic and non-enzymatic molecules. The primary enzymatic scavengers are: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). SOD catalyses the dismutation of superoxide to hydrogen peroxide, which is decomposed into water and oxygen by CAT. In addition, GSH-Px converts peroxides and hydroxyl radicals into non-toxic forms by the oxidation of reduced glutathione (GSH) into glutathione disulfide and further reduced to GSH by glutathione reductase [Birben et al., 2012]. The non-enzymatic antioxidants are molecules such as glutathione, L-arginine, CoQ10, melatonin, albumin and uric acid (85% of antioxidant capacity in plasma) that interact with RONS and terminate the free radical chain reactions [Wu et al., 2013]. Exogenous non-enzymatic antioxidants, supplied through foods, include: ascorbic acid (vitamin C), which scavenges hydroxyl and superoxide radicals; α-tocopherol (vitamin E), which protects against lipid peroxidation of cell membranes; phenolic antioxidants (resveratrol, phenolic acids, and flavonoids), lecithin oil, selenium, zinc, and drugs such as acetylcysteine [Pisoschi and Pop, 2015].

1.4 The oxidative stress theory of ageing and risk of diseases

The free radical theory of ageing originally described by Denham Harman in the 50s [Harman, 1956] and later termed as oxidative stress theory of ageing, is based on the hypothesis that the accumulation of oxidative damage to macromolecules (lipids,

DNA, and proteins) by RONS leads to deleterious progressive age-associated functional losses over time [Beckman and Ames, 1998].

The mechanisms of oxidative stress-induced ageing are not well elucidated, but it is probable that high levels of RONS lead to cellular senescence, a physiological mechanism that irreversibly arrests cellular proliferation in response to damages that occur during replication. Senescent cells gain a permanent senescence-associated secretory phenotype (SASP), characterized by the secretion of soluble mediators (interleukins, chemokines, and growth factors), degradative enzymes like matrix metalloproteases (MMPs), and insoluble proteins/extracellular matrix (ECM) components [Chandrasekaran et al., 2017].

Oxidative stress and cellular senescence are involved in both acute and chronic pathological processes, such as cancer, cardiovascular, renal and neurodegenerative diseases. Given the close relationship between oxidative stress, inflammation and ageing, the oxidation-inflammatory theory of ageing or “oxi-inflamm-ageing” has been proposed: ageing represents a loss of homeostasis due to chronic oxidative stress that mainly disturbs the regulatory systems (nervous, endocrine and immune systems). The resulting activation of the immune system induces an inflammatory state that generates a vicious circle in which chronic oxidative stress and inflammation reciprocally increase in severity, and as a consequence, age-related morbidity and mortality increase (Figure 5) [De la Fuente and Miquel, 2009].

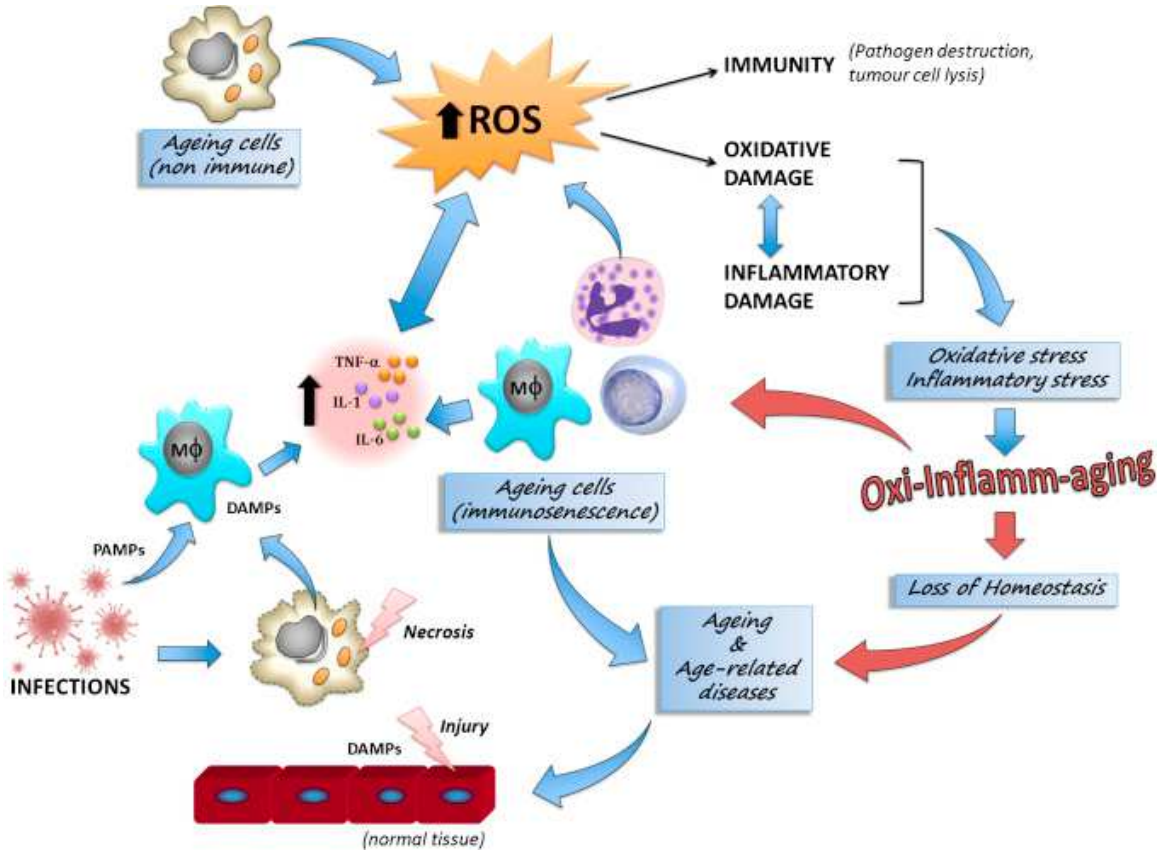


Fig. 5. Oxi-Inflamm-ageing mechanisms [Bauer and De la Fuente, 2016]

1.5 CKD as age related disease

CKD is recognised as a global health problem with a high rates of morbidity, mortality, and elevated healthcare costs [Balasubramanian, 2013]. CKD affects 10-16% of the adult population around the world [Coresh, 2007] with a mortality rate of 109.7 per 1000 patients/years [Kokubo et al., 2009]. A recent meta-analysis of observational studies reported that CKD has a high global prevalence (between 11% and 13%) with a higher percentage in developed areas such as Europe, USA, Canada and Australia, where the elderly population is greater than in developing areas [Hill et al., 2016]. In Italy, the prevalence of CKD is 8.1% in men and 7.8% in women, with a higher prevalence of early stages compared to final stages. Although many young patients are affected by CKD due to congenital disorders (glomerulonephritis and type I diabetes), the risk of CKD increases with age and elderly patients are overrepresented in the dialysis population [Nitta et al., 2013]. Firstly, this is due to the age-related decrease in kidney function due to glomerular, tubular and vascular

changes, and secondly to the fact that kidney-related diseases, such as type II diabetes and generalized atherosclerosis, often affect the elderly [Nitta et al., 2013]. In addition, due to its reduced functional reserve, the ageing kidney is more susceptible to nephrotoxic agents and drugs [Abdel-Kader and Palevsky, 2009]. The main clinical manifestation of CKD is the loss of glomerular filtration rate (GFR), which allows for staging of CKD with progressively decreasing (estimated) GFR. According to the National Kidney Foundation, the Kidney Disease Outcomes Quality Initiative and the Kidney Disease-Improving Global Outcomes convention, CKD is subdivided into 5 different stages (Figure 6): the first two stages have normal (GFR ≥ 90 ml/min/1.73m^2) or mild reduced kidney function (GFR between 60 and 89 ml/min/1.73m^2) while stages 3-5 have a severe reduction of kidney function (GFR 15-29 ml/min/1.73m^2) that lead to end-stage renal disease (ESRD). Because of their diagnostic relevance, the intermediate and most common stage 3 has recently been subclassified into stages 3a and 3b. Notably, early stages 2 and 3 should be rapidly identified and targeted with prophylactic therapies, such as lipid lowering drugs or RAS modifiers, to minimize the progression of CKD [Choudhury et al., 2008].

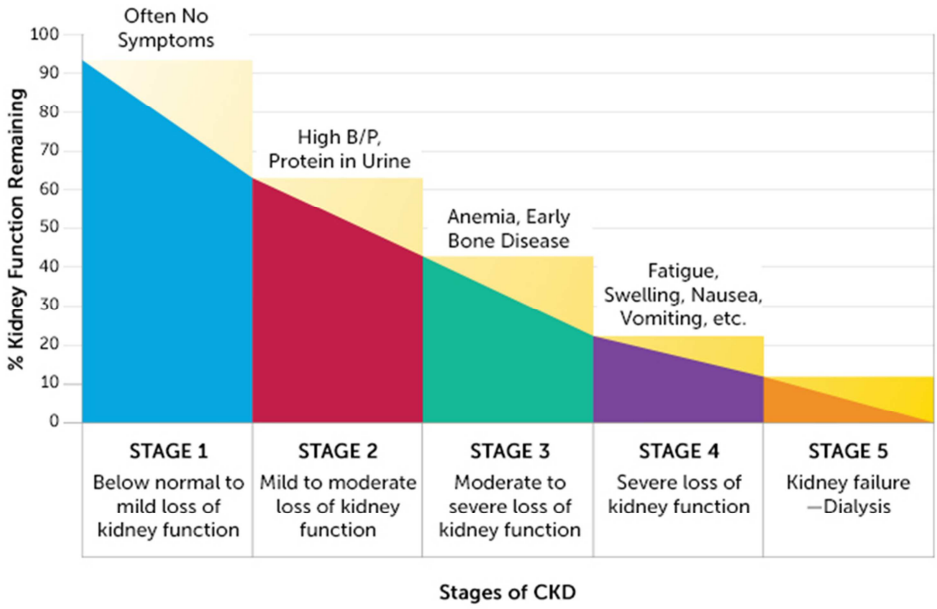


Fig. 6. Progression of CKD

When reaching ESRD, patients are usually prepared for renal replacement therapy. There are three main forms of renal replacement therapy: hemodialysis (HD),

peritoneal dialysis (PD) and kidney transplantation. Although no absolute chronological age limit for renal transplantation can be identified, most elderly patients are either treated with intermittent HD or PD. Dialysis is very efficient in removing uremic toxins and water, as well as in correcting electrolyte and acid-base disorders, but it also increases the risk for homeostatic imbalance. During HD, patients are generally treated three times weekly for 3-4 hours and the blood, by an arteriovenous fistula and an artificial circulation, is cleansed by diffusion through an artificial membrane with a purified solution (dialysate) [Tordoir et al., 2007]. Peritoneal dialysis instead uses the peritoneal membrane as exchange interface by a sterile dialysis fluid installed in the peritoneal cavity. This fluid is exchanged several times either manually or using a mechanical cyclic device. Patients starting on peritoneal dialysis show better initial outcomes and preservation of residual renal function in the first 2 years, compared with patients on haemodialysis but these differences become stable after 2 years [Leurs et al., 2015].

When available, kidney transplantation should be carefully evaluated according to age and comorbidities such as cancer, chronic infections, cardiac or peripheral vascular disease and the risk of medical noncompliance. The half-life of a transplanted kidney is <20 years, making these patients also potential candidates for CKD treatments during their lifespan [Chang, et al., 2012; Allen, et al., 2017].

Chronic kidney disease is defined as kidney damage or decreased kidney function for 3 months or more with or without regard to the presence or absence of the causes of kidney damage [Levey et al., 2003]. The initiating causes of CKD are highly variable since epidemiological studies revealed that in CKD patients unmodifiable and modifiable risk factors can be defined (Figure 7). The first include age, gender, ethnicity, genetic components and low birth weight; the second comprise drug toxicity, inflammation, obesity, oxidative stress, hyperuricemia, hypertension, autoimmune diseases and urinary tract infections [Tanner et al., 2012].

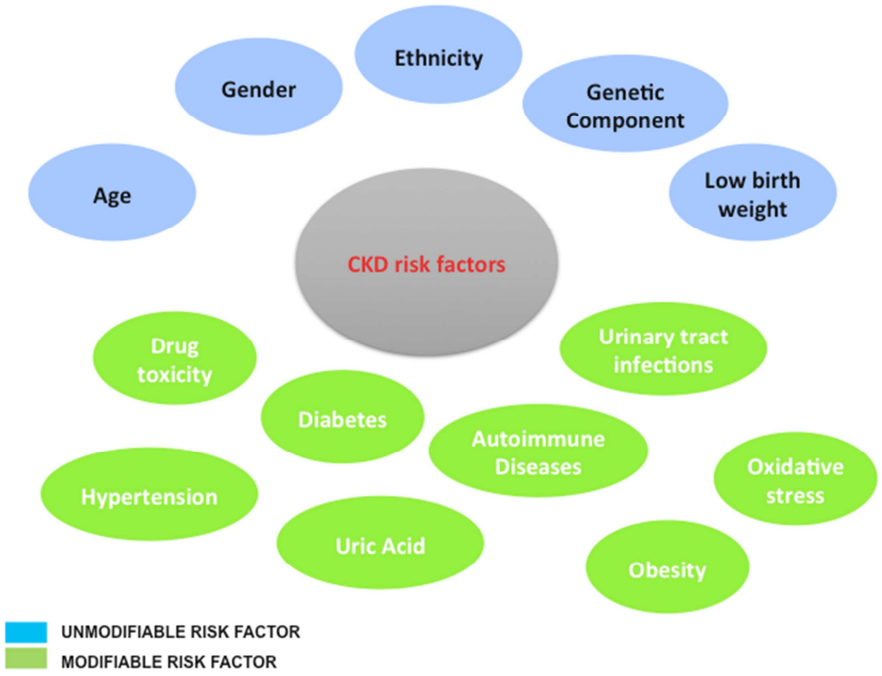


Fig. 7. Modifiable and Unmodifiable CKD risk factors

The pathophysiology of CKD involves two mechanisms (Figure 8): the initial mechanism of the specific underlying aetiology as immune complex glomerulonephritis or exposure to toxins in some renal tubules and interstitial disease, and a series of progressive mechanisms, involving hyperfiltration and hypertrophy of remaining viable nephrons [George and Neilson, 2008]. In addition, inflammation causes the epithelial-mesenchymal transitions of renal tubular cells that move away from the basal membrane and form new interstitial fibroblasts that lead to tissue fibrosis. Interstitial fibrosis seems to drive further nephron injury through the promotion of renal ischaemia [Schnaper, 2017]. Remaining viable nephrons lose the ability to perform autoregulation, resulting in systemic hypertension, which will ultimately be more damageing to the glomerulus and worsen the CKD progression [George and Neilson, 2008].

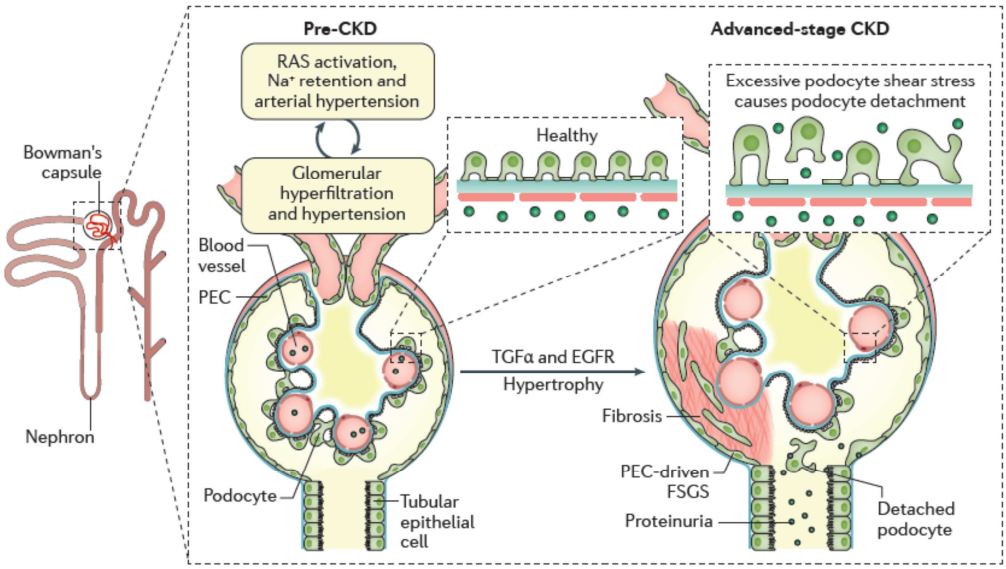


Fig.8. Pathophysiology of CKD [Romagnani et al., 2017]

There are many non-hemodynamic factors that play a role in the CKD progression such as angiotensin II, aldosterone, endothelin, acidosis and oxidative stress. Angiotensin II contributes to the inflammation process by activing cytokines, adhesion molecules, transcription factors and monocytes. Angiotensin II also increases the synthesis of extracellular matrix, hydraulic pressure of the glomerulus and podocyte cell damage. Aldosterone amplifies the glomerular injury by the proliferation of mesangial cells, apoptosis, hypertrophy and podocyte cell damage. Moreover, aldosterone causes structural and functional damage to blood vessels by acting as an angiotensin II-mediator [Agarwal et al., 2004]. Endothelin is a potent vasoconstrictor whose level increases during CKD, and causes higher pressure on efferent blood vessels than on afferent blood vessels, thus resulting in increased glomerular hydraulic pressure. Metabolic acidosis, due to a compromised capacity of the kidney to excrete ammonium or reabsorb bicarbonate, is a common complication of CKD, particularly in patients with a GFR below 20%. The increased ammonia production activates the alternative complement pathway while the acidosis status stimulates the formation of both endothelin and aldosterone that promote renal fibrosis. Oxidative stress accelerates the progression of CKD through cardiovascular

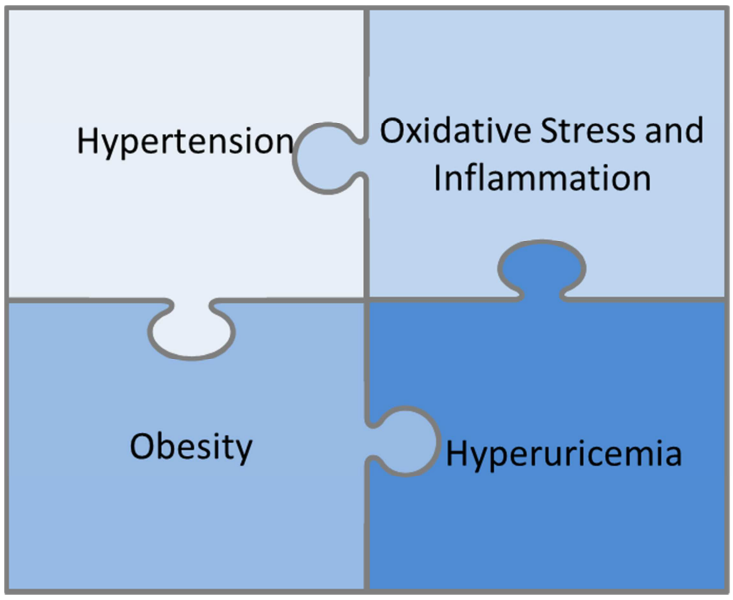
complications, inflammation, fibrosis and apoptosis, as well as glomerular filtration barrier damage [Balasubramanian, 2013]

Chapter 2. Summary of key results

2.1 Introduction to the experimental results

This chapter presents a summary of the key results of the current research (see list of publications on page 35), which are reported in detail in the appended papers.

These research findings are divided into four parts: CKD and Hypertension, Oxidative Stress and Inflammatory status in CKD, CKD and Obesity, CKD and Hyperuricemia.



2.2 CKD and hypertension

Hypertension has long been considered a risk factor for both CKD and ESRD [Kazancioglu, 2013]. Hypertension prevalence increases as renal function declines and may be present in more than 80% of patients with stage 4 to 5 CKD [CDC, 2007], and in Western countries, approximately 20–30% of ESRD cases are attributed to hypertension [Zoccali et al., 2010]. Systemic hypertension is transmitted to intraglomerular capillary pressure leading to glomerulosclerosis and loss of kidney function; thus, variable risk of impaired renal function has been reported among hypertensive subjects [Lea et al., 2002]. Hypertension contributes to the progression of kidney disease as well as to cardiovascular events such as myocardial infarction, congestive heart failure and stroke. One characteristic of hypertension is the remodelling of the arterial wall in response to the increase in blood pressure (BP). Moreover, with time, significant modifications in extracellular matrix composition and in vascular cell phenotype occur in the vasculature [Chen et al., 2004]. Vascular tissue remodelling associated with hypertension might actually create an environment for calcium deposition within the arteries [Moe et al., 2002] and this phenomenon is particularly important in CKD patients. For studying the effects of hypertension on kidney tissue and to clarify its role in the ageing process, both a genetic model of essential hypertension such as the spontaneously hypertensive rat (SHR) for the animal model and centenarian subjects for the super-control group were used.

Studies on centenarians' health history data revealed that the majority of centenarians markedly delay or escape age-associated diseases, especially cardiovascular and renal disorders, and may represent a prototype of successful ageing [Evert et al., 2003]. Currently, there are only limited longitudinal studies investigating the risk profile of centenarians based on hypertension and the other traditional cardiovascular risk factors (age, sex, smoking, dyslipidaemia, diabetes). Therefore, the clinical and prognostic meaning of traditional CVD risk factors in relation to mortality at advanced ages in 355 centenarians were analyzed [Montesanto A, Pellegrino D, Geracitano S, **La Russa D** et al., Geriatrics and Gerontology International, *2019*;

Paper I Appendix]. Our study analyzed data of centenarians carried out between 2002 and 2006 in two European projects, European Challenge for Healthy Ageing (ECHA) and Genetics of Healthy Ageing (GEHA). Mortality was ascertained after about a mean follow-up time of 9 years through the population registers of the municipalities where the long-lived people lived at the time of the interview. Our results indicated that low levels of classic cardiovascular risk factors, usually associated with lower mortality in adults, do not increase survival chances among oldest-old individuals and, in some cases, exert a significant and opposite effect. In particular, we showed that high circulating level of serum cholesterol exerts a significant protective effect in terms of survival in our nonagenarians and centenarians. Mechanisms explaining this reverse epidemiology association are not completely clear, even if the good nutritional status of subjects with normal/high cholesterol positively affects survival. The remarkable result emerging from our study is that hypertension was not significantly associated with mortality in centenarians. Although hypertension is one of the major risk factors for cardiovascular and renal diseases, which influence survival in all age groups, hypertensive centenarians have a greater chance of survival. These findings are extremely interesting and useful for understanding the biological mechanisms underlying human longevity and especially important for preventing age related diseases.

In both humans and animals, essential hypertension acts as a risk factor for subclinical kidney damage and precedes renal dysfunction [Zhang et al., 2016], but mechanisms that correlate hypertension and kidney disease have not been elucidated extensively. Several experimental and clinical data prove that hypertension and oxidative stress are closely related [Majzunova et al., 2014], although it is unclear whether oxidative stress is a cause or an effect of hypertension [Loperena and Harrison 2017]. The important pathophysiological role of ROSs in hypertension development is due, in large part, to oxygen excess and decreased NO bioavailability in vasculature and kidneys [Rodrigo et al., 2013]. Oxidative stress also seems to be a

salient feature in human hypertension; indeed, hypertensive patients show both increased oxidative stress and reduced antioxidant capacity [Rodrigo et al., 2013; Mihalj et al., 2016]. Recent evidence states that oxidative stress is a crucial molecular mechanism involved in the pathogenesis of hypertensive renal damage [Mennuni et al., 2014] and that apoptosis occurs in critical organs (heart, brain, or kidney) during hypertension [Sun et al., 2015]. Therefore, the oxidative balance and organ damage in genetic models of hypertension was analysed [**La Russa D** et al., Clinical Science, 2017; **PAPER II Appendix**]. A genetic model of hypertension, the SHR, derived from the normotensive Wistar–Kyoto (WKY) rat [Okamoto and Aoki, 1963], was used. Besides being the most widely used model for essential hypertension, the SHR is also an excellent model for studying the development of renal damage in the context of human hypertension and hypertensive kidney disease, as highlighted in several recent publications [Hultstrom, 2012; Sironi and Gelosa, 2012]. The timeline of the development of hypertension and renal damage is well known in this experimental model (Figure 9): hypertension develops within the first 10 weeks after birth and remains stable or changes gradually as age increases; vascular remodeling begins very early (4–5 weeks) and appears concurrently with increased renal autoregulatory efficiency; renal function (renal blood flow and glomerular filtration rate) remains constant up to 20 weeks of age; hypertensive kidney damage is not morphologically evident before 30 weeks of age [Hultstrom, 2012]. For our purpose, we used a 20-week-old SHR, when hypertension is fully developed, vascular remodeling has started, whereas kidney function is preserved.

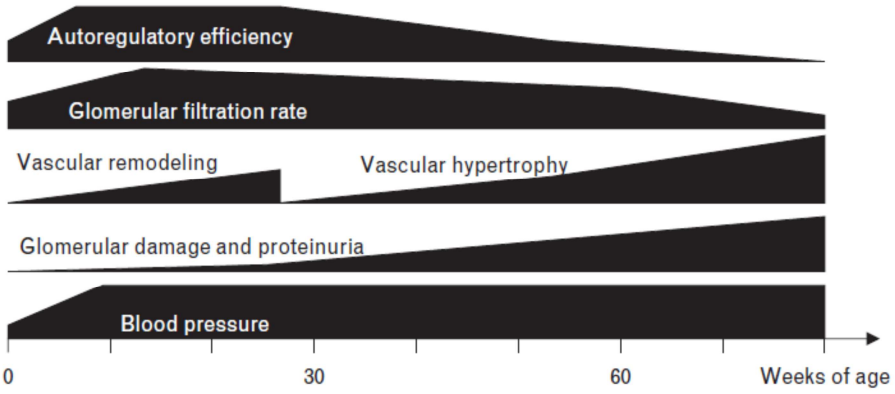


Fig. 9. Time-line of the development of hypertension and signs of kidney damage in SHR between birth and 80 weeks of age

We hypothesized that the increased oxidative stress in genetic models of hypertension may contribute to organ damage through activation of apoptosis signaling pathways. In order to test this assumption, we evaluated [i] plasmatic pro-oxidant/antioxidant status, [ii] tissue lipid peroxidation as an index of oxidative damage, [iii] the expression of cytoplasmic antioxidant enzymes (superoxide dismutase 1, SOD1 and Glutathione S-tranferasi P1, GSTP1), and [iv] extrinsic and intrinsic apoptotic pathways. The kidney was used as a target organ, while liver and skeletal muscle as control tissues, since these organs do not appear particularly susceptible to hypertensive damage [Sun et al., 2015]. Data collected in the present study show that, in addition to a direct effect on Blood Pressure (BP), the redox disequilibrium in both plasma and tissue is extremely important in the hypertensive tissue alteration in terms of both oxidative damage (lipid peroxidation, altered expression antioxidant enzymes) and apoptotic pathway activation (intrinsic/extrinsic). In particular, in kidney SHR tissue an increase in the dimeric proapoptotic form of GSTP1 was not detected, which was higher in SHR liver and skeletal muscle where the intrinsic apoptotic pathway is activated. These results suggest that, in our model, the increase in the dimeric form of GSTP1 can exert its proapoptotic action by activating the intrinsic pathway. Our results highlight the strong causality link between oxidative damage in renal tissue and hypertension and suggest the presence of additional

(genetic and/or acquired) factors intrinsic to the kidney involved in the susceptibility to BP alterations.

2.3 Oxidative stress and inflammatory status in CKD

Oxidative stress has been identified as one unifying mechanism in the pathogenesis of both CKD and CVD. In fact, oxidative stress is closely related to the development of both pathologies and it involves a vicious circle that leads to a progressive deterioration of patients' health. The progression of CKD to CVD, or vice versa, is mediated by inflammation, endothelial dysfunction, and redox perturbations [Small and Gobe, 2013]. Oxidative stress plays a role in the progression of CKD both directly through glomerular damage and renal ischemia or indirectly, being associated with inflammation, hypertension, and endothelial dysfunction [Ueda et al., 2007]. Several lines of evidence indicate that CKD is a pro-oxidant state and factors that cause an increase in oxidative stress in CKD include malnutrition, inflammation, increased phagocytic activity, increased oxidase activity, and decreased antioxidant defense mechanisms [Kuo and Tarng, 2010]. The pathogenesis of oxidative stress in CKD patients is multifactorial and includes several possible causes: uremia-related factors (e.g., hyperhomocysteinemia or AGEs), intravenous iron supplementation and dialysis-related factors (e.g., bioincompatible membranes or endotoxin-contaminated dialysate) [Miyata et al., 2001; Drueke and Massy, 2005]. The measurement of oxidative stress compounds (superoxide radical, hydrogen peroxide and hydroxyl radical) is very difficult because oxidants are highly reactive components with a very short half-life. By contrast, the components oxized by ROS species such as lipids, proteins, carbohydrates, and nucleic acids have a longer half-life, ranging from hours to weeks, and can be used as a marker of oxidative stress [Locatelli et al., 2003]. There is a close relationship between plasma levels of C-reactive protein, a surrogate marker of inflammation, and lipid oxidation in CKD patients supporting the inflammation oxidative stress link [Nguyen-Khoa et al., 2001]. It is thus possible that inflammation promotes both renal deterioration (triggering endothelial dysfunction,

atherosclerosis, and glomerular injury) and cardiovascular mortality. Indeed, several studies reported an association between renal impairment and inflammation. In particular, it has been shown that elevated CRP, IL-6, and fibrinogen are strong predictors of cardiovascular outcomes in patients with CKD [Stenvinkel, 2006]. Although the precise mechanisms that contribute to the high prevalence of inflammation in CKD are not clear, ROS species have been proposed as potential contributors to inflammation, since they are able to activate transcriptor factors such as NF-kB (nuclear factor Kappa-light-chain-enhancer of activated B cells) or, STAT (signal trasducer and activator of transcription), which regulate inflammatory mediator gene expression [Freund et al., 2011]. Moreover, NF-κB has been shown to drive several ageing phenotypes, particularly in the skin, spine and brain [Nasto et al., 2012]. In fact, CKD is not only an age-related disease but also appears to contribute to premature biological ageing process of different organ systems (cardiovascular hypertrophy, vascular calcification, muscle wasting and osteoporosis) [Kooman et al., 2017]. Since CVD's traditional risk factors are poor predictors in patients with late-stage CKD, identifying and intervening against new risk factors is now a health priority [Park et al., 2012]. Even our previous results have highlighted that hypertension and oxidative stress are closely related in determining kidney damage. Therefore, the correlation between oxidative stress and cardiovascular risk in a healthy population was analyzed and, thanks to the collaboration with the Transplantation and Kidney Research Center of The Annunziata Hospital, in hemodialysis patients (HD).

Our research on healthy population [Brunelli E, **La Russa D**, Pellegrino D. Oxid Med Cell Long, 2017; **Paper III Appendix**] was designed to investigate, in the Italian population, whether the oxidative balance is related to traditional cardiovascular risk factors. Through a cross-sectional analysis on 322 healthy subjects, the global plasmatic oxidant/antioxidant ability was evaluated, by measuring reactive oxygen metabolite and biological antioxidant potential by photometric measurement. A significant depletion in the efficacy of total plasma antioxidant

barrier in high cardiovascular risk categories as the first detectable event of a redox disturbance was demonstrated and an age-related alteration of oxidative status was confirmed. This study is of emerging interest in CVD research because the analysis of new biomarkers could improve the predictive role of CVD risk factors.

Our research on HD patients aimed to investigate the role of oxidative stress and inflammation as new CVD biomarkers in this fragile population. The global oxidative balance in HD patients compared to a healthy population was explored [**La Russa D** et al., Oxid Med Cell Long, 2019; **Paper IV Appendix]**. Our study population consisted of 97 HD patients and 95 healthy volunteers as a control group. The main findings are that in the HD sample the oxidative index was significantly lower than in the control group. Moreover, ESRD patients also showed a greater effectiveness in the antioxidant barrier with respect to those observed in the control group. In addition, in our HD group, a strong correlation between oxidative index and blood levels of C-reactive protein emerged, while oxidative index and antioxidant barrier values showed a borderline correlation. Interestingly, when HD patients were stratified according to previous cardiovascular events, subjects with previous acute myocardial infarction were found to have higher values of both oxidative stress and antioxidant barrier in contrast to patients without cardiovascular events. It is remarkable to note that HD subjects with previous cardiovascular events had oxidative stress values comparable to healthy controls while the efficacy of the antioxidant barrier was significantly enhanced compared to all of the groups. What are the possible motivations that giustify this apparent controversial experimental trend? Several authors have reported a profound imbalance between oxidants and antioxidants in CKD. The balance between pro-and antioxidant systems is essential for the regular function of organism’s cells and molecules (Figure 10-A). Many stressors, such as, hypoxia [Stowe et al., 2011], hyperoxia [Soejima et al., 2013] as well as short episodes of ischemia [Blanco et al., 2006] can induce positive oxidative stress (Figure 10-B) via a transiently increased ROS production that is involved in an adaptive response for prophylactic purposes [Bhagatte et al., 2012]. On the contrary, high

levels of ROS (Figure 10-C) would be detrimental to cells and have been thought to contribute to ageing and to the pathogenesis of numerous ageing-related diseases [Sena et al., 2012; Malinin et al., 2011]. Concerning the antioxidant systems, literature data are really complex to discern. Most of the studies on CKD patients focus on the expression/activity of antioxidant enzymes with conflicting results [Poulianiti et al., 2016; Liakopoulos et al., 2017]. In addition, most of the reports focus on a single or a few antioxidants. The stability/activity of enzymatic antioxidant systems is multifaceted: in the case of low/medium oxidative stimulation, enzymatic antioxidant activity can increase, but if oxidative stress is persisting, or its level is very high, the damage caused to proteins becomes profound and a decreased expression/activity may occur via direct oxidative damage of the molecules and/or oxidative-altered gene expression. Interestingly, what emerged from our work was that the HD patients presented a greater effectiveness in the non-enzymatic antioxidant barrier respect to healthy controls, probably to counteract the deleterious effects of ROS overproduction. In addition, this apparent discrepancy can be explained by the fact that the photometric plasmatic antioxidant ability test, quantifies levels of soluble antioxidants such as vitamins (E and C), lipid, glutathione and uric acid whose level increases in the uremic state typical of CKD. Many mediators implicated in inflammatory conditions, such as uric acid crystals [Martinon et al., 2006] have been reported to activate the NLRP3 inflammasoma (NOD-like receptor family pyrin domain containing 3). Being oxidative stress, NLRP3 activation and inflammation interconnected, an investigation on whether genetic variants in NLRP3 could influence the increased generation of mediators of inflammation and oxidative stress and the cardiovascular risk observed in uremic patients was made. Collectively, our results suggested that genetic variant of NLRP3 inflammasoma rs10754558 are related to a higher plasmatic antioxidant barrier and contribute to the increased cardiovascular risk observed in dialysis patients which depends, in turn, on the higher oxidative stress and lower HDL levels [Perri A, La Russa A, Montesanto A, **La**

Russa D et al., Nephrology Dialysis Transplantation 2017; **Published Abstract I Appendix**].

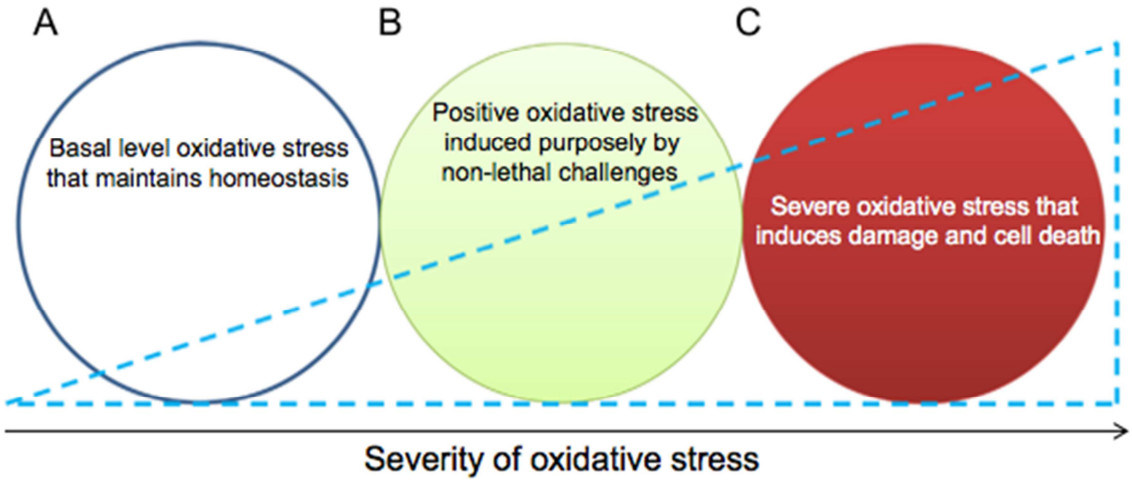


Fig.10. Correlation between oxidative stress levels and effects [Yan et al., 2014]

2.4 CKD and obesity

Obesity has been identified as a potent risk factor for the onset of kidney disease and several population-based studies have shown an association between measures of obesity and both the development and the progression of CKD [Lee et al., 2015; Song et al., 2015]. Some of the deleterious renal consequences of obesity may be triggered by downstream comorbid conditions such as diabetes mellitus or hypertension, but also the endocrine effects of adiposity could impact the kidneys directly via production of adipokines such as leptin [Wolf et al., 2006], adiponectin [Sharma et al., 2009], and resistin [Ellington et al., 2007].

The exact mechanisms whereby obesity may worsen or cause CKD remain unclear but potential mechanisms include the development of inflammation [Ellington et al., 2007], oxidative stress [Furukawa et al., 2004], abnormal lipid metabolism [Ruan et

al., 2009], activation of the renin-angiotensin-aldosterone system [Ruster et al., 2013], and increased production of insulin and insulin resistance (Figure 11) [Oterdoom et al., 2007].

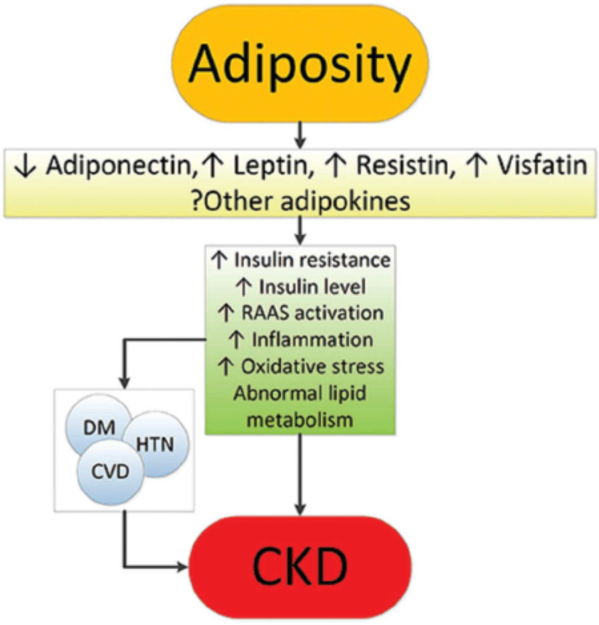


Fig.11. Potential mechanisms of obesity-induced CKD [Kovesdy et al., 2017]

Since adipose tissue mass in obesity contributes to both oxidative stress and inflammation, with a consequent potential renal damage, the oxidative/inflammatory status in 23 obese patients was analysed. These patients were hospitalised at the Bariatric Surgery Unit of Annunziata Hospital (Cosenza) before and 3 months after bariatric surgery [La Russa A, Bonofiglio M, Lofaro D, **La Russa D**, et al. "rs4612666 of NLRP3 polymorphism is associated with oxidative stress in obese patients" *Submitted*; **Paper V Appendix**].

Literature data indicate that weight loss induced by bariatric surgery markedly results in decreasing both systemic oxidative stress and inflammatory state in obesity [Forsythe et al., 2008; Gletsu-Miller et al., 2009].

Our results showed that in only 3 months, bariatric surgery produced a significant reduction in body weigth with concomitant reduction of insulin, triglycerides, C-reactive protein and oxidative stress.

To evaluate the obesity-induced renal damage, an animal model was used, the cafeteria diet-fed rat (CAF), a robust model of human metabolic syndrome. Our study [**La Russa D** et al., Antioxidants, 2019; **Paper VI Appendix]** was designed to evaluate if diet-induced obesity can affects the plasmatic oxidative balance and can influence renal tissue alterations, in terms of both oxidative damage (altered expression antioxidant enzymes) and apoptotic pathways (intrinsic/extrinsic) activation. In parallel, the antioxidant and cytoprotective effects of the Citrus flavonoids from bergamot (BPF) were evaluated. It is known that approaches to reduce oxidative stress with antioxidant supplements may have renoprotective effects against obesity-induced renal damage [Safa et al., 2010; Liu et al., 2010]. The analysis of the plasmatic oxidative balance showed that the CAF diet induces antioxidant barrier depletion when overused. By contrast, BPF administration enhanced the plasmatic ability to neutralize the oxidative insults, mainly in the case of redox disturbance due to the CAF diet. Regarding the apoptosis signaling and antioxidant enzymes expression, our results demonstrated that the CAF diet induces pro-apoptotic effects at renal level by depletion of tissue antioxidant defense. These effects were counteracted by the antioxidant BPF, which proved to be particularly effective in the presence of the oxidative imbalance induced by the CAF diet. In another study [Parafati M, Lascala A, **La Russa D** et al., Nutrients 2018; **Paper VII Appendix**], the extent to which BPF can accelerate therapeutic effects of weight loss induced by a normocaloric standard chow (SC) diet was evaluated. For this purpose, 21 rats fed with CAF diet for 16 weeks to induce non-alcoholic fatty liver disease (NAFLD) with inflammatory features (NASH) were divided into three groups. Two groups were switched to SC diet supplemented or not with BPF (CAF/SC±BPF), while one group continued with CAF diet (CAF/CAF) for 10 weeks. BPF had no effect on SC diet-induced weight loss, but it accelerated hepatic lipid droplets clearance and reduced blood triglycerides. Interestingly, BPF supplementation decreased hepatic inflammation by reducing (*Il6*) mRNA expression and increasing anti-inflammatory interleukin 10 (*Il10*) in CAF/SC+BPF livers compared to CAF/SC

group. These data indicate that BPF mediates a specific anti-inflammatory activity in liver recovering from NASH, while it boosts lipid-lowering and anti-diabetic effects of the dietary intervention. The proautophagic activity of Citrus flavonoids from Bergamot Polyphenol Fraction was mediated by the hydrophobic fraction of acid-hydrolyzed BPF (A-BPF), containing six flavanone and flavone aglycones as identified by liquid chromatography–high-resolution mass spectrometry [Janda E, Salerno R, Martino C, Lascala A, **La Russa D**, e al., Data in Brief 2018**; Paper VIII Appendix**]. Among them, naringenin, hesperitin, eriodictyol and diosmetin were weak inducers of autophagy while Apigenin and Luteolin showed the strongest proautophagic activity.

The exposure of hepatocytes to palmitic acid (PA) causes an accumulation of intracellular lipid droplets and models of non-alcoholic fatty liver disease (NAFLD) *in vitro*. This approach was adopted to measure the proautophagic activity of six main flavonoid aglycones present in A-BPF in the presence and absence of lipotoxic stress. Our data suggests additive as well competitive effects, rather than synergistic effects of tested flavonoids on autophagy.

2.5 CKD and hyperuricemia

Uric acid is a $C_5H_4N_4O_3$ [7,9-dihydro-1H-purine-2,6,8[3H]-trione] heterocyclic organic compound synthesized as the end product of both endogenous purine metabolism and exogenous pool of purines derived largely from animal proteins dietary intake. The endogenous production of uric acid takes place mainly in the liver and intestines but it also involves other tissues such as muscles, kidneys and the vascular endothelium [Chaudhary et al., 2013]. Uric acid has a considerable physiological role since approximately 90% of kidney-filtered uric acid is reabsorbed [Maiuolo et al., 2016]. In humans, according to antioxidant/oxidant uric acid paradox, most of the plasmatic antioxidant capacity [≈60%] derives from uric acid but at intracellular level it can cause oxidative stress, stimulate inflammatory mediators and induce renin-angiotensin system activation [Sautin and Johnson, 2008]. Three urate

transporters, URAT1/SLC22A12, GLUT9/SLC2A9, and ABCG2/BCRP, have been reported to play important roles in the regulation of serum uric acid levels, and their dysfunctions cause alteration in extracellular/intracellular urate balance. The molecular identification of URAT1 as the dominant apical urate exchanger of the human proximal tubule was a landmark event in the physiology of urate homeostasis [Clémençon et al., 2014]. The URAT1 protein is encoded by the *SLC22A12* gene, part of the large SLC22 family of organic ion transporters. GLUT9 membrane transporter is distinct among other members of the glucose transporters family (GLUT or SLC2) due to its substrate specificity and sequence identity. Single nucleotide polymorphisms in the *GLUT9* genes have also been associated with gout, coronary artery disease and myocardial infarction. Regarding *GLUT9*, two isoforms, *SLC2A9a* and *SLC2A9b*, have been identified encoding the two proteins, which are hGLUT9a and b that differ only by the first 29 residues of the N-terminal domains. GLUT9a is ubiquitously expressed, while GLUT9b is restricted to the main organs involved in urate transport, such as liver and kidney. GLUT9-mediated urate transport has been characterized. It is independent of sodium, chloride and other anions, but is voltage dependent and currents have been recorded at physiological pH. Altogether, data provided so far are compatible with a transport model in which GLUT9 is a uniport, without having formally excluded all other possibilities [Clémençon et al., 2014]. Genetic variation in human ABCG2, an ATP-driven efflux pump, has emerged as a major factor in human hyperuricemia. A loss of or reduction in ABCG2-mediated renal urate secretion would lead to increased renal urate reabsorption, given that reduced renal excretion of urate is considered to be the underlying hyperuricemic mechanism in the vast majority of gout patients [Mandal and Mount, 2014]. The biosynthesis of uric acid is catalyzed by the enzyme xanthine oxidase (XO), also known as xanthine oxidoreductase or XOR, coded by the xanthine dehydrogenase (XDH) gene (36 exons) located in the short arm of chromosome 2 [Terao et al., 1997].

XOR is a molybdoflavoprotein hydroxylase that can act as a reductase or oxidase, but

in blood, it mainly acts as oxidase. It is a rate-limiting enzyme in the nucleotide metabolism, which catalyzes degradation of hypoxantine to xanthine and then to uric acid, producing superoxide and hydrogen peroxide during the process [Hille and Nishino, 1995]. In addition, XDH can catalyze the hydroxylation of a wide range of N-heterocyclic and aldehyde substrates, and it can produce nitric oxide under hypoxic conditions from organic and inorganic nitrates and nitrites [Doel et al., 2001]. Consequently, XDH has also been involved in the production of peroxynitrite and in nitric oxide availability.

In the body, uric acid mainly exists as salt or urate, whose blood level is directly correlated with uric acid crystal formation. The normal human blood reference interval of uric acid is 1.5 to 6.0 mg/dL in women and 2.5 to 7.0 mg/dL in men. Kidneys eliminate approximately two-thirds, while the gastrointestinal tract eliminates one-third of the uric acid load. Generally, in adults hyperuricemia is defined as uric acid concentration in blood higher than 7.0 mg/dL in men and 6.0 mg/dL in women. A dramatic rise in uric acid serum levels was originally proposed as a hallmark of gout [Weaver, 2008]. Since then, numerous epidemiologic studies have successively indicated that there is a relationship between elevated uric acid levels and metabolic syndrome [Cirillo et al., 2006], renal disease [Nakagawa et al., 2006], hypertension [Johnson et al., 2005], and CVD [Fang and Alderman, 2000]. Uric acid is not just a potential marker of renal dysfunction [Sonoda et al., 2011], but is also involved in the development of renal disease [Kim et al., 2014;]. Experimental studies have confirmed that the potential mechanisms by which hyperuricemia induces renal injury comprise inflammation, afferent arteriopathy [Mazzali et al., 2002], endothelial dysfunction [Sanchez-Lozada 2012], renin-angiotensin-aldosterone system activation [Sanchez-Lozada et al., 2005], cyclooxygenase-2

expression and oxidative metabolism impairment [Kang et al., 2002].

Increased production of ROS by XDH has been described in experimental models of salt-sensitive [Swei et al., 1999] and glucocorticoid-induced hypertension [Iuchi et al., 2003]. Some studies have suggested that both XDH activity and hydrogen peroxide production are enhanced in hypertensive patients compared to controls [Newaz et al., 1996]. Furthermore, XDH activity and uric acid concentration have a positive correlation with mean arterial blood pressure [Mazzali et al., 2001], while allopurinol treatment, a selective XDH inhibitor, reverses the endothelial dysfunction present in hypertensive type 2 diabetic patients and in smokers [Guthikonda et al., 2003]. Although several polymorphisms have been described along the XDH gene in databases, the potential impact of its variations has not been analyzed. A case-control study demomstrates that two polymorphisms rs206812 and rs2073316 of xanthine oxidoreductase gene are related with blood pressure and oxidative stress in hypertension while no relationship between XDH polymorphisms and uric acid levels has been found in hypertension [Chaves et al., 2007]. The mechanisms involving uric acid in the development and progression of these diseases are not well understood. Some of these mechanisms can be related to the importance of uric acid in the stimulation of vascular smooth muscle proliferation and interaction with NO pathways in vascular, cardiac, and renal tissues [Saavedra et al., 2002]. Many traditional mortality risk factors in patients with CKD have been recognized, such as deep blood pressure alterations, anemia, left ventricular hypertrophy, and smoking [Kovesdy et al., 2013; Nakamura et al., 2015]. In addition, there is growing evidence suggesting a potential role for other modifiable nontraditional risk factors, including hyperuricemia [Madero et al., 2009]. There is conflicting evidence about the role of hyperuricemia as an independent risk factor for mortality in CKD patients

[Beberashvili et al., 2015; Navaneethan and Beddhu, 2009]. Many studies find that high serum uric acid levels can predict risk of mortality [Kowalczyk et al., 2010; Suliman et al., 2006], whereas other studies find that lower serum uric acid levels are associated with and increased risk of mortality [Lee et al., 2009; Latif et al., 2011; Beberashvili et al., 2015]. The association between higher serum uric acid levels and mortality could be explained by several mechanisms: (i) at cellular level, uric acid induces oxidative stress in both control and hyperuricemic rat models [Yu et al., 2010; Sanchez-Lozada et al., 2008]; (ii) hyperuricemia is associated with endothelial dysfunction and lowering uric acid can improve endothelial function [Yelken et al., 2012; Kao et al., 2011]; (iii) uric acid plays an important role in inflammation through inducing T helper 2 cell immunity or Nlrp3 inflammasome [Kool et al., 2011; Kim et al., 2015].

On the basis of evidence found in literature, the correlation between hyperuricemia, oxidative stress and mortality risk in 122 hemodialysis (HD) patients was investigated. In addition, since in the dialysis population the presence of several risk alleles involved in the metabolism of uric acid could exacerbate hyperuricemia, the relationship between serum uric acid levels and the genetic variability of both XDH-rs1042039 and GLUT9-rs11722228 polymorphisms was also explored.

Our results demonstrate that HD patients show a significant change in plasmatic redox balance because the antioxidant barrier is linearly enhanced with the increase of serum uric acid levels and this is in agreement with the uric acid antioxidant/pro-oxidant paradox. In addition, the T allele of rs1042039 in XDH gene was associated with higher values of uric acid (5.9 vs 5.3 mg/dl), while no association was found for GLUT9-rs11722228 polymorphism. Surprisingly, taking advantage of a long follow-up period of about ten years, we found that T carrier patients exhibit a lower survival probability compared to those with the CC genotype. Overall, these results underline

the importance of genetic information in clinical treatment such as the use of allopurinol, an XDH inhibitor, in dialysis patients.

The paper “Functional polymorphisms of uric acid metabolism and long-term survival of patients with chronic kidney disease” is currently in preparation [**La Russa D** et al. “Functional polymorphisms of uric acid metabolism and long-term survival of patients with chronic kidney disease“ *In preparation*].

Appendix

Full Papers

I. Montesanto A, Pellegrino D, Geracitano S, **La Russa D** et al. “Cardiovascular risk factors and all-cause mortality in long-lived people from Southern Italy”. **Geriatrics & Gerontology International,** 19:165–170 (2019). (***if 2.656***)

II. **La Russa D,** Brunelli E, Pellegrino D. “Oxidative imbalance and kidney damage in spontaneously hypertensive rats: activation of extrinsic apoptotic pathways”. **Clin Sci (Lond),** 131(13):1419-1428 (2017). **(*if 5,016)***

III. Brunelli E, **La Russa D**, Pellegrino D. “Impaired oxidative status is strongly associated with cardiovascular risk factors”. **Oxid Med Cell Longev**, 2017:6480145 (2017). **(*if 4,57***)

IV. **La Russa D**, Pellegrino D, Montesanto A et al. “Oxidative imbalance and Inflammation in hemodialysis patients: biomarker of cardiovascular risk?”. **Oxid Med Cell Longev**, 2019: 8567275 (2019). **(*if 4,936***)

V. La Russa A, Bonofiglio M, Lofaro D, **La Russa D** et al. “rs4612666 of NLRP3 gene polymorphism is associated with oxidative stress in obese patients”. *Submitted*

VI. **La Russa D**, Giordano F, Marrone A, Parafati M, Janda E, Pellegrino D. “Oxidative imbalance and kidney damage in cafeteria diet-induced rat model of metabolic syndrome: effect of bergamot polyphenolic fraction”. **Antioxidants**, 8: 66 (2019). **(*if 3,56***)

VII. Parafati M, Lascala A, **La Russa D** et al. “Bergamot polyphenols boost therapeutic effects of the diet on non alcoholic steatohepatitis (NASH) induced

by junk food: evidence for anti-inflammatory activity". **Nutrients,** 10(11) pii: E1604 (2018). **(*if 4.196***)

VIII. Janda E, Salerno R, Martino C, Lascala A, **La Russa D**, Oliverio M. "Qualitative and quantitative analysis of the proautophagic activity of Citrus flavonoids from Bergamot Polyphenol Fraction". **Data in Brief**, 19:1327–1334 (2018).

IX. **La Russa D** et al. "Functional polymorphisms of uric acid metabolism and long-term survival of patients with chronic kidney disease" *In preparation*

Published Abstract

I. Perri A, La Russa A, Montesanto A, **La Russa D** et al. "NLRP3 RS10754558 functional polymorphism increases the susceptibility to renal disease and enhanches the cardiovascular risk". **Nephrology Dialysis Transplantation** 32(3):iii117 (2017). (***if 4,47***)

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Full Paper I

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ORIGINAL ARTICLE

BIOLOGY

Cardiovascular risk profiling of long-lived people shows peculiar associations with mortality compared with younger individuals

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Aim: Centenarians represent a biological model of successful aging because they escaped/postponed most invalidating age-related diseases, such as cardiovascular diseases. The aim of the present study was to clarify whether a favorable cardiovascular risk profile increases the survival chances in long-lived people.

Methods: A total of 355 community-dwelling nonagenarians and centenarians living in Southern Italy were recruited in the study. Patients were classified as at low and high cardiovascular risk on the basis of serum cholesterol, diabetes, hypertension and smoking status. The relationship between cardiovascular risk factors and 10-year mortality was investigated by Cox regression analysis. Splines-based hazard ratio curves were also estimated for total cholesterol, low-density lipoprotein cholesterol, and systolic and diastolic blood pressure.

Results: Low levels of selected cardiovascular risk factors usually associated with lower mortality in adults do not increase survival chances among oldest-old individuals. In particular, after adjusting for age, sex, and cognitive, functional and nutritional status, serum cholesterol >200 mg/dL increased the survival chances during the follow-up period (hazard ratio 0.742, 95% CI 0.572–0.963).

Conclusions: The present results showed that in nonagenarians and centenarians, the clinical and prognostic meaning associated with traditional cardiovascular risk factors is very different from younger populations. Consequently, considering the increase of this population segment, further studies are required to confirm these results and to translate them into clinical practice/primary care. **Geriatr Gerontol Int 2019; 19: 165–170.**

Keywords: cardiovascular diseases, cardiovascular disease risk, centenarians, longevity.

Introduction

Cardiovascular diseases (CVD), the world's primary cause of death and disability, represent a global health problem and involve a great public financial commitment in terms of both inability to work and pharmaceutical costs. It is well known that cardiovascular risk is markedly increased in aged individuals (70–80 years), and CVD represent the most frequent causes of death at these ages.[1] However, several studies questioned the relationship between cardiovascular risk factors and mortality in older and oldest-old people. Centenarians and their offspring have genetic and immune system advantages that reflect a minor likelihood of developing major age-related diseases, such as CVD, hypertension or diabetes mellitus.[2–4] Studies of centenarian health history data revealed three morbidity profiles: survivors, delayers and escapers.[5] Although the majority of centenarians (approximately 60%) markedly delay or escape age-associated diseases, a large proportion of them cope with diseases that would otherwise cause "premature" mortality.[5,6] In fact, few centenarians are disease-free, and the prevalence of specific diseases varies greatly depending on the population examined, confirming the dichotomy genes/environment in successful aging.[5–7]

The aforementioned described scenario accounts for the complex relationship between cardiovascular risk factors and survival in long-lived populations. Indeed, high systolic blood pressure (SBP) was found to be associated with an increased risk of major cardiovascular events, even among subjects aged 85–94 years,[8] but an increased risk of all-cause mortality was recently observed among individuals aged ≥80 years with SBP <120 mmHg.[9] Additionally, current evidence suggests that a genetic predisposition to high low-density lipoprotein cholesterol (LDL-C) levels contributes to mortality, and a beneficial LDL genetic risk profile is associated with familial longevity throughout life up to 90 years of age.[10] Nevertheless, the meaning of lipid profiles in nonagenarians and centenarians is largely to be elucidated. Currently, there are only limited longitudinal studies investigating the risk profile of centenarians based on traditional cardiovascular risk factors,[11] including age, sex, smoking, hypertension, dyslipidemia and diabetes.[12] Individuals with familial longevity (probands, siblings and their children) had low rates of diabetes, chronic pulmonary disease and peripheral artery disease, and showed better lipid profiles.[11] In particular, one of the most consistent differences noted was a lower pulse pressure in individuals with familial longevity, similar to individuals who were aged 5–10 years younger.[11] The limitation of these studies was the smaller sample sizes, therefore caution is required in attributing all differences to genetic factors in that part of the differences might be attributable to the shared environmental component of familial relationships. As a result, more detailed analyses are essential to identify genetic versus environmental components. Therefore, we aimed at investigating the prognostic meaning of traditional CVD risk factors in a population of long-lived community-dwelling individuals.

Methods

Sample

The recruitment of nonagenarians and centenarians analyzed in the present study was carried out between 2002 and 2006, in the

frame of two different European projects: the European Challenge for Healthy Aging and the Genetics of Healthy Aging projects.[13,14] The present study included 155 participants from the European Challenge for Healthy Aging cohort and 200 participants from the Genetics of Healthy Aging cohort, leaving a final sample of 355 participants (145 men and 210 women) aged >90 years (see Appendix S1). Written informed consent was obtained from all subjects at the time of enrollment. The study protocols were approved by the ethics committee of the University of Calabria.

Outcome

The main outcome of the present study was all-cause mortality. The average follow-up duration was 81.6 months (range 64–103 months). Mortality was ascertained through the population registers of the municipalities where the long-lived people lived at the time of the interview. After this period, 138 men (94.5%) and 197 women (91.2%) died.

Measurements

All the enrolled participants underwent a complete multidimensional geriatric assessment with detailed clinical history, including anthropometric measures, smoking and drinking habits, and a set of the most common tests to assess cognitive functioning, functional activity, physical performance and depression. During this interview, participants of the study and their caregivers were asked to present all clinical records (either from a general practitioner or a specialist/hospital) with current medications. Blood pressure was measured on the right arm using a mercury sphygmomanometer with the cuff maintained at the heart level. Blood pressure was measured three times on one occasion, and the mean of three readings was used in the analysis.

Cognitive status was rated by Mini-Mental State Examination.[15] Handgrip strength was measured by using a handheld dynamometer (SMEDLEY's dynamometer; TTM, Tokyo, Japan) while the participant was sitting with their arm close to their body. The test was repeated three times with the stronger hand, and the maximum of these values was used in the analyses. The management of activities of daily living (bathing, dressing, eating, independence in and out of bed) was assessed by using the Katz Index of activities of daily living.[16] Depressive symptoms were assessed using the 15-item Geriatric Depression Scale.[17]

After an overnight fast, a peripheral blood sample was obtained from each participant and delivered to the laboratory of the Italian National Research Center on Aging hospital of Cosenza, where plasma glucose, total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C) and LDL-C were measured using an automatic Biochemical Analyzer (Dimension EXL system; Siemens, Erlangen, Germany).

Study variables

CVD risk factors were defined as follows: high blood pressure was defined as ≥140 mmHg for SBP, and ≥90 for diastolic measurement (DBP) or in presence of an active antihypertensive treatment; diabetes was defined based on the presented clinical records or in the presence of an active antidiabetic treatment; serum cholesterol >200 mg/dL at the time of enrollment or active statin treatment was also included among CVD risk factors. Given that only a minority of participants (n = 49) had serum cholesterol >240 mg/dL and only one participant was taking active statin treatment in our series, we resolved to consider even borderline levels of serum cholesterol (200–239 mg/dL) as a CVD risk factor. Indeed, serum cholesterol >200 mg/dL was considered as a risk factor in a recent study investigating the relationship between serum cholesterol and longevity in a representative population of older people aged 70–90 years.[18]

Age, sex and variables known to affect survival at old ages were included in the analysis. Functional disability (activities of daily living <4), cognitive impairment (Mini-Mental State Examination <18), depression (Geriatric Depression Scale ≥5) and handgrip (<10 kg in women and <20 kg in men) strength were included in the analysis. Body mass index <20 kg/m^2 and hypoalbuminemia (defined as serum albumin <3.5 g/dL) were considered in the analysis as indices of poor nutritional status.

Statistical analysis

First, we compared the sociodemographic and geriatric parameters, as well as the CVD risk factors between the European Challenge for Healthy Aging and Genetics of Healthy Aging cohorts. We used the unpaired t-test for continuous variables, and the χ^2-test for categorical variables. After pooling the two study cohorts, Kaplan–Meier survival curves with the Mantel–Cox log–rank test were separately calculated to compare crude survival of participants in relation to each CVD risk factor. The analysis was also repeated to investigate survival in relation to the cumulative number of CVD risk factors (range 0–4). The time from the enrollment visit through to the day of death was used as the time to failure variable for the model. Survivors were censored on the day of the last follow-up visit. The relative risk of mortality related to each CVD risk factor or cumulative number of risk factors was investigated by Cox regression models. The proportional hazard assumption was tested graphically, plotting the log-minus-log survival function over time. The model was also adjusted for all the variables that were associated with mortality in preliminary analysis (age, gender, geriatric parameters and nutritional status). In order to better understand the effects of each continuous risk factor (total cholesterol, SBP and DBP) on the all-cause mortality, splines-based hazard ratio (HR) curves were estimated taking the mean value of the investigated parameter as the reference value.

Statistical analyses were carried out using survival and *smoothHR* packages[19] of R v3.4.2 statistical language (www.r-project.org).

Results

The mean age of all participants was 96.7 years, and 59.2% were women. Regarding the traditional risk factors for CVD, despite their advanced age, the analyzed sample presented near normal values for total, HDL-C, LDL-C and triglycerides. Smoking habit and the prevalence of diabetes, as well as the malnutrition status, were extremely low throughout the sample (2.8%, 6.2% and 7.7%, respectively), whereas hypertension was highly prevalent (40.8%). More than half (54.6%) reported dependency in activities of daily living (56.6), and more than two-thirds had cognitive impairment (68.7%), whereas depression was much less prevalent (25.9). Handgrip strength values were indicative of a severe physical decline (Table 1).

Kaplan–Meier analysis carried out in pooled cohorts showed that serum cholesterol >200 mg/dL and hypertension were significantly associated with survival, whereas smoking habits and the presence of diabetes did not affect mortality at advanced ages. Additionally, the mortality rate dramatically decreased for increasing number of study risk factors (Fig. 1). Survival analyses also showed that malnutrition, handgrip performance, disability and cognitive impairment were independent predictors of mortality (Fig. 2). These results were almost unchanged, also splitting the dataset according to the analyzed cohorts (Figures S1–S4).

Figure 3 shows the spline-based HR curves with 95% pointwise confidence limits for total cholesterol, LDL-C, HDL-C, SBP and DBP, taking the mean value of the investigated parameter as the reference value. The HR curve shown in Figure 3a reveals that mortality risk increased with decreasing cholesterol below the value of 195 mg/dL (HR 2.70, 95% CI 1.81–6.41 achieved at 104 mg/dL) and decreased with increasing levels >195 mg/dL (HR 0.19, 95% CI 0.05–0.82 achieved at 332 mg/dL). The same pattern can be observed for LDL-C (B) for which that mortality

Table 1 Characteristics of the analyzed sample with the traditional risk factors for cardiovascular disease, geriatric parameters and survival data

	Total sample (n = 355)
Age, years (mean ± SD)	96.64 (3.313)
Women, *n* (%)	210 (59.2)
Total cholesterol, mg/dL (mean ± SD)	195.52 (41.641)
HDL cholesterol, mg/dL (mean ± SD)	58.21 (14.896)
LDL cholesterol, mg/dL (mean ± SD)	113.95 (36.045)
Triglyceride, mg/dL (mean ± SD)	118.27 (52.285)
Systolic blood pressure, mmHg (mean ± SD)	133.63 (15.499)
Diastolic blood pressure, mmHg (mean ± SD)	76.22 (9.169)
Hypertension, *n* (%)	145 (40.8)
Diabetes, *n* (%)	22 (6.2)
Smoke, *n* (%)	10 (2.8)
Grip strength, *n* (%) (<10 kg in women, < 20 kg in men)	169 (54.9)
ADL dependence (≥2 activities)	201 (56.6)
GDS ≥5	92 (25.9)
MMSE (score <18)	233 (68.7)
BMI, kg/m^2 (mean ± SD)	23.25 (4.161)
Malnutrition, *n* (%)	26 (7.7)
Number of deaths, *n* (%)	328 (92.4)
Mean survival time, months (SD)	32.76 (25.399)

BMI, body mass index; GDS, Geriatric Depression Scale; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MMSE, Mini-Mental State Examination.

risk increased with decreasing LDL-C levels <113 mg/dL (HR 2.10, 95% CI 1.51–2.92 achieved at 60 mg/dL) and decreased with increasing levels >113 mg/dL, beyond this it no longer affected survival chances. HDL variability (C) significantly influenced the risk of death during the follow-up period. In fact, mortality risk significantly increased with increasing HDL-C >58 mg/dL. The HR curve for SBP (D) showed that levels of pressure lower than the average value (133 mmHg) were detrimental in terms of survival (its maximum value was 3.03 achieved at 100 mmHg), whereas higher values did not affect the survival chances. Finally, a U-shaped relationship was observed between DBP and mortality (E). In fact, the HR curve for DBP showed that, with respect to the average value, both higher and lower values of DBP negatively affect the survival time during the follow-up period (HR 2.75 achieved at 50 mmHg; HR 2.23 at 103 mmHg of DBP).

After adjusting for age, sex, malnutrition, handgrip performance, disability and cognitive impairment, serum cholesterol >200 mg/dL remained significantly associated with a reduced mortality rate (Table 2). The remaining CVD risk factors (diabetes, smoking habit and hypertension) did not affect survival at old ages. The association between high serum cholesterol and survival remained unchanged, even after further adjusting for enrollment cohort (HR 0.74, 95% CI 0.57–0.97). The analysis including the number of CVD risk factors instead of individual variables showed that the contemporary presence of all four risk factors was not associated with an increased mortality.

Discussion

In the present study, carried out on a representative cohort of nonagenarians and centenarians from Southern Italy, we showed that the clinical and prognostic meaning associated with cardiovascular risk factors is very different from that usually observed in the adult population. In particular, our findings showed that low levels of selected cardiovascular risk factors usually associated with lower mortality in adults do not increase survival chances among oldest-old individuals and, in some cases, exert a significant and opposite effect. Furthermore, adjusting for cognitive, functional and nutritional status confirmed the observed association.

High circulating total cholesterol is known to be an important risk factor for CVD in adult populations, but its predictive value is markedly blunted among people aged >70 years.[18] Mechanisms explaining such a reverse epidemiology association are not completely clear, and studies on this topic involving nonagenarians and centenarians are very limited.[20–22] The present findings show that high circulating serum cholesterol might exert a protective effect in terms of survival. Individuals carrying serum cholesterol >200 mg/dL might have dropped-out from our study population due to a selection effect (demographic selection).[6] On a purely nutritional basis, nonagenarians and centenarians with a good nutritional status (normal-high cholesterol levels) might live longer and better than nonagenarians and centenarians with a

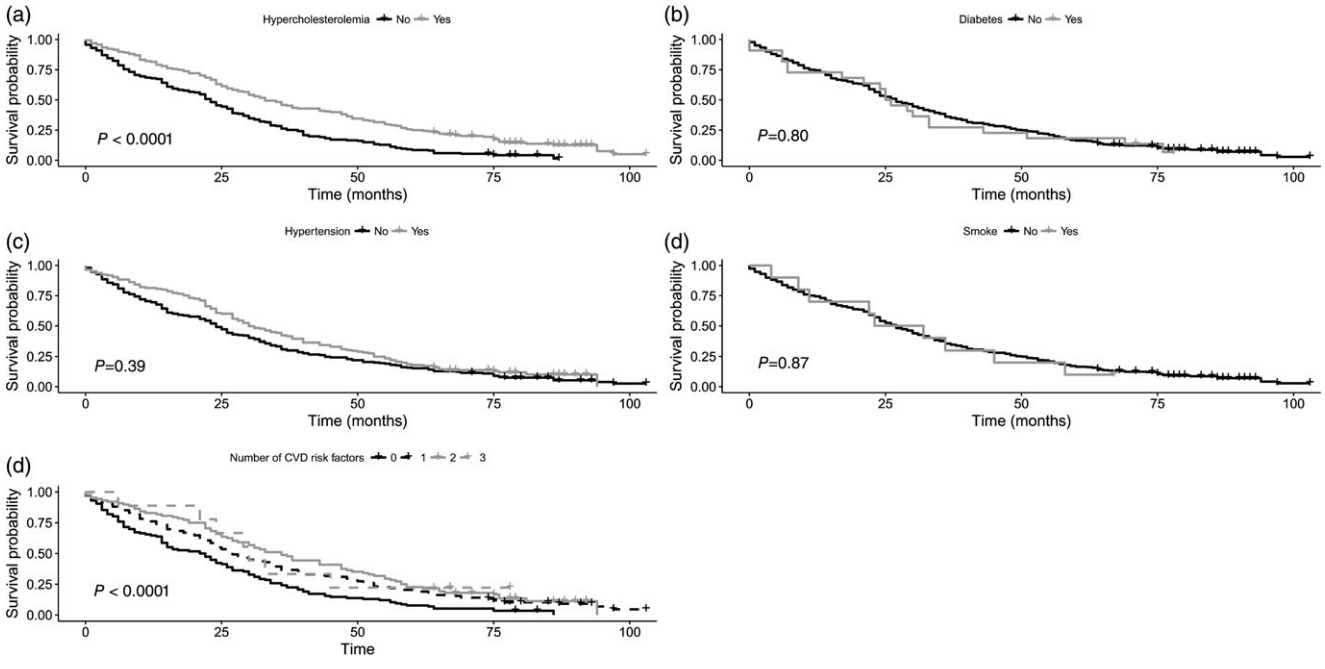

Figure 1 Kaplan–Meier survival curves of participants grouped according to (a) serum cholesterol >200 mg/dL, (b) diabetes, (c) hypertension, (d) smoke and (e) number of cardiovascular disease (CVD) risk factors.

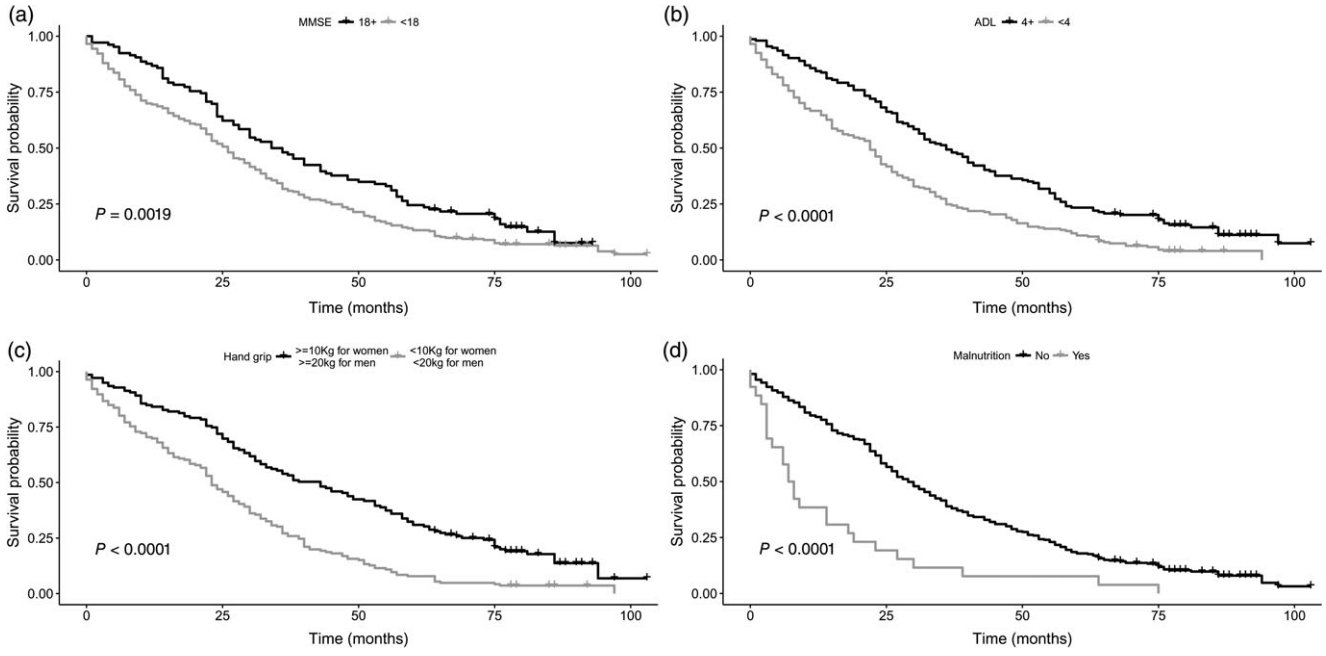

Figure 2 Kaplan–Meier survival curves of participants grouped according to (a) Mini-Mental State Examination (MMSE), (b) activities of daily living (ADL), (c) handgrip (d) and malnutrition.

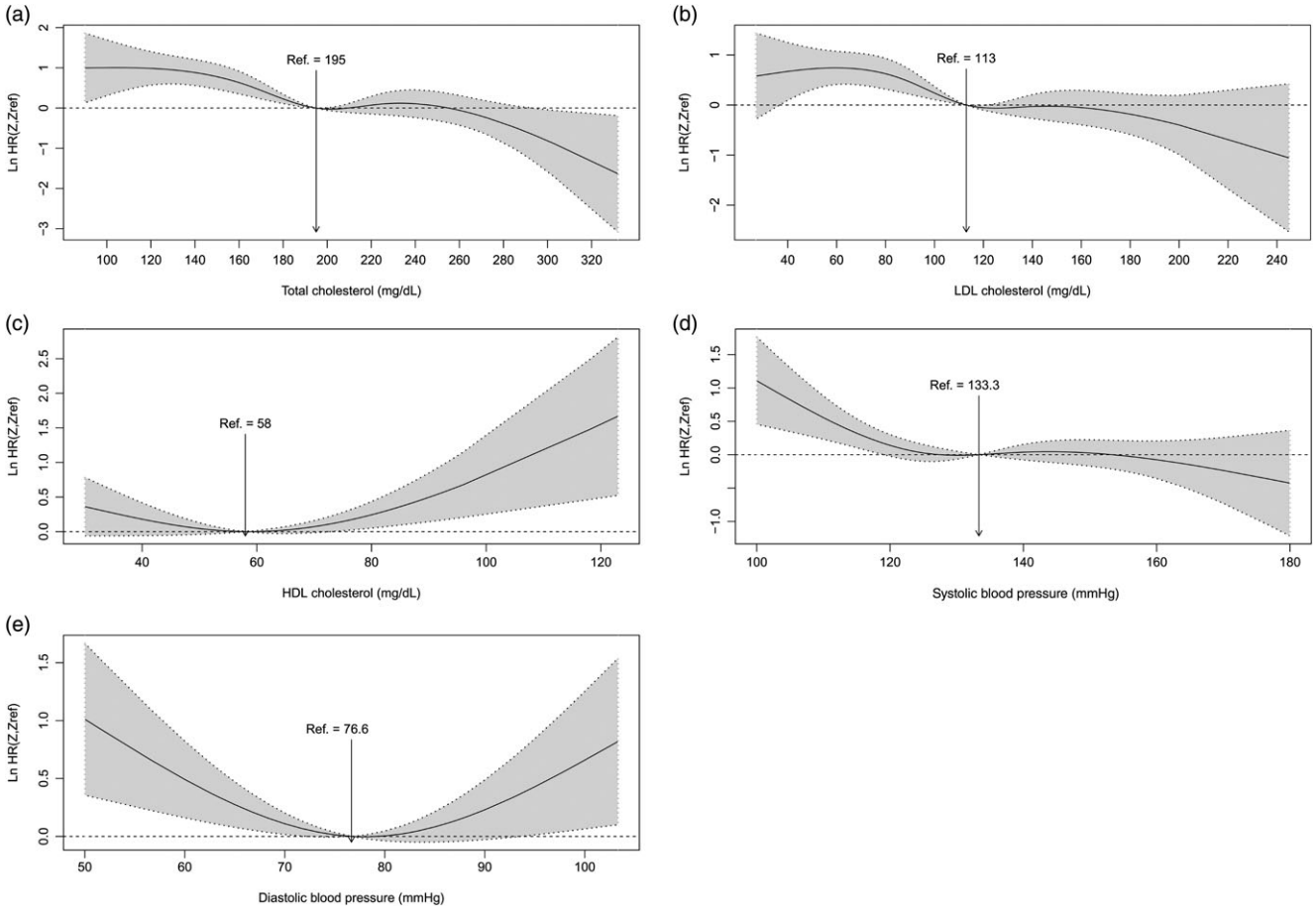

Figure 3 Spline-based hazard ratio (HR) curves with 95% point-wise confidence limits (a) for total cholesterol, (b) low-density lipoprotein (LDL) cholesterol, (c) high-density lipoprotein (HDL) cholesterol, (d) systolic blood pressure and (e) diastolic blood pressure.

poor nutritional status (low cholesterol levels). Alternatively, one can speculate that nonagenarians and centenarians with high levels of total cholesterol could be enriched for protective genetic variants that allow them to escape CVD events, and the observation that genetic variants reducing the risk of atherosclerosis might be more common in centenarians seems to support this view.[23,24] These hypotheses are not mutually exclusive, and probably underline different routes to achieve longevity.

Table 2 Summary of Cox regression analyses of selected risk factors for mortality at old ages

	Model 1			Model 2			Model 3		
Variable	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
High blood cholesterol	0.565	0.453–0.706	<0.001	0.624	0.498–0.783	<0.001	0.742	0.572–0.963	0.025
Hypertension	0.799	0.641–0.996	0.046	0.904	0.723–1.131	0.377	0.903	0.701–1.161	0.426
Diabetes	1.049	0.667–1.650	0.835	1.110	0.704–1.750	0.654	1.256	0.754–2.095	0.381
Smoke	1.073	0.783–1.470	0.662	1.325	0.698–2.517	0.389	1.793	0.868–3.703	0.115
No. CVD risk factors	0.741	0.645–0.852	<0.001	0.813	0.705–0.938	0.004	0.897	0.764–1.055	0.189

Model 1: unadjusted model. Model 2: adjusted for age and sex. Model 3: adjusted for age, gender, malnutrition, handgrip performance, disability and cognitive impairment. CVD, cardiovascular disease.

It is worth noting that the present findings were also confirmed after adjusting for measures of cognitive performance, functional status and malnutrition. Thus, at variance from previous studies among people aged ≥85,[25] geriatric syndromes did not produce any effect modification on the association between high serum cholesterol and increased survival in the present study. The peculiar characteristics of our study population, including exclusively nonagenarians and centenarians, might account for this difference with former studies, and might have contributed to bringing out serum cholesterol >200 mg/dL as an independent survival factor.

Another remarkable detail emerging from the present study is that hypertension was not significantly associated with mortality, and a trend for a U-shaped relationship between DBP and survival was also observed. Several factors might contribute to the observed disruption of the negative prognostic implications of hypertension among nonagenarians and centenarians, which is in keeping with results obtained in recent centenarian studies.[26] Hypertension is known to be among the most prevalent chronic diseases in centenarians, although the percentages of people affected could significantly change as a function of the population studied.[27] Hypertension is a recognized risk factor for cardiovascular and neurodegenerative diseases, and several studies have reported that both DBP and SBP exert a stronger influence on the occurrence and development of cardiovascular diseases.[28,29] SBP was found to be associated with an increased risk of ischemic heart disease and stroke, even among older people, with evidence for an effect modification by age and shallower associations at older ages.[8] Recent studies showed that blood pressure trajectories during aging might significantly impact the relationship between hypertension and outcomes. SBP declined by 12 mmHg among oldest-old individuals during a 5-year follow-up period, and such a decline was found to be associated with incident acute myocardial infarction, baseline antidepressant prescription, new diuretic prescription and increased dependency in personal activities of daily living.[30] Additionally, a terminal decline of SBP during the last 2 years of life suggests that associations of hypotension with higher mortality might be accounted for by reverse causation if participants with lower blood pressure values are closer to the end of life.[9]

Finally, smoking habit and diabetes were not associated with prognosis in the present study. However, the frequency of smoking and diabetes was very low in our study population, which likely prevented us from obtaining a reliable estimate of their prognostic impact.

The main limitation of the present study was the unavailability of information about causes of death, which might be helpful to describe the relationship between study variables and cardiovascular or non-cardiovascular mortality. Additionally, because of the observational design, confounding by indication might be present in our study, and residual confounding by other not measured factors might also represent a relevant issue. Finally, the small sample size could reduce the precision of estimates for the observed associations. This issue prevented us from fully exploring the association between hypercholesterolemia (i.e. serum cholesterol >240 mg/dL) and survival, which warrants further investigations.

However, the long follow-up period and the related mortality allowed an almost optimal exploration of the prognostic impact of the analyzed variables. To the best of our knowledge, this was the first longitudinal study that investigated cardiovascular risk factors and all-cause mortality in long-lived people. Although direct causality cannot be inferred from these correlation studies, the present data provide important support to understand the cross-talk between successful aging and cardiovascular risk factors in order to address further investigations. Living longer than a century appears to be a realizable goal, and there might be multiple routes to achieving exceptional longevity. An understanding of biological mechanisms underlying human longevity can provide deeper insights into extending the healthy lifespan.

In conclusion, the present results showed that in long-lived people the clinical and prognostic meaning associated with traditional cardiovascular risk factors is very different from that observed in younger populations. Consequently, considering the increase of this population segment, further studies are urgently required to confirm these results and to investigate the peculiarity of long-lived individuals in order to translate them into evidence-based clinical practice actions.

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Disclosure statement

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Appendix S1 Sample recruitment.
Figure S1 Kaplan–Meier survival curves of participants grouped according to (a) hypertension, (b) serum cholesterol >200 mg/dL, (c) diabetes, (d) smoke and (e) number of cardiovascular disease risk factors in the European Challenge for Healthy Aging cohort.
Figure S2 Kaplan–Meier survival curves of participants grouped according to (a) hypertension, (b) serum cholesterol > 200 mg/dL, (c) diabetes, (d) smoke and (e) number of cardiovascular disease risk factors in the Genetics of Healthy Aging cohort.
Figure S3 Kaplan–Meier survival curves of participants grouped according to Mini-Mental State Examination, activities of daily living, handgrip and malnutrition in the European Challenge for Healthy Aging cohort.
Figure S4 Kaplan–Meier survival curves of participants grouped according to Mini-Mental State Examination, activities of daily living, handgrip and malnutrition in the Genetics of Healthy Aging cohort.

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Full Paper II

La Russa D, Brunelli E, Pellegrino D. “Oxidative imbalance and kidney damage in spontaneously hypertensive rats: activation of extrinsic apoptotic pathways”. **Clin Sci (Lond),** 131(13):1419-1428 (2017). ***(if 5,016)***

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Research Article

Oxidative imbalance and kidney damage in spontaneously hypertensive rats: activation of extrinsic apoptotic pathways

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In both humans and animals, essential hypertension acts as a risk factor for subclinical kidney damage and precedes renal dysfunction. Several lines of evidence indicate that hypertension and oxidative stress are closely related. The increase in vascular oxidative stress plays a key role in the pathophysiological consequences of hypertension, including kidney disease. Our study examined this issue in spontaneously hypertensive rat (SHR), a reliable model of essential hypertension. We used SHR 20 weeks old when hypertension is stably developed, vascular remodeling started, but kidney function is preserved. We examined plasmatic pro-oxidant and antioxidant status showing a significant alteration in oxidative balance in SHR. As index of oxidative damage, we evaluated lipid peroxidation in kidney, liver, and skeletal muscle, detecting a significant rise in lipid peroxidation levels in all SHR tissues, particularly relevant in kidney. In addition, we analyzed the expression of cytoplasmic antioxidant enzymes, superoxide dismutase 1 (SOD1) and glutatione S-tranferasi P1 (GSTP1). In SHR liver, SOD1 expression slight increased while we have not detected any variation in other tissues. Concerning GSTP1, SHR renal tissues did not display variations in enzyme expression, while in the other tissues, we observed a significant increase in both monomeric and pro-apoptotic dimeric form of the enzyme. By analyzing apoptotic signal, we founded c-Jun N-terminal kinase (JNK) activation in all SHR tissues, but only kidney presented extrinsic apoptotic pathway activation. Our results suggest that, in hypertensive animals with preserved renal function, despite the remarkable oxidative damage of renal tissues, only the extrinsic apoptotic pathway is activated.

Introduction

Renal diseases are a major public health concern and the main consequences including loss in renal function and cardiovascular complications [1]. Moreover, hypertension has been linked with the development of endothelial dysfunction, inflammation, and renal injury [2-4]. In both humans and animals, essential hypertension acts as a risk factor for subclinical kidney damage and precedes renal dysfunction [5], but mechanisms that correlate hypertension and kidney disease have not been elucidated extensively. Although elevated blood pressure (BP) is the major factor contributing to hypertensive organ damage, complex biochemical, hormonal, and hemodynamic mechanisms are also involved in tissue damage [6].

Several experimental and clinical data prove that hypertension and oxidative stress are closely related [7,8], although it is unclear whether oxidative stress is a cause or an effect of hypertension [9,10]. The important pathophysiological role of reactive oxygen species (ROSs) in hypertension development is due, in large part, to oxygen excess and decreased NO bioavailability in vasculature and kidneys [11]. Increased oxidative stress has been revealed in genetic and experimental models of hypertension, although the effectiveness of antioxidant treatments in reducing BP has not been not fully verified [12]. Oxidative stress

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seems to be a salient feature also in human hypertension, indeed hypertensive patients show both increased oxidative stress and reduced antioxidant capacity [11,13]. Another important finding is that the increase in vascular oxidative stress plays a key role in the pathophysiological consequences of hypertension, including kidney disease [6]. Recent evidence states that oxidative stress is a crucial molecular mechanism involved in the pathogenesis of hypertensive renal damage [14] and that apoptosis occurs in critical organs (heart, brain, or kidney) during hypertension [15].

In the present study, we used a genetic model of hypertension, the spontaneously hypertensive rat (SHR), derived from the normotensive Wistar–Kyoto rat (WKY) rat [16]. The SHR, besides being the most widely used model for essential hypertension, is also an excellent model for studying the development of renal damage in the context of human hypertension and hypertensive kidney disease, as highlighted in several recent publications [17,18]. We utilized kidney as target organ and liver and skeletal muscle as control tissues, since these organs do not appear particularly susceptible to hypertensive damage [15].

We hypothesized that the increased oxidative stress in genetic models of hypertension may contribute to organ damage through activation of apoptosis signaling pathways. In order to test this assumption, we evaluated (i) plasmatic pro-oxidant/antioxidant status, (ii) tissue lipid peroxidation as an index of oxidative damage, (iii) the expression of cytoplasmic antioxidant enzymes (superoxide dismutase 1, SOD1 and glutatione S-tranferasi P1, GSTP1), and (iv) extrinsic and intrinsic apoptotic pathways.

Materials and methods

Animals

Twenty-week-old male SHRs and WKY rats (Harlan Laboratories s.r.l. Udine, Italy) were housed under controlled lighting and temperature conditions and fed ad libitum with a standard diet and with free water access. BP measured before each experiment by tail-cuff method was: WKY: systolic BP = 127.565.4 mmHg and diastolic BP = 83.564.5 mmHg; SHR: systolic BP = 181.967.9 mmHg and diastolic BP = 124.366.2 mmHg.

Ethics

The experiments were conducted in accordance with the Directive 2010/63/EU of the European Parliament and Italian law (D.L. 116/92). All surgery was performed under anesthesia and all efforts were made to minimize animal suffering.

Measurement of plasma and tissue oxidative status

Plasma and tissue oxidative status determinations were measured by using photometric measurement kits and a free radical analyzer system with a spectrophotometric device reader (FREE Carpe Diem, Diacron International, Grosseto, Italy), which are routinely used in our laboratory [19]. Plasma oxidative stress was assayed using a diacron-reactive oxygen metabolite (d-ROM) test. The d-ROM test helps to determine the oxidant ability of a plasma sample that measures the presence of reactive oxygen metabolite derivatives, in particular hydroperoxides. By means of an appropriate acidic buffer, transition metal ions (essentially iron), originating by protein, are converted to alkoxy and peroxy radicals that react with hydroperoxides thus forming new radicals; aromatic amine (*N,N*-diethyl-*p*-phenylenediamine) react with these new radicals originating a colored cation radical spectrophotometrically detectable at 505 nm. Results are expressed in Carratelli Units (UC; 1 UC = 0.8 mg/l of hydrogen peroxide). Total plasma antioxidant capacity was assayed using a biological antioxidant potential (BAP) test. The BAP test provides an overall measure of the BAP measuring the blood concentration of antioxidants (such as bilirubin, uric acid, vitamins C and E, and proteins) capable of reducing iron from the ferric to the ferrous form; in fact, when the plasma is mixed with a colored solution (ferric chloride and thiocyanate), a decoloration occurs whose intensity is related to the ability of the plasma to reduce iron ions. The intensity of decoloration is spectrophotometrically detectable at 505 nm. Results are expressed in μmol/l of the reduced ferric ions. Tissue lipid peroxidation was assayed using a LIPO tissue test based on the ability of peroxides to induce the oxidation of Fe^{2+} to Fe^{3+}. The resulting Fe^{3+} binds to thiocyanate, causing a formation of a colored complex that can be measured photometrically. The increase in absorbance is proportional to the concentration of lipoperoxides present in the sample. Briefly, samples (200 mg) were homogenized in 1 ml of distilled water, centrifuged (15 min at 15000 ***g***), and washed twice with distilled water. After removing the supernatant, 2 ml of the indicator mixture (R1) was added, mixed (5 min), and centrifuged (5 min at 1400 ***g***). Then, 10 μl of supernatant or standard 4000 μEq/l terbutilhydroperoxide was added in 1 ml of indicator mixture, followed by the addition of 10 μl of Fe^{2+} (R2 reagent). After incubation (5 min at 37°C), the optical density was read at 505 nm and the concentration of lipoperoxides was calculated and expressed as nanoequivalent hydroperoxydes/g tissue.

Table 1 The d-ROM and BAP values for WKY and SHR rat plasma (*n*=5; *= statistically significant value)

	WKY	SHR
d-ROMs test (U CARR.)		
Mean ± SE	251.6 ± 5.9	292.4 ± 4.1
Range	216–264	282–308
P-value		0,0399*
BAP test (μmol/l)		
Mean ± SE	2417.5 ± 31.7	2132.7 ± 49.5
range	2292–2560	1984–2281
P-value		0.0187*

Western blot and densitometric analysis

Tissue samples (800 mg) were lysed in RIPA buffer (1.6 ml) with the protease inhibitor cocktail (Sigma, St Louis, MO, U.S.A.) and centrifuged at 20817 ***g*** for 20 min at 4°C. Supernatant was collected and protein was quantified with a Bradford reagent kit (Sigma, St Louis, MO, U.S.A.). Samples of supernatants containing 50 μg of proteins were heated for 5 min in Laemmli buffer (Sigma, St Louis, MO, U.S.A.), separated by SDS/PAGE in a Bio-Rad Mini Protean III, and then electroblotted onto nitrocellulose membrane (NitroBind, Maine Manufacturing, Maine, U.S.A.) using a mini trans-blot (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Membrane was blocked with TBS-T buffer containing 5% non-fat dry milk. For immunodetection, the blots were incubated overnight at 4°C with the following antibodies diluted in TBS-T: SOD1 (polyclonal goat antibody, Santa Cruz Biotechnology, Inc.); GSTP1 (monoclonal mouse antibody, Santa Cruz Biotechnology, Inc.); phosphorylated JNK (pJNK) (monoclonal mouse antibody, Santa Cruz Biotechnology, Inc.); poly [ADP-ribose] polymerase 1 (PARP-1) (polyclonal rabbit antibody, Santa Cruz Biotechnology, Inc.); caspase 8 (monoclonal mouse antibody, Santa Cruz Biotechnology, Inc.); caspase 9 (polyclonal rabbit antibody, Santa Cruz Biotechnology, Inc.). Peroxidase-linked secondary antibodies (Santa Cruz Biotechnology, Inc.) were diluted 1:2000. Immunodetection was performed by using an enhanced chemiluminescence kit (ECL Plus, Amersham). Autoradiographs were obtained by exposure to X-ray Films (Hyperfilm ECL, Amersham). Immunoblots were digitalized and the densitometric analysis of the bands obtained was carried out using WCIF ImageJ based on 256 gray values (0 white; 256 black). Quantification of the bands was obtained by measuring (five times on each band) the mean optical density of a square area after the background area had been subtracted. The results of absorbance measurements and the gray values obtained from the densitometric analysis were expressed as means ± SE (standard error) of five determinations for each sample.

Statistical analysis

Data were analyzed using the GraphPad/Prism version 5.01 statistical software (SAS Institute, Abacus Concept, Inc., Berkeley, CA, U.S.A.). Statistical differences were examined using one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test. Data are expressed as the mean ± SE.

Results

Plasma and tissue oxidative status

We analyzed the trend of both oxidative stress (d-ROMs) and antioxidant barrier efficiency (BAP) in plasma of SHR and WKY rats. As shown in Table 1, we found a significant increase in oxidative stress and a remarkable reduction in antioxidant barrier efficiency in SHR compared with normotensive animals. We also evaluated tissue lipid peroxidation (estimated by LIPO tissue test) in kidney, liver, and skeletal muscle. Our results showed a very significant increase in hydroperoxide level in SHR with respect to WKY rats in all analyzed tissues (Figure 1). The hydroperoxide level increase is particularly significant in kidney tissue (nearly 2-fold).

Antioxidant enzymes' expression

We examined the expression of two important cytoplasmatic antioxidant enzymes, SOD1 and GSTP1, in both SHR and WKY rats. We noticed a slight increase in SOD1 expression in SHR liver, while in other tissues (kidney and skeletal muscle) we did not find any variation in hypertensive animals (Figure 2a). Noteworthy, SOD1 expression in liver was significantly higher with respect to other tissues in both WKY and SHR (Figure 2a). Our results showed that in kidney tissue there is no variation in GSTP1 expression in SHR with relation to WKY rats while in both liver and skeletal SHR muscle, we found a significant increase in the expression of both the monomeric (23 kDa) and dimeric

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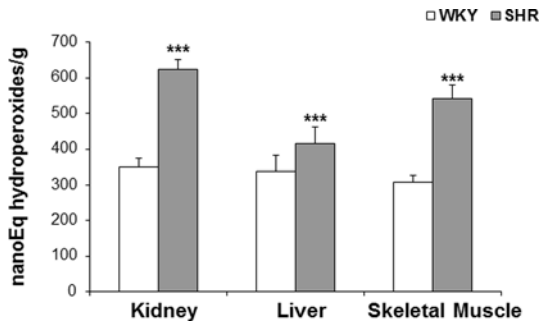

Figure 1. Lipoperoxidation levels in rat tissues.

Evaluation of lipoperoxidation levels in SHR and WKY rat tissues. Data are means ± SE of five determinations for each animal (n=5). Statistical differences were evaluated by one-way ANOVA followed by Bonferroni multiple comparisons test (***P<0.001).

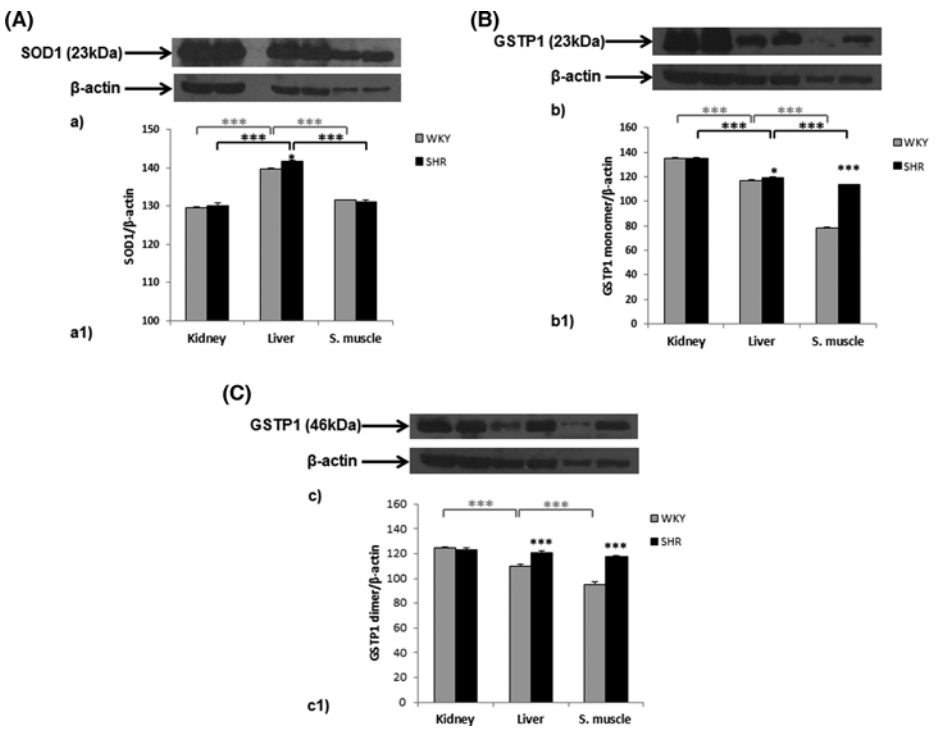

Figure 2. SOD1 and GSTP1 expression in rat tissues.

(**a**) Western blotting of SOD1 in the kidney, liver, and skeletal muscle extracts of SHR and WKY rats; (**a1**) shows the densitometric quantification of the blots. Protein loading was verified by using the anti-β-actin antibody. (**b** and **c**) Western blotting of monomeric GSTP1 form (b) and dimeric GSTP1 form (c) in the kidney, liver, and skeletal muscle extracts of SHR and WKY rats; (**b1**) and (**c1**) show the densitometric quantification of the blots. Protein loading was verified by using the anti-β-actin antibody. Data are means ± SE of five determinations for each animal (n=3). Statistical differences were evaluated by one-way ANOVA followed by Bonferroni multiple comparisons test (***P<0.001).

(46 kDa) enzymatically active form (Figure 2b and c). The basal GSTP1 amount resulted significantly different in all tissues examined and was clearly visible in kidney tissue in both SHR and WKY rats (Figure 2b and c).

Apoptotic pathways

We analyzed apoptosis activation by evaluating the expression of pJNK and PARP-1, apoptotic extrinsic pathways by evaluating the expression of caspase 8, and apoptotic intrinsic pathways by evaluating the expression of caspase 9. Our results showed the apoptosis activation in all SHR tissues examined (kidney, liver, and skeletal muscle) as highlighted by (i) significant up-regulation of pJNK detected as double bands (pJNK1, 46 kDa and pJNK2, 54 kDa; Figure 3a) and (ii) significant up-regulation of PARP-1 active cleaved fragment (89 kDa; Figure 3b). We observed the apoptotic

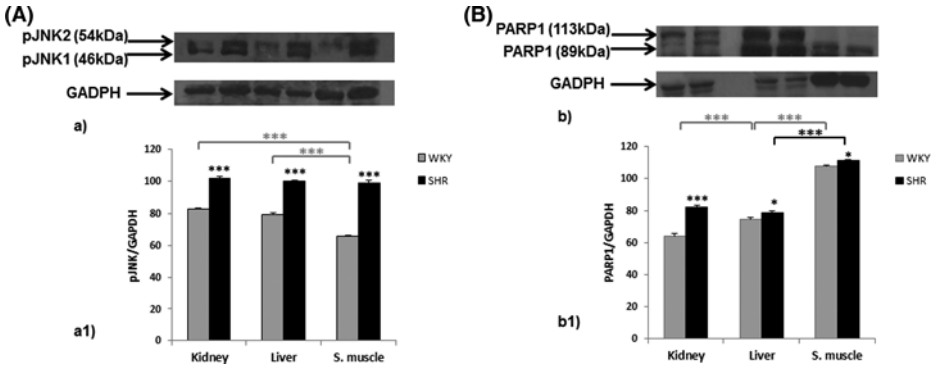

Figure 3. pJNK and PARP-1 expression in rat tissues.

Western blotting of pJNK (**a**) and PARP-1 (**b**) in the kidney, liver, and skeletal muscle extracts of SHR and WKY rats; (**a1**) and (**b1**) show the densitometric quantification of the blots: (a1) pJNK double bands (pJNK1, 46 kDa and pJNK2, 54 kDa); (b1) PARP-1 active cleaved fragment (89 kDa). Protein loading was verified by using the anti-GAPDH antibody. Data are means ± SE of five determinations for each animal (n=3). Statistical differences were evaluated by one-way ANOVA followed by Bonferroni multiple comparisons test (*P<0.05; ***P<0.001).

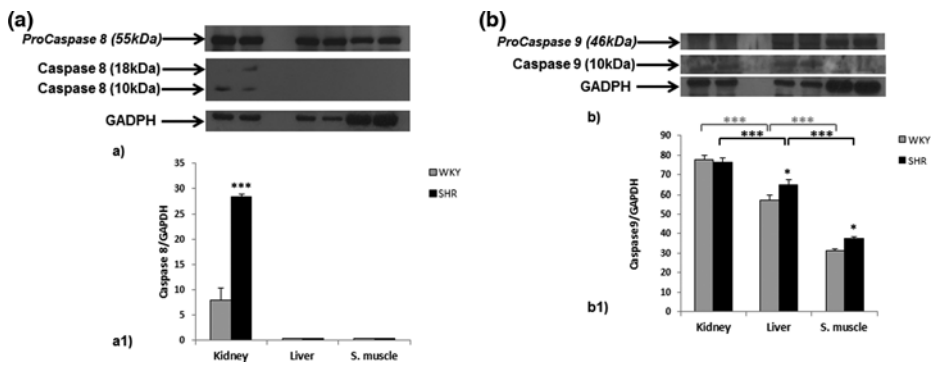

Figure 4. Caspase 8 and Caspase 9 expression in rat tissues.

Western blotting of caspase 8 (**a**) and caspase 9 (**b**) in the kidney, liver, and skeletal muscle extracts of SHR and WKY rats; (**a1**) and (**b1**) show the densitometric quantification of the blots: (a1) caspase 8 active fragments cleaved (10 and 18 kDa); (b1) caspase 9 active fragment cleaved (10 kDa). Protein loading was verified by using the anti-GAPDH antibody. Data are means ± SE of five determinations for each animal (n=3). Statistical differences were evaluated by one-way ANOVA followed by Bonferroni multiple comparisons test (*P<0.05; ***P<0.001).

extrinsic pathway activation only in SHR kidney as highlighted by an increased expression of two caspase 8 active fragments cleaved (10 and 18 kDa) that were absent in other tissues (Figure 4a). We observed the apoptotic intrinsic pathway activation in SHR liver and skeletal muscle as highlighted by an increased expression of caspase 9 active fragment cleaved (10 kDa; Figure 4b).

Discussion

The present study was designed to assess how the alteration of the oxidative balance in the essential hypertension model can influence tissue alterations, in terms of both oxidative damage (lipid peroxidation, altered expression antioxidant enzymes) and apoptotic pathways (intrinsic/extrinsic) activation. Our results are presented in graphical form in Figure 5. We showed significant alterations in plasmatic oxidative balance and tissue oxidative damage in SHR. In particular, we found a particularly relevant lipid peroxidation level in SHR kidney, whereas SOD1 and GSTP1 expression remained unaltered. Concerning the apoptotic signal, we found pJNK and PARP-1 activation in all SHR tissues tested, while we found intrinsic apoptotic pathway activation in liver and skeletal muscle and extrinsic apoptotic pathway activation only in kidney tissue.

Hypertension acts as an important risk factor for renal diseases as highlighted by several studies in animal models of hypertension, in particular in spontaneously hypertensive animals [17,18]. In our study, we used a genetic model of essential hypertension that represents the most popular model of hypertension with over 20,000 articles in PubMed. Furthermore, the SHRs are currently widely used as an important model in order to analyze the intricate link between

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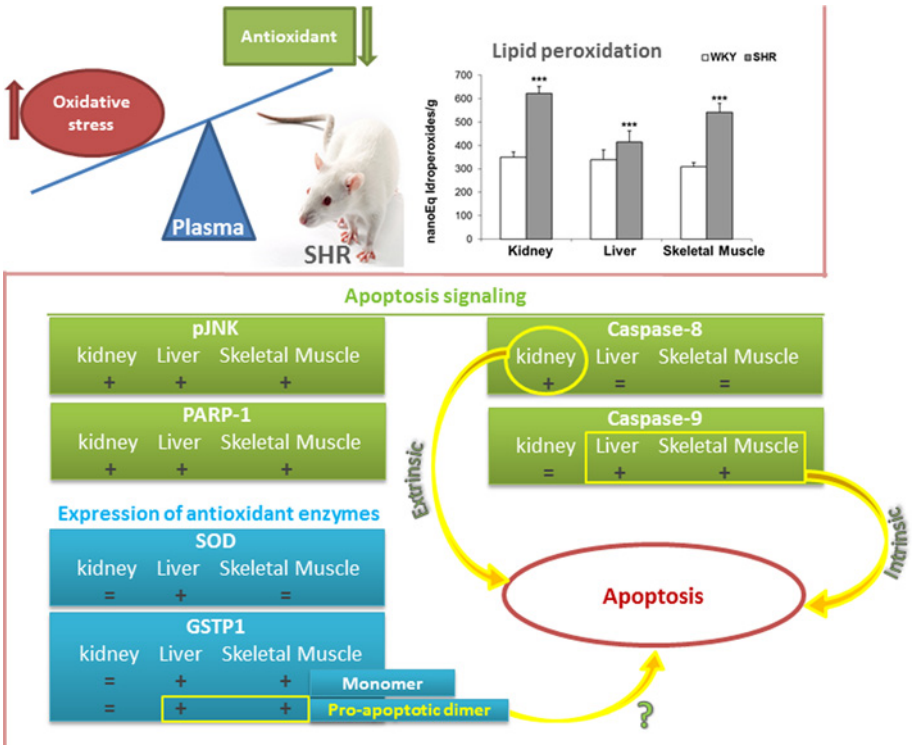

Figure 5. Key findings in graphical form

BP and kidney function [17,18,20]. The timeline of the development of hypertension and renal damage is well known in this experimental model: hypertension develops within the first 10 weeks after birth and remains stable or changes gradually as age increases; vascular remodeling begins very early (4–5 weeks) and appears concurrent with increased renal autoregulatory efficiency; renal function (renal blood flow and glomerular filtration rate) remains constant up to 20 weeks of age; hypertensive kidney damage is not morphologically evident before 30 weeks of age [17]. For our purpose, we used 20-week-old SHR, when hypertension is fully developed, vascular remodeling has started, but kidney function is preserved.

The first stage of this research examined the plasma and tissue oxidative status in SHR showing significant alterations in plasmatic oxidative balance and tissue oxidative damage, which was particularly relevant in kidney tissue [21,22]. To detect total plasma redox balance, we used a simple and standardized methodology [19] that allowed us to analyze both oxidative stress (d-ROM) and antioxidant capacity (BAP). The analysis of the overall redox balance does not allow us to identify the alterations in the systems, but it provides a global vision that reflects the balance created after the perturbation of the individual factors. Our results clearly show a significant increase in oxidative stress values and a significant depletion in the efficiency of total plasma antioxidant barrier in SHR, while in normotensive WKY we detected normal values of oxidative balance, similar to those in humans [19,23]. In SHR, the plasmatic oxidative stress increase has been already detected at 2 weeks of age, which is when the rats are still normotensive [24]. Our results confirm literature reports in both animal models and humans [11,13]. To quantify the tissue damage by oxidative stress, we detected lipid peroxidation. Lipids that contain unsaturated fatty acids with more than one double bond are particularly susceptible to the deleterious action of free radicals with consequent impairment of the biological membranes' structure and function. In our study, we detected a significant increase in tissue LIPO levels (kidney, liver, and skeletal muscle), as markers of lipid peroxidation, in SHR in contrast with WKY animals. This increase appears particularly relevant in kidney tissue, where the lipid peroxidation level results 2-fold in the hypertensive animal compared with the normotensive one. It is well known that the kidney is a target organ for hypertensive oxidative stress, and several studies have confirmed that oxidative damage appears as a primary abnormality in the kidney before development of hypertension [24]. Interestingly, in the liver, commonly used as an indicator for systemic oxidative stress [25], we detected a lower degree of lipid peroxidation compared with other tissues examined. Probably, being the main center of detoxification, the liver detoxifies various ROS by efficient antioxidant defense mechanisms.

In the second part of our work, we examined the expression of two important antioxidant enzymes, SOD1 and GSTP1. The high ROS levels determine a depletion of non-enzymatic antioxidants since the ROS species neutralization implies their consumption [10]. Concerning enzymatic antioxidants, the issue is more complex: in the case of low/medium oxidative stimulation, enzymatic antioxidant activity can increase, but if oxidative stress is persisting, or its level is very high, the damage caused to proteins becomes profound and a decreased expression/activity may occur via direct oxidative damage of the molecules and/or oxidative-altered gene expression. Hypertensive patients experience reduced activity and decreased content of antioxidant enzymes, including SOD1, the most important preventive antioxidants that catalyze the dismutation reaction of superoxide anion to the more stable hydrogen peroxide [11,26]. However, in experimental models of hypertension, it has been reported either as a reduction in antioxidant enzyme expression/activity or as an adaptive increase in antioxidant enzyme activities [27-29]. In addition, also physiological post-translational oxidative modification can influence the stability/activity of SOD1, as recently highlighted [30]. In our study, we showed a slight increase in SOD1 expression only in liver tissue. Noteworthy, SOD1 expression in the liver was significantly higher when compared with other tissues in both WKY and SHR. These results highlight the importance of SOD1 as the first line of cellular defense against oxidative injury. Indeed, in liver tissue, we found an increase in SOD1 expression and a low rate of lipid peroxidation, thus confirming the important protective role of SOD1 against oxidative damage. The GST family comprises dimeric isoenzymes widely expressed in mammalian tissues that catalyze the conjugation of reduced glutathione with a wide variety of electrophiles, including various oxidative stress products such as oxidized DNA and lipid [31]. In addition, several lines of evidence suggested a non-catalytic role for GSTP1 as an integral determinant for stress response cellular pathways, proliferation, and apoptosis in both humans and rodents [32,33]. In particular, although GSTs are dimeric proteins, the monomeric form of GSTP1 acts as a c-Jun N-terminal kinase (JNK)-proliferative pathway activator [34]. Several studies have shown that GSTP1 acts as a stress response protein that multimerizes through disulfide cross-links, if affected by oxidative stress, and loses its ability to bind JNK, causing an increase in JNK-apoptotic pathways [32]. Our results showed that in kidney tissue, where GSTP1 resulted markedly expressed, there is no variation in SHR respect to WKY while in both liver and skeletal muscle, we found a significant increase in both monomeric and dimeric forms of the enzyme in hypertensive animals. Despite the lack of remarkable difference in SOD1 and GSTP1 expression, SHR shows a significant increase in lipid peroxidation; therefore, future studies should be directed at measuring the enzymatic activities of both SOD1 and GSTP1 in hypertension.

In the final stage of our research, we examined the apoptotic signal showing a significant increase in both pJNK and PARP-1 in all SHR tissues tested with respect to WKY controls. Apoptosis signaling has been widely classified into extrinsic (initiated by death receptors) and intrinsic (initiated by mitochondrial events) pathways, and pJNK plays a central role in both of these pathways [35,36]. JNK, an important member of the mitogen-activated protein kinase (MAPK) family, plays a critical physiological and pathophysiological role in cells and a complex role in apoptosis. Several lines of evidence suggest that JNK is an important mediator in oxidative stress-induced apoptotic cell death, particularly significant in the pro-apoptotic state associated with hypertension [37]. Gomes and co-workers [37] suggest that enhanced vulnerability to oxidative stress-induced apoptosis in SHR renal cells may result from impaired catalase activity and JNK hyperactivation. Our result confirmed this finding, indeed in our SHR tissues (including kidney) we detected a specific and robust pJNK2 activation, and we support the hypothesis that enhanced JNK activity is a vital mechanism underlying the apoptotic response to oxidant injury in hypertension. The activation of the extrinsic pathway is mediated by caspase-8 [38], and the activation of the intrinsic pathway is mediated by caspase-9 [39]. Caspase-mediated apoptotic cell death is accomplished through the cleavage of key proteins required for cellular functioning/survival, and PARP-1 is one of caspase cellular substrates. Cleavage of PARP-1 by all the caspases is considered to be a hallmark for apoptosis [40]. Accordingly, all SHR tissues analyzed by us showed the activation of the mechanisms leading to cell death. Apoptosis occurs in critical organs (heart, brain, kidney, liver, and skeletal muscle) during hypertension [15,41-43], and emerging data confirms that excessive ROS induced by hypertension can damage tissue components including lipids, proteins, and ultimately lead to cellular loss via apoptosis or necrosis [41,44]. Indeed, adult SHRs present a significant increase in renal interstitial fibrosis and apoptosis [45], while in newborn animals apoptosis was minor in SHRs' kidneys compared with their normotensive controls [46]. In the present study, by analyzing both the extrinsic and intrinsic apoptotic pathways, we found that only in kidney tissue extrinsic apoptotic pathway activation was present while liver and skeletal muscle showed intrinsic apoptotic pathway activation. In the rat model for kidney disease, the apoptotic process is associated with both intrinsic and extrinsic pathways [47]. Also in the ventricle of hypertensive rats, caspase−8 and caspase−9 activity was substantial compared with that in the control group [48]. In our model, in which hypertension is stably developed whereas kidney function is preserved, the remarkable renal oxidative damage (i.e. lipid peroxidation) in SHR does not lead to the activation of the mitochondrial-mediated intrinsic apoptotic pathway. Furthermore, in SHR tissue we did not detect an increase

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in the dimeric pro-apoptotic form of GSTP1, which was higher in SHR liver and skeletal muscle where the intrinsic apoptotic pathway is activated. These results suggest that, in our model, the increase in the dimeric form of GSTP1 can exert its pro-apoptotic action by activating the intrinsic pathway.

In conclusion, data collected in the present study show that, in addition to a direct effect on BP, the redox disequilibrium in both plasma and tissue is extremely important in the hypertensive tissue alteration in terms of both oxidative damage (lipid peroxidation, altered expression antioxidant enzymes) and apoptotic pathways (intrinsic/extrinsic) activation. Furthermore, our results highlight the strong causality link between oxidative damage in renal tissue and hypertension and suggest the presence of additional (genetic and/or acquired) factors intrinsic to the kidney involved in the susceptibility to BP alterations.

Clinical perspectives

- Hypertension is complex systemic condition resulting from anatomical, physiological, and molecular events, which can cause an anomalous environment for cells and tissues. The aim of our study was to assess the relationship between the oxidative balance alteration and the tissue alterations, in terms of both oxidative damage (lipid peroxidation, altered expression antioxidant enzymes) and apoptotic pathway (intrinsic/extrinsic) activation. For our purpose we utilized SHR, a useful model of essential hypertension.

- Our results showed significant alterations in plasmatic oxidative balance and tissue oxidative damage in SHR. In particular, despite the lack of remarkable difference in SOD1 and GSTP1 expression, SHR shows a significant increase in lipid peroxidation, particularly relevant in kidney. Concerning the apoptotic signal, we found pJNK and PARP-1 activation in all SHR tissues tested, but only kidney tissue experienced extrinsic apoptotic pathway activation.

- A deeper understanding of the mechanisms underlying the relationship between hypertension and progression of renal tissue damage can provide new treatment possibilities for both renal and cardiovascular diseases.

Author Contribution

D.P. developed and led the project. All of the authors designed, performed the experiments, and analyzed the results. D.P. wrote the paper with input from E.B. and D.L.R.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

BAP, biological antioxidant potential; BP, blood pressure; d-ROM, diacron-reactive oxygen metabolite; GSTP1, glutatione S-tranferasi P1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PARP-1, poly [ADP-ribose] polymerase 1; pJNK, phosphorylated JNK; ROSs, reactive oxygen species; SHR, spontaneously hypertensive rat; SOD1, superoxide dismutase 1; WKY, Wistar–Kyoto rat.

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Full Paper III

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Research Article

Impaired oxidative status is strongly associated with cardiovascular risk fac

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Research Article

Impaired Oxidative Status Is Strongly Associated with Cardiovascular Risk Factors

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The main target of primary prevention is the identification of cardiovascular risk factors aimed at reducing of the adverse impact of modifiable factors, such as lifestyle and pharmacological treatments. In humans, an alteration of the oxidative status has been associated with several pathologies, including diabetes and cardiovascular diseases. However, the prognostic relevance of circulating oxidative stress biomarkers remains poorly understood. Our epidemiological study explored, in a healthy population (322 healthy Italian volunteers of both sexes, age 25–69 years), the putative relationship between oxidative status and cardiovascular risk factors. Plasmatic prooxidant and antioxidant determinations were performed by photometric measurement kits (Diacron Int., Italy). In the present study, we were successful in demonstrating that plasmatic oxidative status of healthy subjects is significantly associated with traditional cardiovascular risk factors. In detail, we revealed a significant depletion in the efficacy of total plasma antioxidant barrier in high cardiovascular risk categories and we confirmed an age-related alteration of oxidative status. Moreover, the efficacy of total plasma antioxidant barrier is significantly depleted in relation to metabolic disorders (such as diabetes, obesity, and dyslipidemia). Interestingly, the cholesterol imbalance is the main factor in depleting the efficacy of total plasma antioxidant barrier; in fact, a minimal increase in both total serum cholesterol and total serum cholesterol/HDL has a highly significant influence on the oxidative status. The oxidative status is also influenced by hypertension, and a slight increase in systolic blood pressure is able to determine a highly significant effect. Our results show that the first detectable event of a redox disturbance is the repairing intervention of the antioxidant barrier that is thus decreased as overutilized.

1. Introduction

Cardiovascular diseases (CVDs) are a group of diseases that share the principal risk factors and often the aetiology. The main manifestations of CVDs are coronary heart disease and stroke that represents the world's primary cause of death and disability and the most important cause of premature death, in agreement with the World Health Organization. CVDs represent a major health problem worldwide that causes a great public financial effort due to both inability to work and higher pharmaceutical expenditure. Therefore, for their broad and well-recognized importance, strategies to prevent CVDs should be considered as a priority for all citizens and healthcare systems.

The main target of primary prevention is the identification of cardiovascular risk factors aimed at reducing of the adverse impact of modifiable factors, such as lifestyle and pharmacological treatments. Furthermore, the evaluation of early and reliable risk factors can be used to identify high-risk subjects before the irreversible effects of the disease (early diagnosis). A growing number of scientific evidence suggests that effective prevention strategies are feasible and useful, also from the economic viewpoint [1].

A series of risk factors with pathogenic implication for CVDs have been identified and summarized in the Framingham study [2]. The main risk factors included smoking, hypertension, dyslipidemia, and diabetes. Over the years, several epidemiological studies validated the prediction models of cardiovascular diseases based on these risk factors, thus contributing to a steady decrease in CVD mortality [3], and the prediction models based on Framingham risk score are still used all over the world. Since the publication of

results from Framingham study [2], other important predisposing factors with pathogenic implication for CVDs have been identified, including a high-fat diet, low physical activity, obesity, and genetic influences [4].

Currently, the ongoing studies are aimed at improving the risk algorithms through the individuation of new biomarkers strongly associated with CVDs (even if devoid of a direct relationship with these pathologies) also in order to define the appropriate preventive therapy of asymptomatic individuals [5, 6].

There are several clinical and experimental evidences supporting the hypothesis of a link between the oxidative status alteration and the development and progression of many health problems, such as neurodegenerative conditions, cardiovascular and inflammatory diseases, and cancer [7–9]. The predictive value of circulating oxidative stress biomarkers is poorly understood, despite the modified oxidative status has been associated with over 100 diseases. In particular, the ability of oxidative stress biomarkers to predict CVDs has been widely studied but remains largely unclear [10].

Oxidative stress is referred to the disproportion between free radicals and antioxidant system to counteract or detoxify their detrimental effects. The direct detection of free radicals is made complex by the nonspecificity and the high reactivity of these molecules. It takes, therefore, evaluating oxidative damage by measurement of secondary products, although the limited evidence that it reflects is oxidative status *in vivo* [11]. Epidemiological investigations have considered just a few of the numerous oxidant species as a biomarker relating them with cardiovascular dysfunctions, such as homocysteine, nitrosated tyrosines, and isoprostanes [12].

An alternative approach to investigate oxidative imbalance is the assessment of antioxidant enzymes (superoxide dismutase, catalase, and ascorbate peroxidase) and antioxidative defense, as well as nonenzymatic ascorbate, glutathione, flavonoids, tocopherols, and carotenoids [12, 13]. However, the predictive ability of these biomarkers and their usefulness to the definition of cardiovascular risk scores are underinvestigated. In the last years, several researchers are using two simple methods for detecting in vivo reactive oxygen species (ROS) using derivatives of reactive oxygen metabolites (dROMs) and biological antioxidant potential (BAP) [14–17]. For instance, in Japanese and Korean epidemiological trials, a significant correlation between oxidative balance and lifestyle-related diseases was found through these new methods [18, 19]. Hence, it is evident that there is a need for more extensive studies on large cohorts and under different clinical situations, including preclinical stages.

In view of this background, our research was designed to investigate, in a Mediterranean population, whether the oxidative balance is related to traditional cardiovascular risk factors. We evaluate, through a cross-sectional analysis on 322 healthy subjects, the global plasmatic oxidant/antioxidant ability by measuring reactive oxygen metabolite and biological antioxidant potential by photometric measurement. This study is of emerging interest in CVD research since the analysis of new biomarkers could improve the predictive role of CVD risk factors.

2. Subjects and Methods

2.1. Subjects. Our study involved 322 healthy Italian volunteers (work suitable) of both sexes and aged between 25 and 69 years (190 males, mean age: 51.42 ± 11.08 years and 132 female subjects, mean age: 46.11 ± 10.40 years) recruited from University of Calabria (UNICAL) staff during the annual visit performed by "UNICAL Prevention and Protection Service". The volunteers were subjected to a "health check" by filling in a form (information on health status and lifestyle), by physical measurements (body mass index, systolic, and diastolic blood pressure), and by blood tests (blood glucose, lipoprotein panel, prooxidant, and antioxidant status). All subjects were studied in the morning and in a fasting state. Blood samples were taken from the antecubital vein and immediately centrifuged ($2500g$ for 15 min at 4°C), and the plasma obtained was stored at 4°C until measurements (maximum 6 hours of venous blood collection). Baseline characteristics of the cohort are shown in Table 1.

2.2. Cardiovascular Risk Chart. The cardiovascular risk charts, based on the global absolute risk, are a simple and verified way of assessing the probability of experiencing a first major cardiovascular event (myocardial infarction or stroke) over the following ten years, by using the values of six risk factors: gender, diabetes, smoking, age, systolic blood pressure, and total serum cholesterol. When applied to the population from which they derive, they provide the best estimate of CVD risk. Therefore, in this study, we used Italian cardiovascular risk chart of The CUORE Project (http://www.cuore.iss.it). The risk charts are four: diabetic man, nondiabetic man, diabetic woman, and nondiabetic woman. For each of these four categories, the charts are further divided into smokers and nonsmokers, and the risk is calculated on the basis of age decade, serum cholesterol, and arterial pressure values. Six cardiovascular risk categories were constructed, called MCV (from I to VI): the CVD risk category indicates how many persons out of 100 with the same characteristics will fall ill over the next 10 years.

2.3. Oxidative Status and Biological Antioxidant Potential Measurements. Oxidative status and biological antioxidant potential determination were performed by using photometric measurement kits and a free radical analyzer system provided with spectrophotometric device reader (FREE Carpe Diem, Diacron International, Grosseto, Italy). All analyses were performed on ice-stored samples within maximum 6 hours of venous blood collection to prevent auto-oxidation phenomenon. We used Diacron reactive oxygen metabolite (dROM) and biological antioxidant potential (BAP) tests to evaluate plasma levels of reactive oxygen metabolites and antioxidant capacity. The dROM test helps to determine the oxidant ability of a plasma sample measuring the presence of reactive oxygen metabolites derivatives, in particular, hydroperoxides. By means of an appropriate acidic buffer, transition metal ions (essentially iron), originating by protein, are converted to alkoxy and peroxy radicals that react with hydroperoxides thus forming new radicals; aromatic

Table 1: Baseline characteristics of the cohort ($n = 322$; data are expressed as mean ± SD).

		Normal range
Age (years)	49.24 ± 11.10	
BMI (body weight/height^2)	25.95 ± 9.19	18.50–24.99
Systolic blood pressure (mmHg)	123.07 ± 16.08	<120
Diastolic blood pressure (mmHg)	76.31 ± 9.44	<80
Blood glucose (mg/dL)	97.22 ± 22.54	70–99
Total cholesterol (mg/dL)	206.12 ± 40.45	<240
HDL cholesterol (mg/dL)	54.76 ± 14.37	>60
LDL cholesterol (mg/dL)	132.03 ± 32.22	<115
Triglycerides (mg/dL)	119.01 ± 61.76	<150
Smokers	55 (17%)	

amine (N,N-diethylparaphenylene-diamine) reacts with these new radicals originating a colored cation radical spectrophotometrically detectable at 505 nm [14, 15]. Results are expressed in Carratelli units (UC; 1 UC = 0.8 mg/L of hydrogen peroxide). The BAP test provides an overall measure of the biological antioxidant potential measuring the blood concentration of antioxidants (such as bilirubin, uric acid, vitamins C and E, and proteins) capable of reducing iron from ferric to the ferrous form; in fact, when the plasma is mixed with a colored solution (ferric chloride and thiocyanate), a decoloration occurs whose intensity is related to the ability of the plasma to reduce the ions of iron [16, 17]. The intensity of decoloration is spectrophotometrically detectable at 505 nm. Results are expressed in μmol/L of the reduced ferric ions.

2.4. Statistical Analysis. Data have been analyzed using GraphPad/Prism version 5.01 statistical software (SAS Institute, Abacus Concept Inc., Berkeley, CA, USA). Differences between groups were examined using the unpaired t-test, or the Mann–Whitney test, or the Dunn's test, or the Kruskal-Wallis test, or the ANOVA test. A p value of < 0.05 was considered to be statistically significant. Data are expressed as the mean ± standard deviation.

2.5. Ethics Statement. All investigations have been conducted according to the Declaration of Helsinki principles and have been approved by Local Ethical Committee (n°8/2016, Regione Calabria, Sezione Area Nord). All subjects have provided written informed consent that, as guarantor, is retained by the corresponding author.

3. Results

Our study population consists of 322 subjects (190 males and 132 females) aged between 25 and 69 years (25–39, $n = 79$; 40–49, $n = 71$; 50–59, $n = 107$; and 60–69, $n = 65$).

Baseline characteristics of the cohort are shown in Table 1. These data are comparable to the results of the second population survey of Cardiovascular Epidemiologic Observatory (The CUORE Project—Istituto Superiore Sanità—Italy) relative to a population sample from Calabria monitored in the period 2008–2012 (http://www.cuore.iss.it/eng/factors/south.asp). In the whole sample, oxidative status and antioxidant barrier efficacy values are the following: dROM test = 333.80 ± 72.94 UC and BAP test = 1968.96 ± 412.21 μmol/L. By suitable statistical tools, we analyzed the trend of both oxidative status and antioxidant barrier efficacy in order to identify possible correlations with MCV and traditional cardiovascular risk factors: gender, diabetes, smoking, age, systolic blood pressure, and total serum cholesterol. We also considered further determinants that may predispose to cardiovascular risk as menopausal status, obesity, and the ratio of total cholesterol to HDL (high-density lipoproteins).

3.1. MCV. We calculated the total CVD risk of our cohort using the Italian cardiovascular risk chart of The CUORE Project. MCV category (from I to VI) has been assigned to subjects aged between 40 and 69 years ($n = 243$) based on parameters described in http://www.cuore.iss.it/eng/assessment/risk_assessment.asp. We analyzed the trend of both oxidative status and antioxidant barrier efficacy by comparing subjects with low (MCV I-II; $n = 180$) medium (MCV III-IV; $n = 45$), and high (MCV V-VI; $n = 18$) total CVD risk. We found no significant differences in ROM values between MCV categories (Figure 1(a)), while we showed a significant decrease in antioxidant barrier efficacy in high (MCV V-VI) CVD risk categories (Dunn's test, $p < 0,005$; Figure 1(b)).

3.2. Cardiovascular Risk Factor: Gender. We found a significant difference between males ($n = 190$) and females ($n = 132$) in the values of both oxidative status (Mann–Whitney test, $p < 0.0001$; Figure 2(a)) and antioxidant barrier efficacy (Mann–Whitney test, $p < 0.001$; Figure 2(b)). In particular, females present high ROM (364.70 ± 85.96 UC) and BAP (2035.74 ± 412.28 μmol/L) values, while males show ROM values close to the normal values (312.00 ± 52.30 UC) and low BAP values (1915.03 ± 406.64 μmol/L). Within the female group, no significant difference was observed in premenopausal ($n = 87$) and postmenopausal ($n = 45$) subjects (ROM values: premenopausal 366.49 ± 50.83 UC and postmenopausal 361.22 ± 45.92 UC; BAP values: premenopausal 2079.32 ± 441.73 μmol/L and postmenopausal 1951.49 ± 337.15 μmol/L).

3.3. Cardiovascular Risk Factor: Diabetes and Obesity. In our study population, only 14 people, all males, had a diagnosis of diabetes and were undergoing insulin or oral hypoglycemic agent treatment. Therefore, we analyzed the values of oxidative status and antioxidant barrier efficacy by comparing nondiabetic males ($n = 176$) with respect to diabetic males ($n = 14$). Despite the small sample size, we found a significant decrease in antioxidant barrier efficacy in diabetic subjects (Mann–Whitney test, $p < 0.05$; Figure 3(b)) while no differences were evidenced in oxidative status (Figure 3(a)). Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify underweight, overweight, and obesity in adults. To analyze the trend of both oxidative status and antioxidant barrier efficacy in relation to

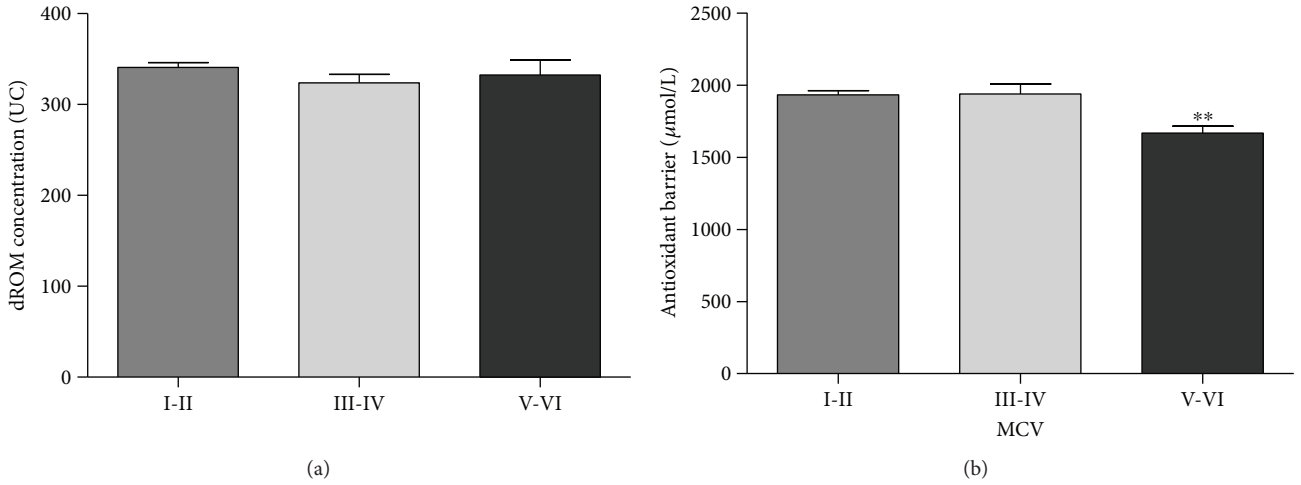

Figure 1: Values of dROM (a) and BAP (b) tests by MCV (data are expressed as mean ± SE; Dunn's test, $^{**}p < 0.005$).

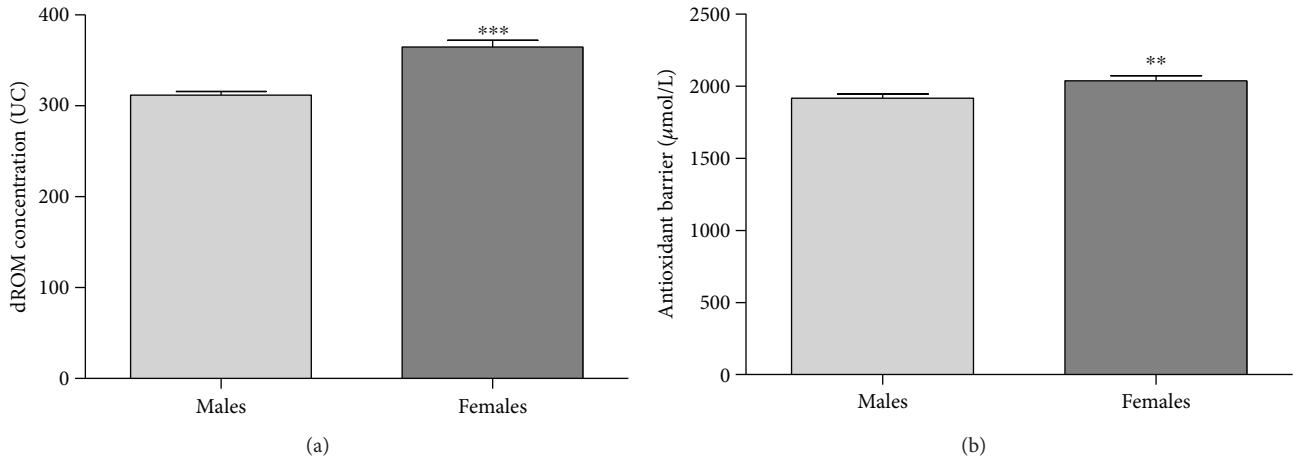

Figure 2: Values of dROM (a) and BAP (b) tests by gender (data are expressed as mean ± SE; Mann–Whitney test, $^{***}p < 0.0001$; $^{**}p < 0.005$).

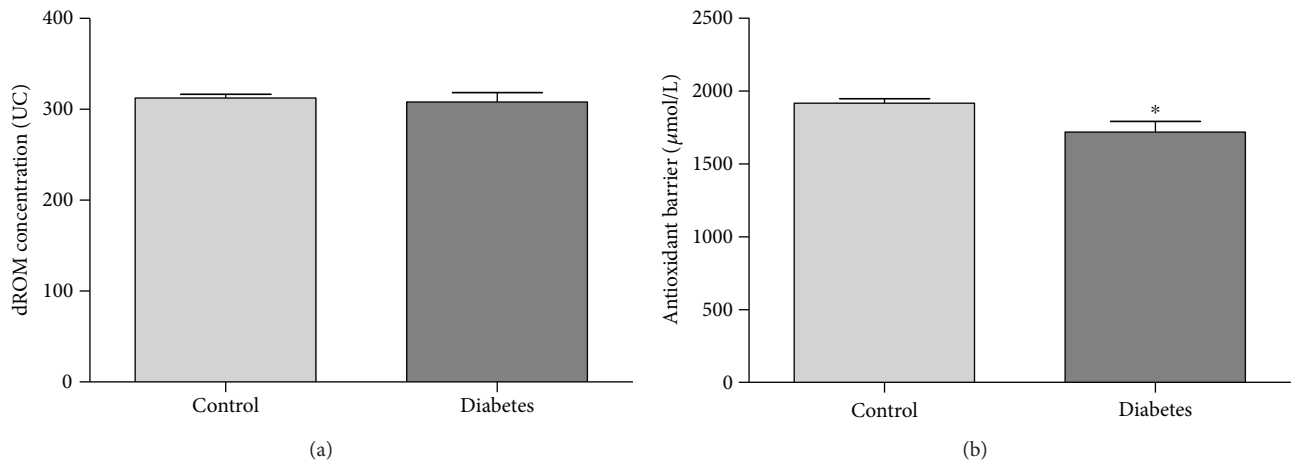

Figure 3: Values of dROM (a) and BAP (b) tests by diabetes status (data are expressed as mean ± SE; Mann–Whitney test, $^{*}p < 0.05$).

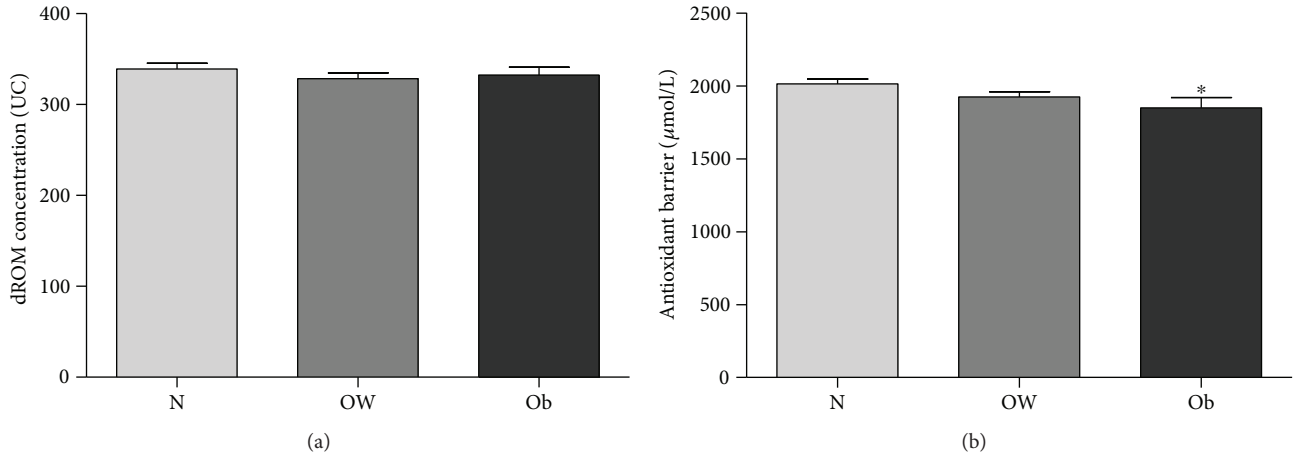

Figure 4: Values of dROM (a) and BAP (b) tests by BMI (data are expressed as mean ± SE; Kruskal-Wallis test, $^{*}p < 0.025$).

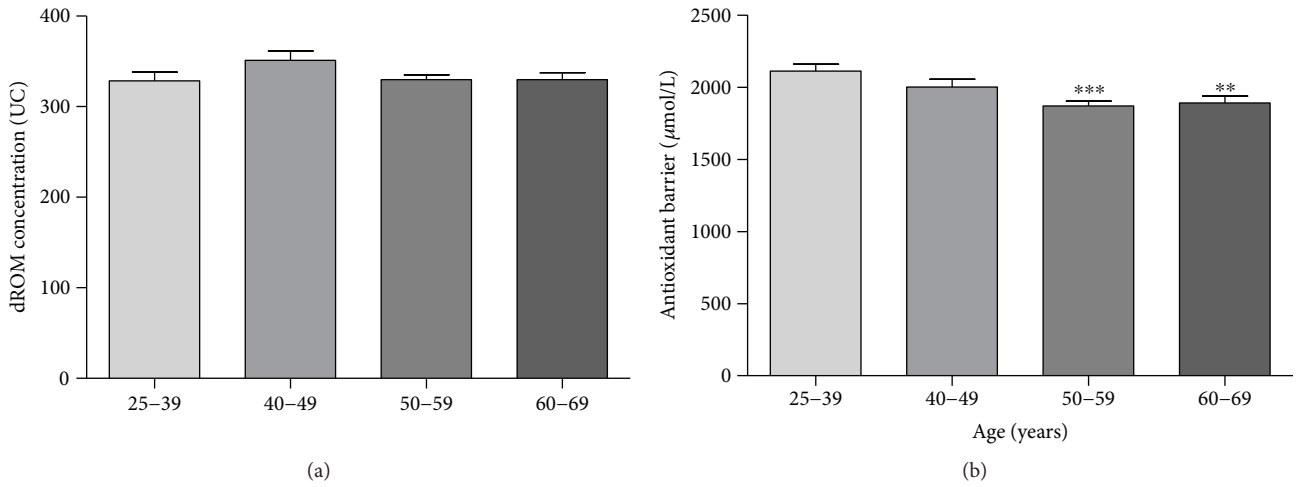

Figure 5: Values of dROM (a) and BAP (b) tests by age (data are expressed as mean ± SE; Kruskal-Wallis test, $^{***}p < 0.0005$; $^{**}p < 0.005$).

BMI, we divided our study population according to this international classification: underweight (UW, <18.50 BMI, $n = 0$); normal range (N, 18.50–24.99 BMI, $n = 150$); overweight (OW, ≥25.00 BMI, $n = 126$); and obese (Ob, ≥30.00 BMI, $n = 35$). Our results showed that there were no differences in ROM values among N, OW, and Ob groups (Figure 4(a)) as a decrease in antioxidant barrier efficacy can be observed already in OW subjects, reduction statistically significant in Ob subjects (Figure 4(b); Kruskal-Wallis test, $^{*}p < 0.025$).

3.4. Cardiovascular Risk Factor: Smoking. Concerning smoking habits, equally distributed between sex (17%), we found no significant differences between nonsmoker ($n = 267$) and smoker ($n = 55$) subjects in the values of both oxidative status and efficacy of antioxidant barrier (ROM values: nonsmokers 335.03 ± 76.23 UC and smokers 329.05 ± 55.06 UC; BAP values: nonsmokers 1968.14 ± 415.10 μmol/L and smokers 1944.09 ± 405.46 μmol/L). However, it should be noted that the smoker sample consists mainly of moderate smokers (less than ten cigarettes daily).

3.5. Cardiovascular Risk Factor: Age. We divided our study population into four age groups: the first group (25–39 years, $n = 78$) not provided for cardiovascular risk chart and used herein as controls and three groups according to the age decades of cardiovascular risk chart (40–49 years, $n = 71$; 50–59 years, $n = 105$; and 60–69 years, $n = 63$). The ROM values remain at a constant level in all groups (Figure 5(a)), while a constant reduction of antioxidant barrier efficacy was observed with increasing age (Figure 5(b)). This reduction is remarkable and highly statistically significant starting from 50 years of age (Kruskal-Wallis test, 50–59 years $p < 0.0005$ and 60–69 years $p < 0.005$).

3.6. Cardiovascular Risk Factor: Systolic Blood Pressure. To analyze the systolic blood pressure values in relation to oxidative status and antioxidant barrier efficacy, we divided our study population into three groups according to the range of cardiovascular risk chart (90–129 mmHg, $n = 174$; 130–149 mmHg, $n = 103$; and 150–169 mmHg, $n = 26$). The ROM values remain at a constant level in all groups (Figure 6(a)), while a statistically significant reduction of

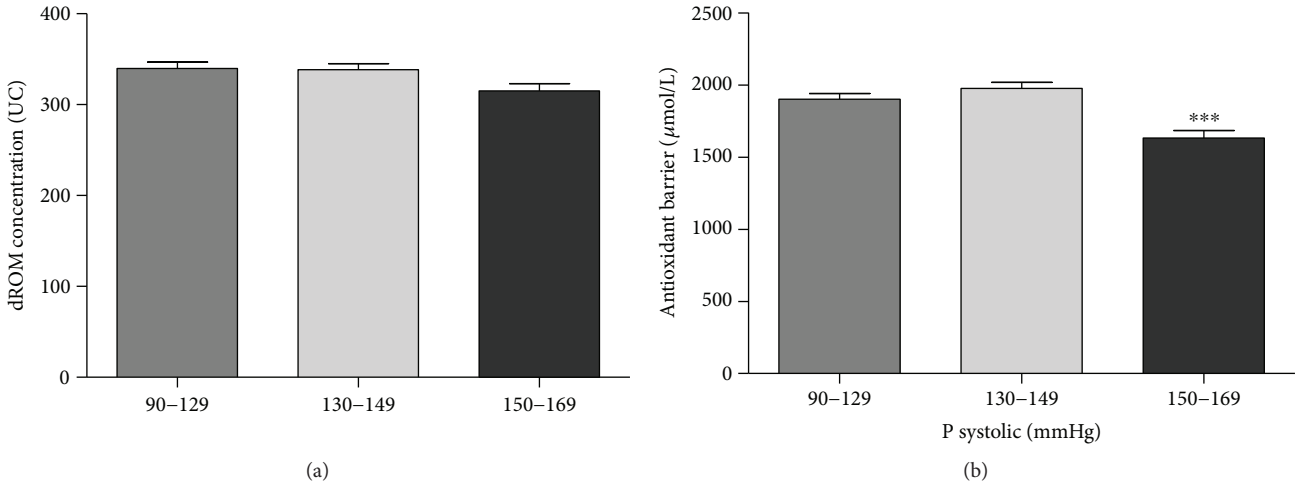

Figure 6: Values of dROM (a) and BAP (b) tests by systolic pressure (data are expressed as mean ± SD; Kruskal-Wallis test, $^{***}p < 0.0005$).

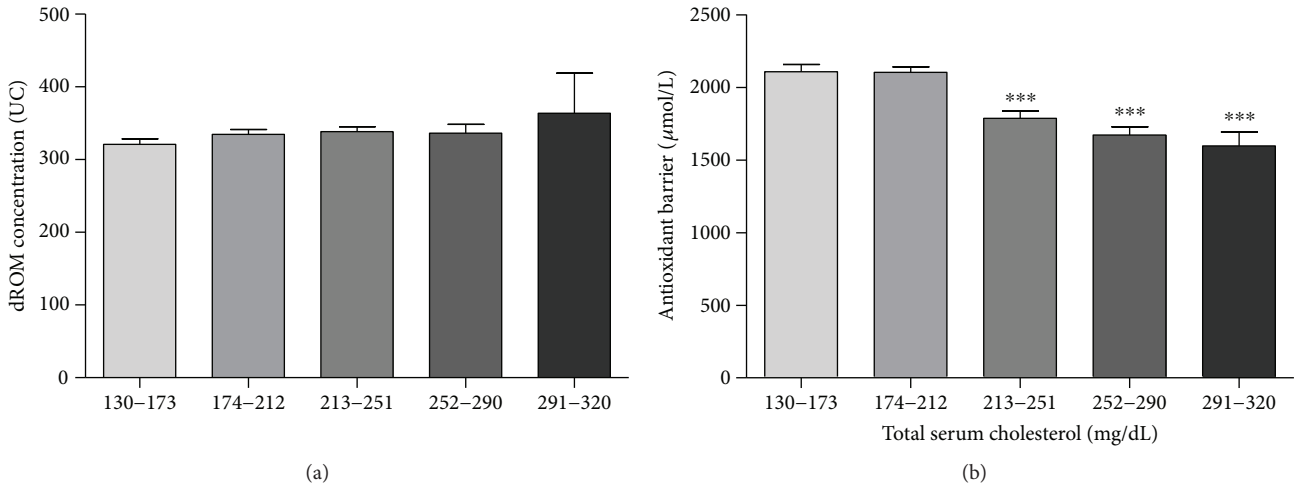

Figure 7: Values of dROM (a) and BAP (b) tests by total serum cholesterol (data are expressed as mean ± SD; Kruskal-Wallis test, $^{***}p < 0.0001$).

antioxidant barrier efficacy was observed starting from 150 mmHg of systolic blood pressure values (Figure 6(b); Kruskal-Wallis test, $p < 0.0005$).

3.7. Cardiovascular Risk Factor: Total Serum Cholesterol and Ratio of Total to HDL. We analyzed both total serum cholesterol values and the ratio of total to HDL fraction in relation to oxidative status and antioxidant barrier efficacy. In relation to total serum cholesterol values, we divided our cohort in five groups according to the range of cardiovascular risk chart (130–173 mg/dL, $n = 56$; 174–212 mg/dL, $n = 124$; 213–251 mg/dL, $n = 91$; 252–290 mg/dL, $n = 30$; and 291–320 mg/dL, $n = 9$). The ROM values remain at a constant level in all groups (Figure 7(a)), while a constant reduction of antioxidant barrier efficacy was observed with increasing total serum cholesterol values (Figure 7(b)). This reduction is remarkable and highly statistically significant starting from 213 mg/dL of total serum cholesterol (Kruskal-Wallis test, $p < 0.0001$). Concerning the ratio of total cholesterol to HDL fraction, we divided the study population into five groups: ratio of less than 3 (<3, $n = 59$); ratio of less than 4 (<4, $n = 107$); ratio of less than 5 (<5, $n = 93$); ratio of less than 6 (<6, $n = 33$); and ratio greater than 6 (>6, $n = 7$). Even in this case, the ROM values remain at a constant level in all groups (Figure 8(a)), while a remarkable and highly statistically significant reduction of antioxidant barrier efficacy was observed with increasing ratio of total cholesterol to HDL fraction (Figure 8(b); Kruskal-Wallis test, $p < 0.0001$).

4. Discussion

Our research focused on identifying the putative relationship between oxidative imbalance and cardiovascular risk factors through a cross-sectional analysis on a large healthy population. We clearly showed that the oxidative status is significantly associated with MCV, diabetes, obesity, age, high systolic blood pressure, serum cholesterol, and total cholesterol/HDL. In particular, we reported, for the first time, that

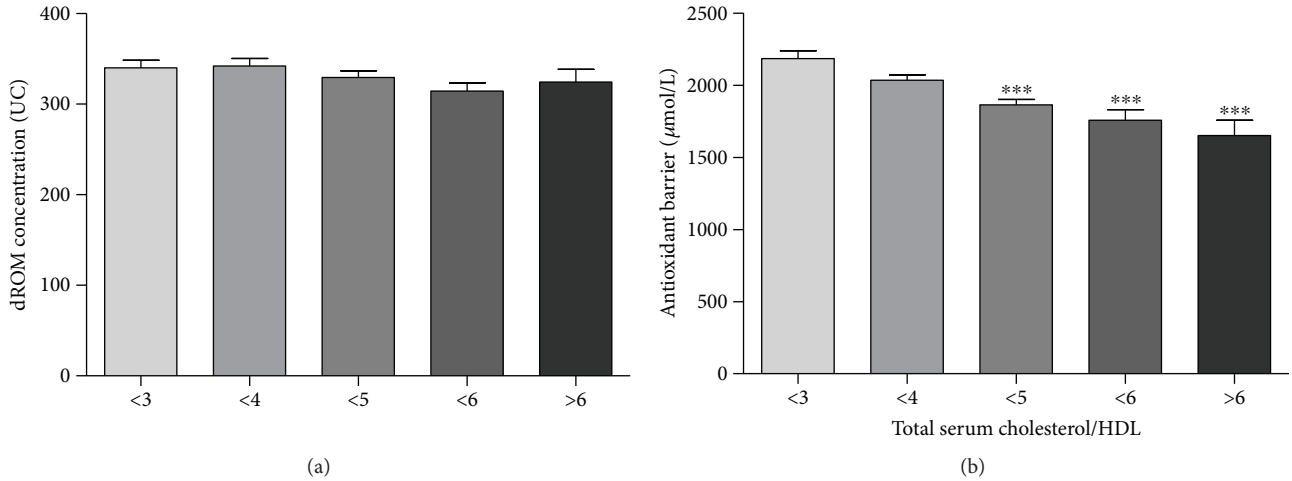

Figure 8: Values of dROM (a) and BAP (b) tests by total serum cholesterol/HDL (data are expressed as mean ± SD; Kruskal-Wallis test, $^{***}p < 0.0001$).

the early warning alteration at a systemic level is the reduction of antioxidant capacity.

It is noteworthy that these results have been achieved using a noninvasive method to detect total plasma redox balance, which is of particular importance when analyzing the healthy subject. The analysis of the overall redox balance does not identify the impaired system/s, but it provides an evaluation of the imbalance induced by the alteration of individual parameters.

To the best of our knowledge, this is the first cross-sectional study that investigated, on a healthy cohort, the oxidative status taking into account all the cardiovascular risk factors. Although a direct causality cannot be inferred from such kind of correlative investigations, our data provide an important contribution to understanding the cross-talk between oxidative imbalance and cardiovascular risk factors also representing a point of departure to address further investigation.

4.1. MCV. Global comparative risk assessment and associated health effect studies have estimated that hundreds of thousands or millions of CVD deaths are attributable to established CVD risk factors and other putative, emerging, risk factors that are the subject of extensive research. In particular, several studies suggested a positive correlation between CVDs/CVD risk factors and an increased oxidative stress [7–9]. However, information on healthy populations are scarce, and most of the available data derive from trials conducted on subjects with very high cardiovascular risk [20]. In these situations, it becomes difficult to establish a clear understanding of the relative influence of the different factors in determining oxidative imbalance and then to evaluate the synergic or independent action of CVD risk factors.

Because traditional risk factors account for only a fraction of CVDs, the importance of alternate and additional predictors is evident [21, 22]. Our results, exploring the oxidative status of healthy subjects (without previous cardiovascular events), show a significant association between the high cardiovascular risk (MCV V-VI) and the depletion in the efficacy of total plasma antioxidant barrier despite the normal values of oxidative status. Recently, some authors found a significant correlation between ROM values and age [18] or lipid profile [19], but our study remains the only considering overall CVD risk factors.

Antioxidant deficiencies may be the result of a decreased antioxidant intake, a reduced synthesis of endogenous enzymes, or an increased antioxidant utilization [22]. Since the antioxidant species are numerous and they operate synergistically, evaluating the activity of each antioxidant species may underestimate the association among different effects and probably do not reflect the physiological conditions. Moreover, for each antioxidant compound, a specific test is needed thus making the evaluation of antioxidant capacity extremely complex [23, 24].

We recognize that there are currently no validated methods for quantifying the oxidative status but certainly, the total antioxidant capacity is indicative of both organism antioxidant protection and oxidative stress amount.

4.2. Gender. According to our previous findings [25], we showed that the oxidative status was significantly higher in females than in males and we also demonstrate that this result is not related to the level of circulating hormones since no differences have been detected between pre- and post-menopausal women. The physiological significance of this gender-related difference remains unclear, and research on both animals and humans have shown contrasting results ([25] and references therein). Another interesting finding in our study was the more effective antioxidant barrier detected in females compared to males suggesting that, in healthy subjects, the altered oxidative status is balanced by an enhancement in the antioxidant barrier effectiveness.

4.3. Diabetes and Obesity. Obesity is an important cause of CVDs, and it promotes a cluster of risk factors including dyslipidemia, type 2 diabetes, and hypertension [26]. Several pieces of evidence support the role of oxidative stress in obesity and diabetes metabolic perturbations (and subsequent

cardiovascular pathogenesis) [27, 28]. The proinflammatory and prooxidant effects of an increased adiposity represent a potential link between obesity and CVDs, even in the absence of other risk factors [29, 30]. The positive association between indices of obesity and oxidative stress biomarkers is well acknowledged [20], but the underlying mechanisms are complex and not yet fully identified. In the obese-diabetic patients, the excessive uric acid has been shown to induce CVDs through the generation of ROS and subsequent endothelial dysfunction [31]. Recent studies have emphasized the importance of antioxidant defense in type 2 diabetes patients. In these subjects, the excessive ROS stimulation leads to a progressive deterioration of the antioxidant system that tends to crumble [31]. In our cohort, we found a significant decrease in antioxidant barrier efficacy in both diabetic and obese subjects; these results contribute to emphasize the importance of antioxidant barrier effectiveness in countering the deleterious effects of ROS overproduction.

4.4. Smoking. Smoking is an important risk factor for cardiovascular disease development. Cigarette smoke is a complex mixture of chemical compounds, containing many free radicals and oxidants [32, 33], and it can be associated with oxidative stress in smokers [34, 35]. It has also been highlighted a direct correlation between oxidative index and number of cigarettes smoked [36]. In our cohort, we found no significant differences in the oxidative status between nonsmokers and smokers. However, the smoker sample in our study was small and consisted mainly of moderate smokers (less than ten cigarettes daily). Moreover, the ex-smokers were very few and all of them had stopped smoking for more than ten years.

4.5. Age. The oxidative stress theory of aging postulates that reactive oxygen species play a key role in the aging process through an age-related accumulation of oxidative damages in macromolecules, resulting in a progressive loss of cellular function and senescence [37]. Over the past two decades, several lines of evidence supported this theory [38] and a number of experimental studies, in both humans and animals, showed a linear correlation between age and oxidative stress [39]. In our study, we detected no difference in ROM values in the different age groups whereas a regular reduction of antioxidant barrier efficacy was observed with increasing age. It is well known that antioxidants delay or protect against the damage produced by free radical reactions and are consumed during this process. In fact, global antioxidant status is even used to indirectly evaluate free radical activity [24].

4.6. Blood Pressure. Endothelial dysfunction, the initial stage in the pathogenesis of several cardiovascular diseases including hypertension, is associated with increased vascular ROS production, oxidative stress, and vascular inflammation [40]. Clinical studies, in patients with essential hypertension, demonstrated that systolic and diastolic blood pressure correlate positively with oxidative stress biomarkers [41, 42], and similar results have been found in rats [43]. Direct measurements of ROS vascular production in hypertensive subjects demonstrated higher levels of O_2 and H_2O_2 and an enhanced angiotensin II-stimulated redox signaling compared with cells from normotensive counterparts [44, 45]. In our study, we divided the healthy population into three groups, according to the range of cardiovascular risk charts (90–129 mmHg; 130–149 mmHg; and 150–169 mmHg), and we did not observe any change in the ROM values. On the contrary, we found a statistically significant reduction of antioxidant barrier efficacy in the group with systolic blood pressure higher than 150 mmHg. Several observational studies have reported an inverse relationship between blood pressure and antioxidant levels [46–48]. A decreased antioxidant activity and reduced levels of ROS scavengers might contribute to induce oxidative stress in hypertensive subjects, but also an increase in vascular ROS production has been hypothesized to reduce the antioxidant efficacy [8].

4.7. Lipidic Profile. Lipid metabolism disorders are associated with the overproduction of reactive oxygen species and have been shown to affect the antioxidant status and the lipoprotein levels in different organs [49, 50]. Dyslipidemia in combination with endothelial damage is a crucial event in the most common pathological processes underlying CVDs [51, 52]. In addition, endothelial dysfunction can be started/supported by several factors, including an excess of ROS and the exposure to harmful agents such as oxidized LDL [53]. In our study, we did not find a significant relationship between changes in oxidative status and total cholesterol values whereas a regular and significant reduction of antioxidant barrier efficacy was observed in subjects with pathological cholesterol values (total serum cholesterol values > 213 mg/dL).

We observed a similar result by relating oxidative status with total cholesterol/HDL cholesterol ratio. According to evidence from large observational studies, total cholesterol/HDL cholesterol ratio seems to be a more powerful risk predictor than isolated parameters used independently ([54] and references therein). Indeed, both diagnosis and treatment of dyslipidemia, including instruments for calculating cardiovascular risk factors, nowadays include the lipoprotein ratios that, in view of the evidence-based results, present greater predictive power [54].

Interestingly, in a recent paper, Yagi and colleagues [55] demonstrated that BAP was strongly correlated with carotid artery IMT suggesting that it may be considered a suitable risk marker for carotid atherosclerosis; moreover, they postulate that the measurements of BAP may be superior to the measurements of glutathione peroxidase, superoxide dismutase, catalase, and total antioxidant status for the assessment of antioxidant potential. Our results emphasize that the first detectable event of a redox disturbance is the repairing intervention of the antioxidant barrier that is thus decreased as overutilized.

5. Conclusion

In the present study, we showed through a cross-sectional analysis on a large healthy population that a reduced antioxidant capacity is significantly associated with cardiovascular

risk factors. In epidemiological studies, the magnitude of the cohort is a key factor for the validity of the results. Our numbers reach an average value; therefore, our research can be considered as a pilot study and as the first application of a protocol aimed to verify the validity of the experimental design. Moreover, we assessed the oxidative status through indirect determinations thus providing an overall measure of many oxidants/antioxidants, also without identifying the molecules involved in the perturbation of normal homeostasis. The assessment of both validity and reproducibility of such indirect determinations is important as they represent an analytical tool not many invasive and easy to perform which allows an application on a large scale. Further studies are needed to clarify better how these new putative biomarkers and the traditional risk factors are related and how they can improve the prediction of cardiovascular risk.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

D. Pellegrino developed and led the project. All of the authors designed, performed the experiments, and analyzed the results. D. Pellegrino wrote the paper with input from E. Brunelli and D. La Russa. E. Brunelli and D. La Russa equally contributed to this research.

Acknowledgments

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Full Paper IV

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Research Article

Oxidative Balance and Inflammation in Hemodialysis Patients: Biomarkers of Cardiovascular Risk?

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During chronic kidney disease, the progressive deterioration of renal function induces several biological/clinical dysfunctions, including enhancement of synthesis of inflammation/oxidative stress mediators. Impaired renal function is an independent cardiovascular risk factor; indeed, cardiovascular complications dominate the landscape of both chronic kidney disease and end-stage renal disease. The aim of this study is to explore the correlation between the global oxidative balance in hemodialysis patients and both inflammatory markers and cardiovascular events. Using photometric tests, this study explored plasmatic oxidative balance in 97 hemodialysis patients compared to a healthy population. In the hemodialysis patients, we showed that oxidative stress values were significantly lower than in controls while effectiveness in the antioxidant barrier was significantly increased in the hemodialysis group. Furthermore, we highlighted a strong correlation between oxidative index and blood levels of C-reactive protein. When patients were divided into two groups based on previous cardiovascular events, we found that subjects with previous cardiovascular events had higher values of both oxidative stress and antioxidant barrier than patients without cardiovascular events. Our results indicated that in hemodialysis patients, the clinical and prognostic significance of oxidative status is very different from general population. As cardiovascular complications represent a strong negative factor for survival of hemodialysis patients, the research of new cardiovascular risk biomarkers in these patients takes on particular importance in order to translate them into clinical practice/primary care.

1. Introduction

Chronic kidney disease (CKD) is a major public health problem worldwide, and its main consequences include loss of renal function leading to end-stage renal disease (ESRD), increased risk of cardiovascular disease (CVD), significant increase in morbidity and mortality, and a decrease in health-related quality of life [1–3]. A recent meta-analysis of observational studies reported that CKD has a high global prevalence (between 11 and 13%) with percentage prevalence higher in developed areas such as Europe, USA, Canada, and Australia, where the populations of elderly is greater than in developing areas [4]. Indeed, the risk of CKD increases with age and elderly patients are overrepresented in the dialysis population [5]. However, there seems to be a complex relationship between aging and CKD, as geriatric complications are highly detectable in younger patients with ESRD [6]. This has led to the hypothesis of a premature biological aging process of different organ systems associated with CKD [7].

During CKD, the progressive deterioration of kidney function has important systemic effects due to its central role in body homeostasis and induces several biological and clinical dysfunctions including alteration in cellular energetic metabolism, change in nitrogen input/output, protein malnutrition, resistance to insulin, and considerable enhancement

of synthesis of inflammation/oxidative stress mediators. Inflammatory markers such as C-reactive protein and cytokines increase with renal function deterioration suggesting that CKD is a low-grade inflammatory process [8] comparable to the "inflammaging" phenomenon, in which aging tissues exhibit low-grade, chronic, systemic inflammation, in the absence of overt infection [9]. A number of factors can be involved in triggering the inflammatory process including oxidative stress. Several authors have reported that in CKD, even in the early stage, there is an abundant production of reactive oxygen species and hemodialysis exacerbates oxidative stress [10]. However, it remains unclear at which stage of renal insufficiency the redox imbalance becomes more profound [11]. Knowledge on causes and consequences of oxidative stress in CKD is rapidly expanding [12], but the factors influencing the oxidative status have not been characterized in these patients as well as the prognostic importance of circulating oxidative stress biomarkers remains poorly understood.

Impaired renal function is an independent cardiovascular risk factor; indeed, cardiovascular complications dominate the landscape of both CKD and ESRD [13]. The complex relationship of CKD with CVD is likely due to both traditional (age, hypertension, diabetes, and dyslipidemia) and CKD peculiar risk factors (volume overload, mineral metabolism abnormalities, proteinuria, malnutrition, oxidative stress, and inflammation). CKD patients can display opposite associations with traditional CVD risk factors as obesity, hypercholesterolemia, and hypertension that paradoxically appear to be protective features, in contrast to the general population [14]. Indeed, death associated with these pathologies cannot be attributed only to complications of atherosclerotic disease (myocardial infarction, stroke, and heart failure), but other specific processes contribute to cardiovascular morbidity and mortality in these patients [15]. As traditional risk factors are poor predictors in patients with late-stage CKD, identifying and intervening against new risk factors are priorities.

The aim of this study is to explore the global oxidative balance in ESRD hemodialysis (HE) patients compared to a healthy population. Since inflammatory stress and cardiovascular complications represent two very frequent conditions in hemodialysis subjects, the correlation between the oxidative parameters and both inflammatory markers and cardiovascular events was also investigated.

2. Methods

2.1. Subjects. The study involved 97 patients older than 18 years of both sexes with ESRD on regular hemodialysis therapy for at least 6 months (HD, mean age 67.25 ± 1.66) recruited from the Department of Nephrology, Dialysis and Transplantation, Kidney and Transplantation Research Center, Annunziata Hospital of Cosenza (Italy), between August 2017 and December 2017. The hemodialysis prescription consisted of 4 hourly, thrice weekly sessions, using polysulfone membrane and bicarbonate-buffered dialysate. The blood flow rate was 300 mL/min and low molecular weight heparin was used as anticoagulant. Hemodialysis adequacy was determined by using Kt/V formula. Table 1 reports the biochemical profiles of hemodialysis patients.

As controls for patient group, a recruitment campaign focused on the staff of the University of Calabria within the frame of the prevention and protection program adopted by the same university. In particular, 95 healthy volunteers age- and sex- matched with the ESRD group (CTR, mean age 63.7 ± 0.33) were recruited between August 2017 and December 2017. They underwent through a structured interview during which sociodemographic, anthropometric, and clinic information was collected. The lifestyle was evaluated using self-administered questionnaires, which included items on smoking, exercise habits, and drinking habits. Anthropometric measurements included measurements of weight and height. Body mass index (BMI) was calculated as weight (kg)/height (m^2), while blood pressure was measured in the sitting position using an automatic sphygmomanometer (Microlife BP A2). The biochemical measurements included glucose, total cholesterol, triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), HbA1c, uric acid (UA), and creatinine levels. Blood tests also included the evaluation of prooxidant, and antioxidant status. Exclusion criteria included a family history of ESRD or an estimated glomerular filtration rate (eGFR) lower than 60 mL/min/1.73 m^2, calculated by CKD-EPI equation [16].

All subjects (patients and controls) were studied in the morning and in a fasting state. Blood samples were taken from the antecubital vein and immediately centrifuged (2500 g for 15 min at 4°C), and the plasma obtained was stored at 4°C until measurements (maximum 6 hours of venous blood collection). Standard blood tests were performed at the clinical laboratory of Annunziata Hospital, Cosenza; prooxidant and antioxidant status were determined at the Analysis and Research on Oxidative Stress Laboratory (LARSO) of the University of Calabria.

2.2. Oxidative Status and Biological Antioxidant Potential Measurements. Oxidative stress and biological antioxidant potential determination were performed by using photometric measurement kits and a free radical analyzer system provided with spectrophotometric device reader (FREE Carpe Diem, Diacron International, Grosseto, Italy). The d-ROM test helps to determine the oxidant ability of a plasma sample measuring the presence of reactive oxygen metabolite derivatives, in particular, hydroperoxides (oxidative index). Results are expressed in Carratelli units (UC; 1 UC = 0.8 mg/L of hydrogen peroxide). The BAP test provides an overall measure of the biological antioxidant potential measuring the blood concentration of antioxidants (such as bilirubin, uric acid, vitamins C and E, and proteins) capable of reducing the iron from ferric to the ferrous form (antioxidant barrier). Results are expressed in μmol/L of the reduced ferric ions.

2.3. Statistical Analysis. Differences between groups were examined using the independent sample t-test. The correlation between continuous variables was assessed using Pearson's coefficient. Data have been analyzed using R statistical language (http://www.r-project.org/).

Table 1: Biochemical profiles of the hemodialysis patient cohort.

	Mean±SE
Albumin (g/dl)	6.567 ± 3.27
Cholesterol, total (mg/dl)	154.9 ± 4.14
HDL cholesterol (mg/dl)	43.65 ± 1.42
LDL cholesterol (mg/dl)	79.38 ± 3.88
C reactive protein (mg/dl)	12.06 ± 2.10
Uric acid (mg/dl)	5.73 ± 0.16
Urea (mg/dl)	64.73 ± 2.55
Iron, total (μg/dl)	68.53 ± 4.83
Transferrin (mg/dl)	150 ± 5.17
Ferritin (ng/mL)	509.1 ± 41.46
Fibrinogen (mg/dL)	397.6 ± 22.39
Insulin (μIU/mL)	26.65 ± 4.19
White blood cell (106/mL)	6.595 ± 0.25
Platelets (106/mL)	192.1 ± 9.4
Protein, total (g/dL)	6.01 ± 0.12

3. Results

Our study population consists of 97 hemodialysis patients and 95 healthy volunteers as control group. Table 2 reports the demographic characteristics of the analyzed samples together with the oxidative and inflammatory parameters and CVD prevalence.

We found that in the HD sample, the oxidative index was significantly lower than in CTR (304.25 vs 332.4, P value = 0.002). Moreover, ESRD patients also showed a greater effectiveness in the antioxidant barrier than CTR subjects (2127.4 vs 1888.39, P value = $1.24^{*}10^{-5}$). The relationship between oxidative index and antioxidant barrier values showed a borderline correlation ($r = 0.121$; P value = 0.094; Figure 1). In healthy subjects, literature data showed significant gender and age differences in oxidative stress biomarkers [17]. In our hemodialysis patients, by linear regression analysis, we verified that these results are not related to both age and sex.

It is well known that CKD subjects present a significant increase of both inflammation and oxidative stress mediators. In our HD group, we found a strong correlation between oxidative index and blood levels of C-reactive protein ($r = 0.440$; P value = 2.43*10-5; Figure 2), while antioxidant barrier efficacy was not correlated with this inflammatory marker.

To explore the putative relationship between oxidative status and classic cardiovascular risk factors in ESRD, linear regression analysis was performed. In particular, we observed that gender, diabetes, age, and lipid profile (total cholesterol and LDL and HDL cholesterol) were not related to both oxidative index and antioxidant barrier efficacy. Concerning smoking habits, it has not been possible to analyze any correlations given the small number of smoking subjects. Interestingly, when HD patients were stratified according to previous CVD events, we detected that subjects with previous acute myocardial infarction (CVD+, $n = 18$) had higher values of both oxidative stress and antioxidant barrier than patients without cardiovascular events (CVD-; $n = 79$; Figures 3(a) and 3(b)). It is remarkable to note that HD subjects with ?previous CVD events had oxidative stress values comparable to healthy controls while the efficacy of the antioxidant barrier was considerably and significantly enhanced compared to all the groups.

4. Discussion

Our research focused on the evaluation of the overall redox state on a significant hemodialysis population. Our results clearly showed that the hemodialysis patients presented oxidative stress values significantly lower and antioxidant barrier effectiveness significantly higher compared to healthy controls. Despite the good plasmatic redox status found in all hemodialysis patients, we highlight a strong correlation between oxidative index and C-reactive protein blood levels. In addition, we reported that, in our cohort of hemodialysis patients, subjects with previous acute myocardial infarction had higher values of both oxidative stress and antioxidant barrier respect to patients without cardiovascular events. It is noteworthy that these results have been realized using noninvasive method to detect total plasma redox balance, which is of particular importance when analyzing the frail hemodialysis patients. Indeed, to detect plasmatic redox status, we utilized two simple methods widely used in recent years, the d-ROM and the BAP tests [17–20]. The predictive ability of these biomarkers and their usefulness in cardiovascular and renal disease has been tested by our research group on both human and mammalian models [21–23].

Several authors reported a profound imbalance between oxidants and antioxidants in CKD [24]. The balance between pro- and antioxidant systems is essential for the regular function of organism's cells and molecules. Transiently increased concentrations of reactive oxygen species (ROS) perform specific physiological roles in keeping the organism's homeostasis in the redox-related signaling and also in the immune defense system, as they are produced in high amounts in inflammation [12, 25, 26]. Since CKD is characterized by a steady, low-grade inflammation, the long-lasting high ROS levels can lead to oxidation of DNA, lipids, and proteins with consequent cellular damage in CKD patients [12]. In addition, an impaired renal function leads to the accumulation of toxins and waste metabolites inducing an imbalance in redox homeostasis [11]. However, it remains unclear at which stage of renal insufficiency the redox imbalance becomes more profound and if dialytic treatment increases redox imbalance [11, 27]. Studies on various plasma and erythrocyte parameters of oxidative stress (free radicals, ROS, and lipid peroxidation products) and antioxidant defense have shown conflicting results [24]. Dursun and coworkers [28] described a high oxidative stress and an impaired antioxidant response in both hemodialysis and predialysis uremic patients. These authors ascribed in part this redox imbalance to antioxidant enzyme deficiency, but in parallel, they found an increased protein

Table 2: Demographic characteristics and redox profile of the cohort.

	Control group	Hemodialysis patients	P value
Age (mean±SE)	63.737 ± 0.33	67.25 ± 1.66	0.0355
Oxidative index (UC)	332.4 ± 5.74	304.25 ± 6.66	0.002
Antioxidant barrier (μmol/L)	1888 ± 39.14	2127.4 ± 46.77	$1.24^{*}10^{-5}$
Cardiovascular events (n, %)	—	18 (18.5%)	—

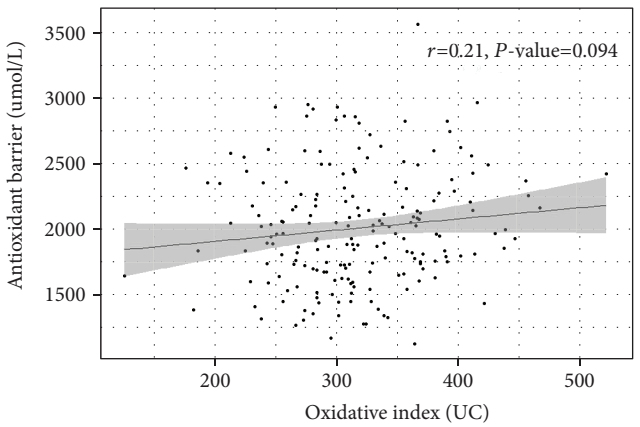

Figure 1: Correlation between oxidative index and antioxidant barrier values in HD patients (r = Pearson correlation coefficient).

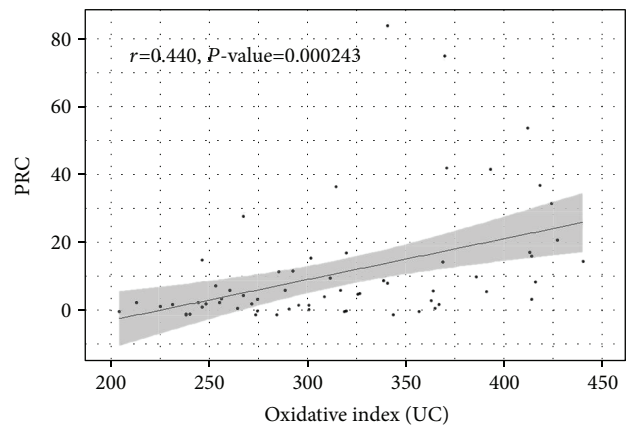

Figure 2: Correlation between oxidative index and PCR (C-reactive protein) values in HD patients (r = Pearson correlation coefficient).

carbonyl content, an indicator of oxidative protein damage, in predialysis patients but not in hemodialysis subjects thus supporting the hypothesis of high oxidative stress due to uremic state [28].

In our cohort of hemodialysis patients, we detected oxidative stress values significantly lower compared to healthy controls. As index of oxidative stress, we did not analyze the oxidative damage of single macromolecules but the plasmatic oxidant ability by measuring the presence of reactive oxygen metabolite derivatives, in particular, hydroperoxides. Actually, the most direct approach for lipid peroxidation evaluation is the quantification of the primary products, hydroperoxides, rather than the secondary or end products derived from hydroperoxides such as malondialdehyde. Plasmatic hydroperoxides are a sensitive and specific index that the presence of oxidative stress in vivo providing a global assessment of the imbalance induced by the alteration of the individual parameters [21]. Indeed, various serum markers of lipid and protein oxidation have been tested for the evaluation of oxidative stress in vivo, but the contrasting results obtained clearly indicate that a single marker does not have the ability to indicate the real redox state of the subjects [28–31].

Concerning the antioxidant systems, literature data are really complex to discern. Most of the studies in CKD patients have analyzed expression/activity of antioxidant enzymes with conflicting results [11, 27]. In addition, most of the reports concentrate on a single or a few antioxidants. The stability/activity of enzymatic antioxidant systems is multifaceted: in the case of low/medium oxidative stimulation, enzymatic antioxidant activity can increase, but if oxidative stress is persisting, or its level is very high, the damage caused to proteins becomes profound and a decreased expression/activity may occur via direct oxidative damage of the molecules and/or oxidative-altered gene expression. An alternative approach to investigate the antioxidative defense is the assessment of nonenzymatic ascorbate, glutathione, flavonoids, tocopherols, and carotenoids [32]. Unlike antioxidant enzymes, the ROS stimulation can determine a depletion of nonenzymatic antioxidants since the ROS species neutralization implies their consumption [33]. Interestingly, our results clearly showed that the hemodialysis patients presented a greater effectiveness in the nonenzymatic antioxidant barrier respect to healthy controls. This could be explained by the fact that we have measured the plasma total antioxidant capacity and not a single antioxidant specie. Since the antioxidant species are numerous and they operate synergistically, evaluating the activity of each antioxidant species may underestimate the association among different effects and probably do not reflect the physiological conditions [34]. Furthermore, cellular antioxidants are under homeostatic control and a decrease in a particular antioxidant can be compensated by an increase in a different one [35, 36]. Our results contribute to emphasize the importance of total antioxidant barrier effectiveness in countering the deleterious effects of ROS overproduction in hemodialysis patients, as suggested by several authors who reported antioxidant therapy as an early intervention to stop premature cardiovascular disease in CKD [12].

CKD is a low-grade, chronic, systemic inflammation with considerable enhancement of synthesis of inflammation mediators such as C-reactive protein and cytokines [8].

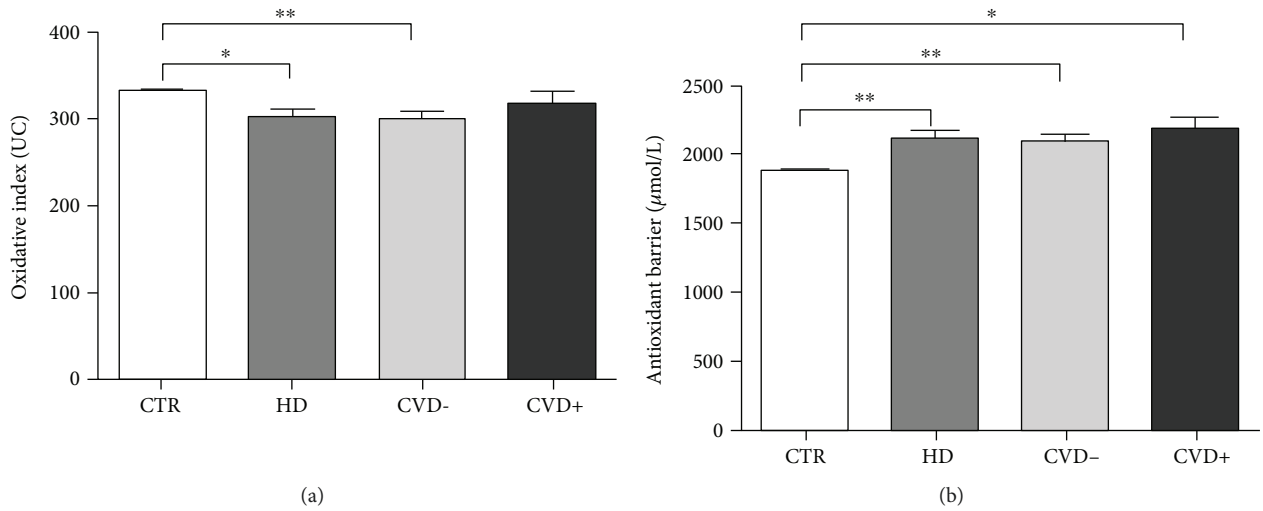

Figure 3: Values of d-ROM (a) and BAP (b) tests in a healthy population (CTR), all hemodialysis patients (HD), hemodialysis patients without cardiovascular events (CVD-), and hemodialysis patients with previous CVD events (CVD+). Data are expressed as mean±SE (Tukey's multiple comparison test, $^{*}p < 0{,}005$; $^{**}p < 0,0005$).

Literature data showed a significant increase of plasmatic C-reactive protein also in pediatric patients on hemodialysis [37], and this continuous inflammatory status is related to adverse outcomes mainly for cardiovascular events [7]. A number of factors can be involved in triggering the inflammatory process including oxidative stress [7, 29]. Despite the good plasmatic redox status found in our hemodialysis patients, we highlight a strong correlation between oxidative index and C-reactive protein blood levels, confirming that the inflammatory status is an important factor relating to oxidative stress in hemodialysis patients.

In recent decades, numerous studies and meta-analyses have highlighted that impaired renal function is an independent cardiovascular risk factor; indeed, cardiovascular complications dominate the landscape of both CKD and ESRD [13]. Traditional cardiovascular risk factors are poor predictors in patients with late-stage CKD; indeed, death associated with these pathologies cannot be attributed only to complications of atherosclerotic disease (myocardial infarction, stroke, and heart failure), but other processes peculiar to CKD can contribute to cardiovascular morbidity and mortality in these patients [15]. In our study, we detected that patients with previous acute myocardial infarction had higher values of both oxidative stress and antioxidant barrier respect to HD patients without cardiovascular events. This result is of a particular significance as it shows that in HD patients, the "normal range" of oxidative stress levels (calculated in healthy subjects) is a signal of cardiovascular risk. Interestingly, the HD patients with previous cardiovascular event also have an enhanced antioxidant barrier, considerably and significantly higher than all other groups. The underlying mechanism for this plasmatic antioxidant capacity increases was not investigated in the present study; we speculate that there may be a protective compensatory response as already reported in the CKD for the enzymatic antioxidant component [38, 39].

5. Conclusions

Our results suggest that in HD patients, the clinical and prognostic significance of oxidative status associated with cardiovascular risk factors is very different from the general population. Although a direct causality cannot be inferred from such kind of correlative investigations, our data provide an important contribution to understanding the cross-talk between oxidative imbalance and cardiovascular risk in CKD also representing a point of departure to address further investigation. As cardiovascular complications represent a strong negative factor for hemodialysis subject survival, the preservation of an optimal global redox balance through effective methods may be considered as a potential target for therapies aimed at preventing cardiovascular complications during CKD progression.

Data Availability

The hematochemical data used to support the findings of this study are included within the article.

Ethical Approval

All investigations have been conducted according to the Declaration of Helsinki principles. Control study has been approved by the Local Ethical Committee (n°8/2016, Regione Calabria, Sezione Area Nord). The patients were submitted to the standard biochemical measurements according to that provided for the follow-up of patients in end-stage renal disease. All subjects have provided written informed consent that, as guarantors, is retained by the corresponding author (controls, University of Calabria) and by the last author (patients, Annunziata Hospital-Cosenza).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

D.L.R., D.P., A.M., P.G., A.P., A.L.R., and R.B. are responsible for the study design. P.G., A.P, A.L.R., and R.B. are responsible for the patient sampling. DLR and DP are responsible for the control sampling and execution of the study. A.M. is responsible for the statistical analysis. D.P. is responsible for the manuscript writing. D.L.R., D.P., A.M., P.G., A.P., A.L.R., and R.B. are responsible for reviewing the manuscript.

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Full Paper V

La Russa A, Bonofiglio M, Lofaro D, **La Russa D** et al. “rs4612666 of NLRP3 gene polymorphism is associated with oxidative stress in obese patients”. *Submitted*

rs4612666 of NLRP3 GENE POLYMORPHISM IS ASSOCIATED WITH OXIDATIVE STRESS IN OBESE PATIENTS

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ABSTRACT

In this study we have investigated, in obese patients candidate to sleeve-gastrectomy (SLG), the association of genetic variants of NLRP3 (rs4612666; rs10754558) and CARD8 (rs204321), with the inflammation and oxidative stress before and after SLG. To this aim, 23 obese patients underwent, before (T0) and after three months form SLG (T3), to: body-composition (BIA) assessments; oxidative stress (d-ROM-and BAP-test), biochemical measurements, extraction of DNA from peripheral blood lymphocytes and genotyping by RFLP analysis. Biochemical measurements and BIA, at T0 and T3, evidenced that SLG improved inflammation and BIA parameters, as we registered a significant reduction of fat mass and an increase of lean mass. We observed that in patients carrying rs4612666 functional C variant of NLRP3 gene, d-ROM test values were higher respect to non-carriers. In vivo studies, performed using adipose visceral tissue isolated during SLG, evidenced higher expression levels of NLRP3, IL-6 and MCP-1 mRNA in rs4612666 C variant carriers, particularly in C allele homozygous. No significant correlation were found with rs10754558, rs204321 variants of NLRP3 and CARD8.

Collectively**,** our results suggested that in obesity patients, rs4612666 C variant of NLRP3 gene is associated with a worse oxidative stress, although SLG improve biochemical and BIA in all patients, independently by their genotype.

Keywords: obesity, polymorphisms, NLRP3-inflammasone, oxidative stress, sleeve gastrectomy, body composition

1.1 INTRODUCTION

Inflammation and oxidative stress, affecting obese patients, occur when the energy supply exceeds the storage capacity of adipocytes, leading to adipose tissue hypertrophy (Klöting and Blüher, 2014). Hypertrophic adipocytes, as well as the infiltrated macrophages, release higher amount of pro-inflammatory cytokines, triggering, concomitantly, an imbalance of redox system (Grant, 2008). Yu et al reported that the release of mitochondrial reactive oxygen species (ROS), occurring as consequence of mitochondrial stress, strongly contribute to the activation of NLRP3 inflammasome, leading to the production of IL-1β or IL-18 (Yu and Lee, 2016). NLRP3 is the major player in the multi-protein complex known as the inflammasome, and interacts with several adaptor proteins such as apoptosis-associated speck-like protein (ASC), pro-caspase-1, and other proteins that contain caspase activation and recruitment domains (CARD) a cytoplasmic multi-protein complex (Yu and Lee, 2016). The components of NLRP3 inflammasome are highly expressed in adipose tissue of obese patients, particularly in infiltrated macrophages, whose abundance seems to contribute to the onset of obesity-associated complications, as insulin-resistance (Jager et al, 2007). In addition, Vandanmagsar et al reported that NLRP3 inflammasome activation plays also a role in macrophage infiltration in adipose tissue, leading to sustained levels of chronic inflammation in obesity (Vandanmagsar et al, 2011).

Bariatric surgery improves long-term weight loss and is accompanied by a reduction in adipose tissue pro-inflammatory state (Stienstra et al, 2010). Interestingly, Mocanau et al demonstrated that in obese Sprague-Dawley rats, Roux-en-Y gastric bypass (RYGB) reversed inflammation by suppressing NLRP3 inflammasome activation, particularly in visceral adipose tissue, that represents the main source of inflammatory mileu, suggesting that RYGB surgery could serve also as an effective anti-inflammatory treatment. (Mocanau et al, 2015). However, recent in vivo study reported that loss of NLRP3 did not protect mice from western diet-induced adipose tissue inflammation, suggesting that *NLRP3* deletion could just alleviate western diet-induced metabolic and cardio-vascular complications (Ringling et al, 2016). Therefore, we can state that the molecular mechanisms by which obesity

promotes adipose tissue and systemic inflammation, as well as the role exerted by NLRP3, remain poorly understood.

Studies reported in literature showed that some *NLRP3* functional polymorphisms, in particular the variants rs4612666 and rs10754558, as well as those found in *CARD8*, are associated with several autoimmune and inflammatory diseases and type-2 diabetes, as they enhance *NLRP3* mRNA stability or its expression, leading to the formation of an hyperactive NLRP3 inflammasome (Hitomi et al, 2009; Liu et al, 2015).

To our knowledge, there have been no studies exploring the contribute of *NLRP3* and *CARD8* variants in obesity. Therefore, in this study we investigated the effects of laparoscopic sleeve gastrectomy on body composition, inflammation and oxidative stress. Concomitantly, we investigated, in vitro and in vivo, the potential association of *NLRP3* variants, rs4612666 and rs10754558, and *CARD8* variant, rs204321, with inflammation and oxidative stress observed in obese patients before and after sleeve-gastrectomy.

1.2 MATERIALS AND METHODS

1.2.1 Participants

23 consecutive obese patients affected by severe obesity and having criteria established by the SICOB guidelines to bariatric surgery (Foschi et al, 2016) were enrolled at the Unit of Bariatric Surgery of Annunziata Hospital of Cosenza, Italy. Preparation of patients consisted in a thorough examination by different teams involving areas such as psychology, surgery, nutrition, clinical medicine, endocrinology, gastroenterologist and anesthesiology. In all patients, electrolytes, vitamin D, vitamins B, nutritional protein energy supplements (Ensure plus Advance Abbott, 220 ml/die; Fortifit Nutricia, 30 g/die; Abound Abbott, 1 medicine sachet/die) were prescribed as an oral supplementation for the entire period of observation. Furthermore, each patient was submitted to the protocol of the Italian Society of Surgery of Obesity SICOB for dietary therapy after Sleeve Gastrectomy. All patients, before surgery, (T0) and after three months from surgery (T3) were subjected to (i) nutritional assessment: medical patient history, anthropometric measurements and bioelectrical impedance analysis; (ii) collect serum for determination of oxidative stress and antioxidant barrier activity; (iii) body composition analysis; (iv) Measurements of fasting plasma glucose, insulin, total cholesterol, triglyceride, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), PCR and creatinine. Peripheral blood sample for DNA extraction was performed only at T.

Weight and height of all subjects were collected by a nutritionist. The data was collected immediately before the procedure (T0) and 3 months after bariatric surgery (T3). Participants were weighed without shoes and wearing light clothing on a Seca® mechanic scale with a 150-kg capacity and 50-g precision. Height was measured in centimeters with the height rod of the same digital scale. Waist circumference (cm) was measured at the midpoint between the last rib and the iliac crest, with a tape meter Seca® for determining circles.

Bioelectrical impedance analysis was performed before surgery (T0) and after three months from surgery (T3), using the Body Composition Monitor of Fresenius Medical Care. It is a stand-alone device that allows to separate excess fluid (over-hydration) from the lean and adipose tissue components of the body on the basis of a unique body composition model: Body weight = Lean Tissue Mass (LTM) + Adipose Tissue Mass (ATM) + Over-hydration (OH). The three compartments (LTM, ATM and OH) are identified from measurements of body weight, height, intracellular (ICW) and extracellular water (ECW) determined by whole body bioimpedance spectroscopy.

The evaluation of plasmatic oxidative status of subjects has been evaluated before surgery (T0) and after three months from surgery (T3) by using photometric measurement kits and a free radical analyzer system (FREE Carpe Diem, Diacron International, Grosseto, Italy). The analyses were carried out at the Laboratory of Analysis and Research on Oxidative Stress (DiBEST, University of Calabria). We used Diacron reactive oxygen metabolite (dROM), and biological antioxidant potential (BAP) tests to evaluate plasma levels of reactive oxygen metabolites and antioxidant capacity. The d-ROMs test help to determine the oxidant ability of a plasma sample measuring the presence of Reactive Oxygen Metabolites derivatives, in particular, hydroperoxides. Total plasma antioxidant capacity was assayed using a biological antioxidant potential (BAP) test that provides an overall measure of the biological antioxidant potential measuring the blood concentration of antioxidants (such as bilirubin, uric acid, vitamins C and E and proteins) capable of reducing iron from the ferric to the ferrous form. BAP test, in healthy subjects, assumes a value above 2200 µmol /L of the reduced ferric ions. Lower values are indicative of oxidative stress condition by lowering of antioxidant defenses.

1.2.2 Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA), following the manufacturer's instructions. At a later stage, DNA was quantified by spectrophotometric reading absorbance at 260 nm with Eppendorf

BioSpectrometers. Genotyping was carried out by Polymerase Chain Reaction and digestion reaction using restriction enzymes yet described in literature (Zheng et al, 2013; Fontalba et al, 2007).

Total RNA isolation from adipose tissue sections of genotyped patients was performed using TRI Reagent (Invitrogen) method. Around 100 mg of frozen adipose tissue was homogenized in TRI Reagent solution (1 ml) and incubated at room temperature for 5 minutes. 200µl of chloroform were then added, the tubes were shake vigorously for 15 second and then the mixture was incubate at room temperature for 15 minutes. Subsequently a centrifugation step at 12000 g at 4°C for 10 min was performed, the aqueous phase was transferred in a new tube, then RNA was precipitated using isopropanol, washed and resuspended in DNAse/RNAse free water. Quantity and quality were assessed both spectroscopically and electrophoretically. RNA was then reversed transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Applera Italia, Monza, Milano, Italy).

Analysis of NRLP3, IL1β, IL-6 and MCP1 was performed using Real time PCR as previously described (Vizza et al, 2013). β-actin was utilized as internal loading.

Primers used:

NLRP3 Fw: 5'-ATGCCAGGAAGACAGCATTG-3'; NLRP3 Rv: 5'-TCATCGAAGCCGTCCATGAG-3'

IL1β Fw: 5'-GGATATGGAGCAACAAGTGG-3'; IL1β Rv: 5'-ATGTACCAGTTGGGGAACTG-3'

MCP-1 Fw: 5′-CATAGCAGCCACCTTCATTCC- 3′; MCP-1 Rv: 5′-TCTGCACTGAGATCTTCCTATTGG-3'

IL-6 Fw: 5′-GGTACATCCTCGACGGCATCT-3′; IL-6 Rv: 5′-GTGCCTCTTTGCTGCTTTCAC-3′

β-actin Fw: 5'-AGAAAATCTGGCACCACACC-3'; β-actin Rv: 5'-AGAGGCGTACAGGGATAGCA-3'

The relative gene expression levels were normalized as previously described (Vizza et al, 2013).

1.2.3 Statistical analyses

Patients general characteristics and measured parameters are presented as mean ± SD for numerical values and number (%) for categorical values. Comparison of BIA, anthropometric and biochemical parameters before and three months after surgery was performed by ANOVA for repeated measures. dROMs and BAP test results in patients with and without the relevant risk variants were compared using Multilevel growth model to test the interaction between surgery and polymorphism. All in vitro experiments were performed in three independent experiments. Real Time RT-PCR results are presented as fold induction over basal condition. All results are presented as mean ± SD of data from three combined experiments. Data were analyzed by unpaired t-test (between two groups) or one-way analysis of variance with Tukey or Dunnett post-test analysis (for three or more groups) using R (Version 3.2.3, R Core Team); a p value < 0.05 was considered significant.

1.3 RESULTS

1.3.1 Sleeve-gastrectomy significantly improves body composition

We enrolled 23 consecutive patients affected by severe obesity at Unit of Bariatric Surgery of Annunziata Hospital of Cosenza, Italy (4 males and 19 females, mean age 43.15 ± 14.36, mean BMI 42.59 ± 10.26). 30.4% was affected by hypertension (21.7% used anti-hypertension drugs), 17.4 by diabetes mellitus treated with oral anti-diabetic drugs and 13% by dyslipidemia (none swallowed statins). As reported in Figure 1, we observed that in all patients SLG significantly reduced body mass index (BMI), waist (WC), hip (HC), arm (AC) and thigh (TC) circumferences ($p < 0.05$). To evaluate changes in body composition, we performed, at both T0 and T3, the bioelectrical impedance analysis, using a multi-frequency instrument that, respect to that commonly used, is able to measure both extracellular and intracellular water. We observed that at T0 all patients have a marked dehydration status (OH: -1.8 ± 1.5; ECW %: -8 ± 7.5), that resulted significantly reduced after SLG (OH: -0.1 ± 1.3, $p = 0$; ECW %: -0.5 ± 6.8, $p < 0.0001$). As reported in Figure 2, at T3 we observed a significant reduction of TBW (38.8 ± 5.2 (T3) vs 44.3 ± 7.2 (T0) $p < 0.0001$), together with a normalization of E/I ratio (0.94 ± 0.09 (T3) vs 0.9 ± 0.1 (T0), $p = 0.003$).

The evaluation of body composition parameters performed at T3, showed, respect to T0, a significant increase of lean mass percentage (LTM % $p = 0.032$), with a concomitant reduction of fat mass percentage (Fat % $p = 0.002$). In addition, we observed a significant reduction of lean and fat tissue index (LTI $p = 0.021$ and FTI $p < 0.0001$). Finally, we observed a significant reduction of adipose tissue inclusive of hydro-component (ATM Kg; $p < 0.0001$) (Fig. 3). We did not observed a significant change of active cellular mass (BCM Kg) ($p = 0.618$). As reported in Table 1, our results evidenced that in all patients SLG produced a significant reduction of insulin ($p < 0.001$), triglycerides ($p = 0.001$) and protein C Reactive (PCR) ($p<0.002$) levels respect to T0. On the contrary, we did not registered changes in others biochemical parameters (data not shown).

1.3.2 rs4612666 NLRP3 variant is associated with higher oxidative stress

All patients were subjected to genotyping for *NLRP3* variants, rs4612666 and rs10754558, and *CARD8* variant, rs204321. Interestingly, in patients carrying rs4612666 *C* variant of *NLRP3* gene, oxidative stress measured al baseline with dROM test was higher respect to that detected in non-carriers, (Fig. 4 p = 0.04). Concomitantly, we observed that at T3, all patients showed a significant reduction of d-ROM test values (Table 2 p = 0.001) and that the decrease was not associated with rs4612666 *C* variant (Table 2 p=0.11). In addition, we did not find a difference of B-PAP test values after three months (Table 2 p=0.26). Finally, there were no differences on oxidative stress trend between carriers and non-carriers of rs4612666 *C* variant (Table 2). No statistically significant correlation was found for SNPs rs10754558 and rs2043211 variants of *NLRP3* and *CARD 8* genes, respectively (data not shown).

1.3.3 NLRP3 inflammasome, IL-1β, IL-6 and MCP-1 mRNA expression in visceral adipose tissue

Visceral adipose tissue (VAT) biopsy was performed without any complication in all patients. We did not fractioned VAT into macrophage, non-macrophage and adipocyte populations, therefore we evaluated the genic expression of NRLP3, IL1β, IL-6 and MCP1 in total VAT. As expected, the expression of NLRP3 was significantly higher in VAT of patients carrying *C* allele of rs4612666 variant of NLRP3 gene respect to that detected in VAT of patients with TT genotype ($p < 0.01$), although we did not find a significant difference between patients heterozygous and homozygous for the C allele (p=0.5) (Fig.5A). Next, our results evidenced that mRNA levels of IL-1β, IL-6 and MCP1 (Fig. 5B, 5C, 5D respectively) resulted significantly higher in patients carrying *C* variant ($p<0.01$), and, more interestingly, we observed a strong expression in homozygous for the C allele respect to heterozygous ($p<0.001$).

1.4 DISCUSSION

In this study we observed, for the first time, that in severe obese patients, the *C* variant rs4612666 of *NLRP3* gene was associated with a higher oxidative stress respect to that observed in non-carriers. Concomitantly, we found that SLG, together with an early nutraceutical supplementation post-surgery, promoted a restoration of normal body composition, reducing inflammation and improving metabolic parameters.

Bariatric surgery is an effective tool in the management of obesity and its associated comorbidities (Rubino et al, 2010). Previously, LSLG was used as a first step surgery procedure to reduce morbidity in high risk patients (Regan et al, 2003). However, as it has proven as an effective and safe procedure, showing several advantages respect to other surgery bariatric approaches, currently it represent a stand-alone procedure in the management of morbid obesity. Indeed, SLG is associated with less Dumping syndrome and, mainly, decreases the risk of nutritional deficiencies following to bariatric surgery (Benaiges et al, 2015). Undoubtedly, the endpoints of bariatric surgery should be both preservation of free fat mass, and restoration of normal body composition, as it is well known that patients affected by severe obesity, show a pathological increase of fat mass concomitantly with a pathological decrease of free fat mass, leading to sarcopenia and frailty (Angrisani et al, 2017). The introduction of early nutraceutical supplementation, as (Beta-Hydroxy-Beta-Methyl-Butyric Acid (HMB), Essential and Non-essential Amino Acids, Polyunsaturates Fatty Acid (including omega 3 and/or ALA and DHA/EPA, omega 6), Carbohydrates, Vitamin D, Vitamin B12 , Folic Acid, Iron), has substantially improved the complications due to the nutritional deficiencies following bariatric surgery (Dogan et al, 2014; Smelt et al, 2017), which incidence and time of occurrence are not clear, therefore, the efficacy of supplementation remains questionable. Our patients were early supplemented starting from two days post-surgery and it was decisive in avoiding nutritional deficiencies for each patient. Furthermore, the nutritional supplementation have been able to increase muscle mass levels and improving hydration state, after only three months from bariatric surgery. Finally, our patients

have maintained a good body composition over time and, therefore, have not had any disease from vitamin or nutritional deficiency.

Adipose tissue undergo to a significant changes in its cellular composition as well as secretory profile during the onset and progression of obesity, producing a pro-inflammatory mileu, leading to a systemic low-grade inflammation. Stienstra et al demonstrated that NLRP3 inflammasome activation is strongly involved in pathophysiology of obesity, as it exerts a crucial role in the pro-inflammatory cytokines production by adypocites and, mainly, by macrophage infiltrated. Indeed, the authors reported that mice deficient in inflammasome components are protected from high-fat-diet associated body-weight gain, adipocyte hypertrophy and hyperinsulinemia. Concomitantly, they observed a reduced MCP-1 production in adipose tissue, a key molecule that mediates macrophage infiltration (Stienstra et al, 2011). Interestingly, the in vivo study of Mocanau et al revealed that bariatric surgery reversed inflammation in visceral adipose tissue by suppressing NLRP3 inflammasome activation (Mocanau et al, 2015). However, although animal models established the importance of inflammasome in the development of obesity, to date there are few studies that have investigated the role of inflammasome activation in adipose tissue of patients affected by obesity. In addition, to our knowledge, although it has been reported that functional NLRP3 polymorphisms are associated with autoimmune and inflammatory diseases (Lee et al, 2016) to date, there are no studies that have investigated their role in inflammation and oxidative stress observed in obese patients. Genotyping analysis performed in our population, evidenced that 83% of patients carried rs4612666 *C* variant of *NLRP3* gene. Previous functional analyses regarding this polymorphism showed that it influenced higher mRNA expression by altering expression, enhancer activity or mRNA stability (Zheng et al 2013; Hitomi et al, 2009; Villani et al, 2009). Our real-time PCR results revealed that in VAT obtained from patients carrying *C* rs4612666 variant of *NLRP3* gene, the expression levels of NLRP3, as well as of IL-6 and MCP-1, were higher respect to that detected in non-carriers. In addition, we found that the genic expression of IL-6 and MCP-1 resulted particularly up-regulated in homozygous for the C allele. These results led us to postulate that this variant may predispose VAT to produce greater amount of some cytokines both from hypertrophic adipocytes and infiltrated macrophage, summoned

in loco by higher levels of MCP-1. To support our hypothesis, as it is well known that inflammasome activation is involved in the higher mitochondrial ROS production by adipose tissue (Zhou et al, 2011), our findings revealed that, although at baseline all patients showed elevated levels of oxidative stress, it was significantly higher in patients carrying *C* variant respect to non-carriers. Concomitantly, we observed that although SLG strongly improved oxidative stress in all patients, this effect was regardless of genotype, indeed we did not observed a significant differences between rs4612666 *C* variant carriers and no-carriers. This result may be not surprising, since it is reasonable that the importance of SLG on inflammation and oxidative stress reduction is prevalent respect to that exerted by polymorphism alone, although is the variant is functional. On the contrary, as it is well known that VAT is the key source of inflammatory *mileu* in obese patients, leading to severe comorbidities, we believe that patients carrying this variant are exposed to a more severe inflammation. Therefore, these subjects should be subjected early to bariatric surgery.

In conclusion, although further studies are need to increase the sample, our preliminary results show that rs4612666 variant of NLRP3 is associated with an higher oxidative stress, suggesting that genotyping may be included in the diagnostic assessment of obese patients.

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ETHICS STATEMENT

All investigations have been conducted according to the Declaration of Helsinki principles, and have been approved by Local Ethical Committee (Calabria Region Nord Area Section). All subjects have provided written informed consent that, as guarantor, is retained by the corresponding author.

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DECLARATION OF CONFLICTING INTEREST

The authors declare that they have no conflict of interest.

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TABLE 1. Laboratory findings before (T0) and after (T3) SLG.

	TIME (MONTHS)		
	T0 mean ± SD (minimum– maximum)	T3 mean ± SD (minimum– maximum)	*P value*
Glucose (mg/dL)	92.00 (89.00-99.00)	89.00 (85.00-101.00)	0.306
Insulin (μUI/ml)	16.80 (11.35-20.70)	10.80 (6.30-15.95)	0.004
Total Cholesterol (mg/dL)	179.00 (154.50-205.00)	161.00 (145.00-183.00)	0.235
HDL Cholesterol (mg/dL)	44.00 (40.50-49.50)	44.00 (38.00-49.00)	0.843
LDL Cholesterol (mg/dL)	106.00 (87.50-124.00)	95.00 (83.10-107.50)	0.312
Triglycerides (mg/dL)	128.00 (111.50-171.00)	108.00 (87.50-129.50)	0.017
C-reactive Protein (mg/L)	3.55 (2.95-4.05)	1.70 (0.64-3.00)	0.036

SD, standard deviation.

TABLE 2. Multilevel growth model to evaluate the association between surgery, rs4612666 *C NLRP3* gene variant and their interaction with levels of oxidative stress measured with dROM and BAP test.

	VARIABLE	β	SE	p value
dROM test	Intercept	428	22.174	< 0.0001
	Time	-26.519	6.811	0.0009
	NLRP3 variant	-109.25	52.003	0.044
	Time by NLRP3 variant	26.185	15.973	0.116
BAP test	Intercept	2126.978	92.504	<0.0001
	Time	0.890	39.343	0.982
	NLRP3 variant	233.522	216.942	0.288
	Time by NLRP3 variant	-142.057	92.269	0.139

SE, standard error.

LEGEND OF FIGURES

Figure 1: anthropometric measures change before (T0) and after 3 months (T3) from SLG.

BMI: body mass index; , waist (WC), hip (HC), arm (AC) and thigh (TC) circumferences. Bold points and line represents the value trend between T0 and T3.

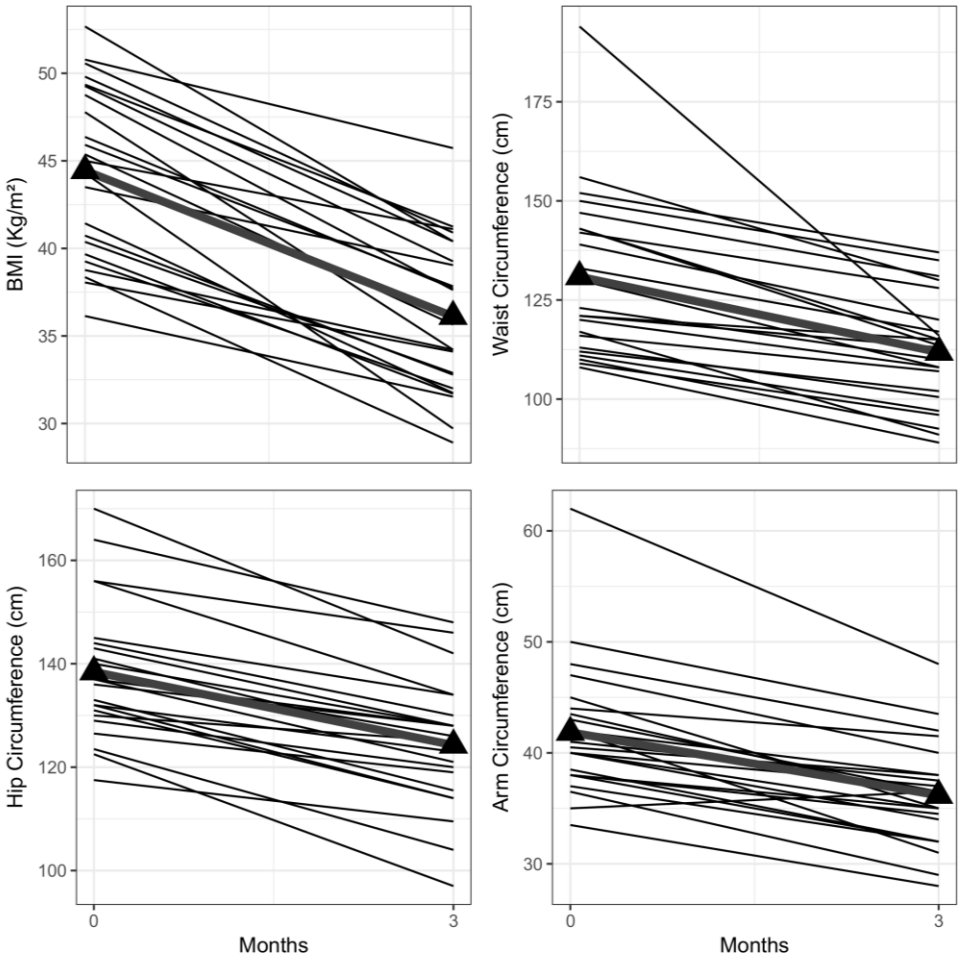


Figure 2: hydration status change of patients before (T0) and after 3 months (T3) from SLG.

TBW: total body water; E/I ratio: extracellular water/intracellular water. Bold points and line represents the value trend between T0 and T3.

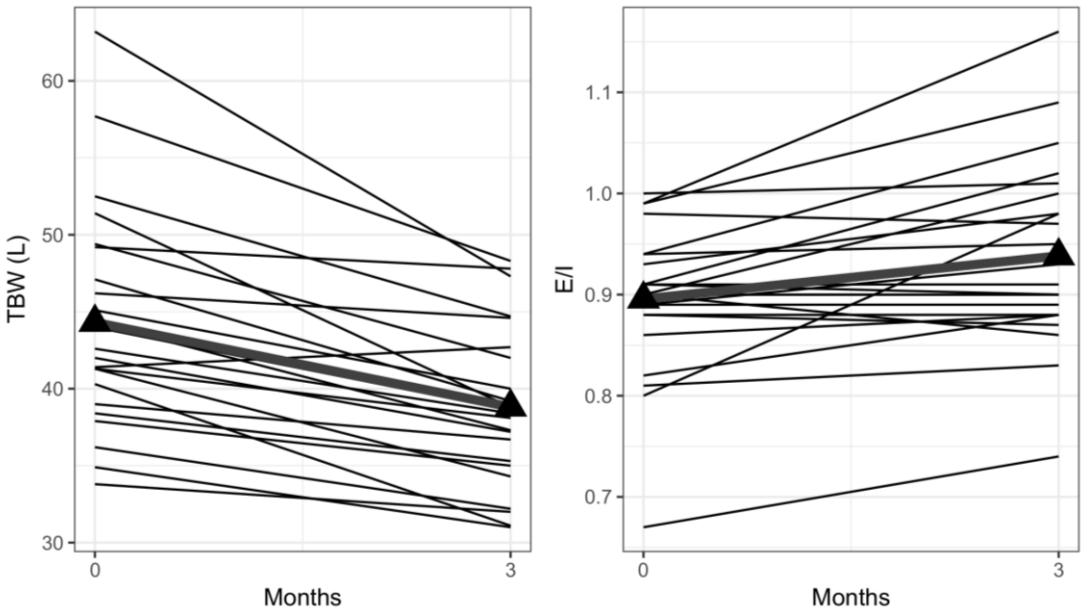


Figure 3: body composition parameters changes before (T0) and after 3 months (T3) from SLG.

LTM %: lean mass percentage; Fat %: fat mass percentage; LTI: lean tissue index; FTI: fat tissue index; ATM: adipose tissue inclusive of hydro-component; BCM: active cellular mass. Bold points and line represents the value trend between T0 and T3.

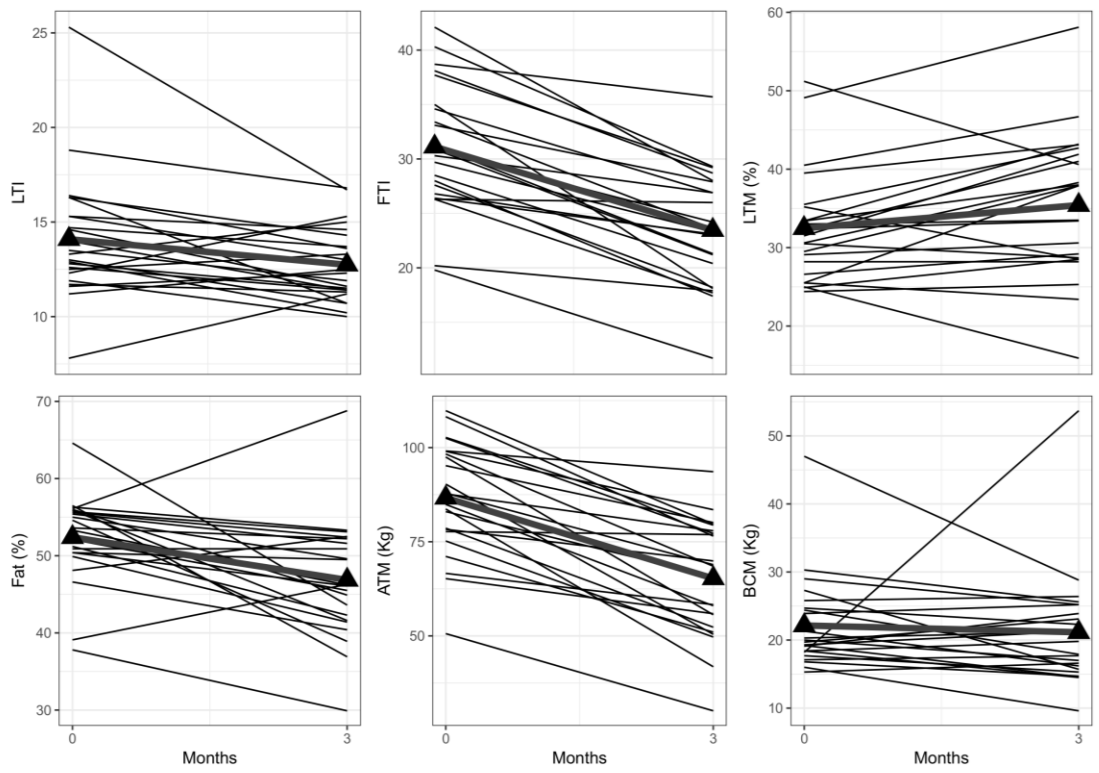


Figure 4: Oxidative stress mean values between carriers and non-carriers of rs4612666 C variant of NLRP3 gene detected at baseline (T0).

BAP (upper panel) and dROM test (lower panel) measured in patients by rs4612666 C variant of *NLRP3* gene. Results are reported as median values (black lines), interquartile range (boxes) and data within 1.5 × IQR of the 1st and 3rd quantile respectively. Data beyond the end of the whiskers are outliers plotted as points.

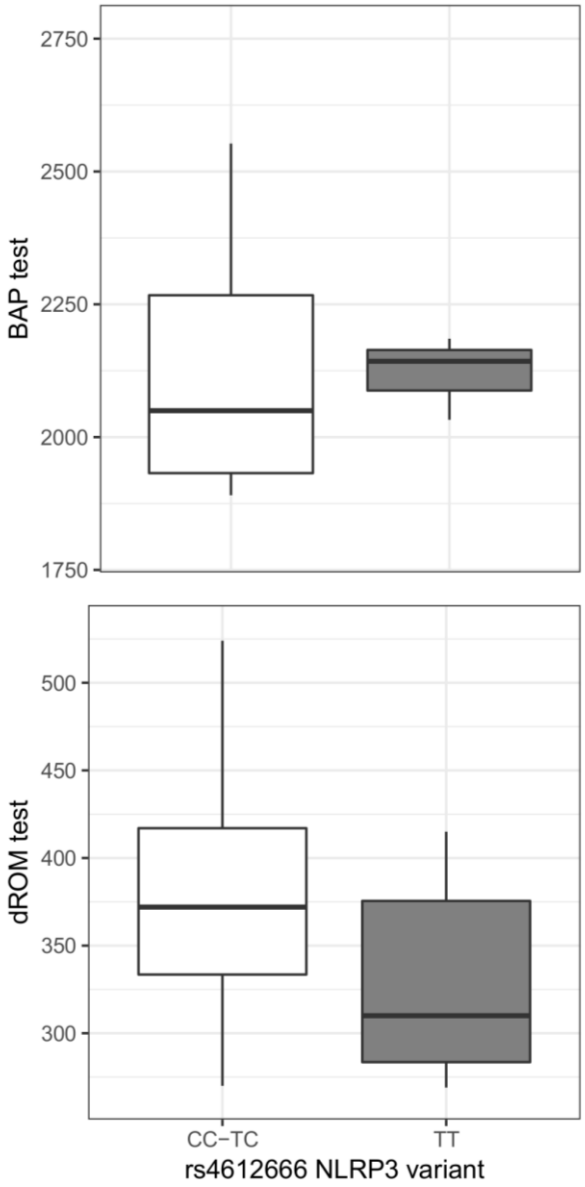
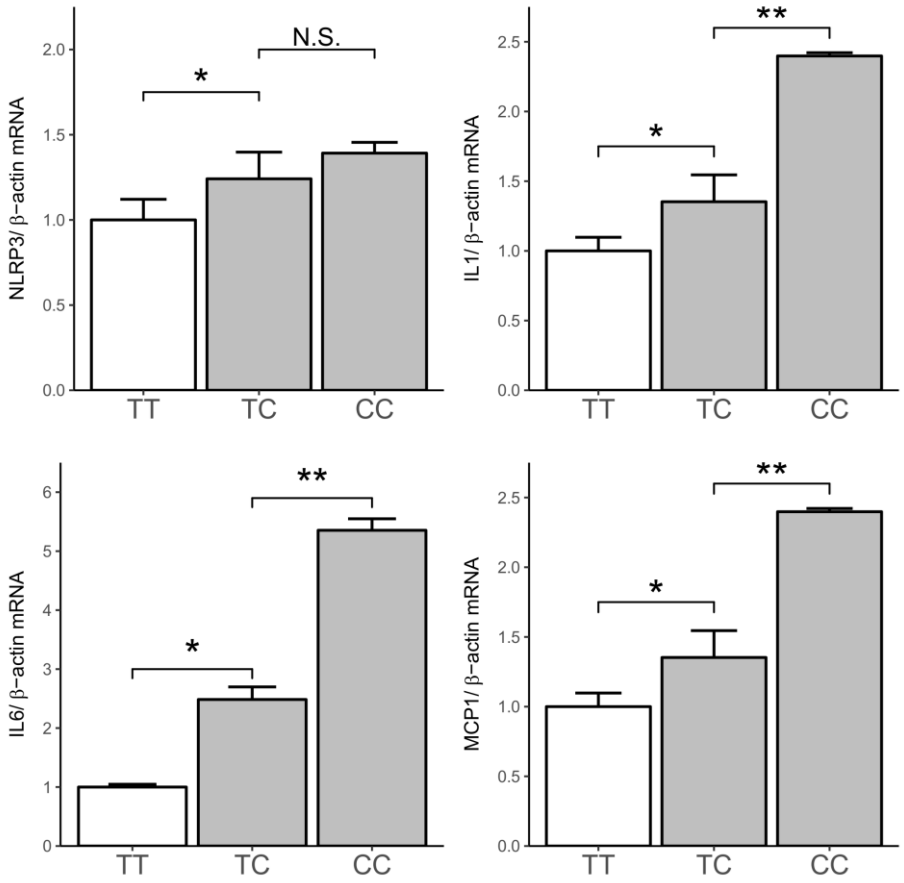


Figure 5: NLRP3, IL-1β, IL6 and MCP-1 mRNA expression levels in visceral adipose tissue of patients.

Quantitative real time PCR analysis of NLRP3, IL-1β, IL6 and MCP-1 mRNA extracted from visceral adipose tissue of obese patients at baseline (T0). Each sample was normalized to its β-actin mRNA content. *p<0.05 *vs* TT genotype of rs4612666 variant of *NLRP3* gene; **p<0.05 vs TC genotype of rs4612666 variant of *NLRP3* gene; *ns* = not significant. The histograms show the quantitative representation of data (mean ± SD) of three independent experiments each performed in triplicate.



Full Paper VI

La Russa D, Giordano F, Marrone A, Parafati M, Janda E, Pellegrino D. "Oxidative imbalance and kidney damage in cafeteria diet-induced rat model of metabolic syndrome: effect of bergamot polyphenolic fraction". **Antioxidants**, 8: 66 (2019). (***if 3,56***)

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Article

Oxidative Imbalance and Kidney Damage in Cafeteria Diet-Induced Rat Model of Metabolic Syndrome: Effect of Bergamot Polyphenolic Fraction

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Abstract: Obesity is a potent risk factor for kidney disease as it increases the possibility of developing diabetes and hypertension, and it has a direct impact on the development of chronic kidney disease and end-stage renal disease. In this study, we tested the effect of bergamot polyphenolic fraction in a cafeteria with diet-fed rats, an excellent experimental model for studying human metabolic syndrome, as it is able to induce severe obesity with insulin resistance and high plasma triglyceride levels more efficiently than a traditional lard-based high-fat diet used in rodent models. We analyzed the plasmatic oxidative balance by photometric tests, and the expression of cytoplasmic antioxidant enzymes (superoxide dismutase 1 and glutatione S-tranferasi P1) and apoptotic markers (Caspase 8 and 9) in kidney tissues by Western blot analysis. Our results clearly showed that the cafeteria diet induces a marked pro-oxidant effect: significant reduction of plasmatic antioxidant capacity; downregulation of cytoplasmic antioxidant enzymes expression; and activation of apoptotic pathways. All these hallmarks of redox disequilibrium were mitigated by treatment with polyphenolic fraction of bergamot, highlighting its antioxidant effect in the metabolic syndrome. Our data show that the link between obesity and renal damage could be represented by oxidative stress.

Keywords: oxidative stress; biological antioxidant potential; kidney damage; cafeteria diet; bergamot polyphenolic fraction

1. Introduction

The prevalence of overweight and obesity is constantly growing worldwide, even in low and middle income countries [1]. The most alarming aspect of this pandemic of obesity is that increasingly involves adolescents and children [2]. These findings highlight the necessity for development of effective food-policy actions focused on the key determinant of obesity, a healthy diet. Epidemiologic studies have highlighted the strong correlation between high body mass index (BMI) and several chronic diseases, including cardiovascular disease [3,4], cancers [5], and renal diseases [6–8]. Chronic kidney disease (CKD) represents a major public health concern and the main consequences include loss in renal function and cardiovascular complications [9].

Obesity is a potent risk factor for the development of new-onset kidney disease as it increases the possibility of developing diabetes and hypertension, and it has a direct impact on the development of CKD and end-stage renal disease (ESRD). The mechanisms whereby obesity may cause or exacerbate CKD remain unclear. It has been hypothesized that, to meet the high metabolic needs of the increase in body weight, a compensatory mechanism of hyperfiltration occurs with consequent increase in intraglomerular pressure that can damage the kidney structure and raise the risk of developing CKD in the long-term [6]. Indeed, observational studies showed that metabolically healthy obese subjects (without diabetes or hypertension) present a high risk of developing CKD, suggesting that obesity per se may cause renal dysfunction and kidney damage [10]. In particular, higher visceral adipose tissue has been associated with renal damage and great mortality in CKD patients, highlighting a direct role of visceral adiposity in organ damage [11–13]. Interestingly, also non-alcoholic fatty liver disease (NAFLD), a pathology characterized by fat accumulation in the liver in the absence of significant alcohol intake, was associated with an increased risk of CKD [14]. This harmful effect is probably due to the endocrine activity of adipose tissue via production of several adipokines involved in the development of inflammation, oxidative stress, abnormal lipid metabolism, increased production of insulin and insulin resistance [6]. Increased ROS production was observed in obesity and is closely linked to the development of metabolic syndrome and diabetes [15]. In addition, adiposity may be a potent, independent amplifier to the inflammatory and oxidative milieu already present in CKD [16].

Several studies have investigated the beneficial properties of various phytochemicals with particular attention to both antioxidant and anti-inflammatory activities. To date, existing studies appear encouraging, but results are premature to translate into clinical practice [17]. In particular, flavonoids, a family of polyphenols found abundantly in fruits, vegetables, nuts, whole-grains and vegetable oils consumed by humans, have been extensively studied and their beneficial effects have been documented in many diseases [18–25]. Citrus fruits and their juices show a high content of flavonoids and their nutraceutical efficacy has been shown in many studies. Bergamot (*Citrus bergamia Risso et Poiteau*) and bergamot-derived extracts, such as bergamot polyphenol fraction (BPF) have attracted a considerable attention due to its peculiar composition and the highest content of Citrus flavonoids, such as naringin, hesperidin and neoeriocitrin [26,27]. Its lipid-lowering, anti-diabetic, anti-inflammatory, and autophagy-stimulating activity have been confirmed in both animal models and clinical studies [28–32]. Although the metabolic effects of BPF suggest an underlying redox-balancing activity, this property has not been properly evaluated and documented so far. In addition, some authors have proposed a new mechanism of how food antioxidants exert their health-protective effects, i.e., the oxidative activation of the Nrf2 signaling pathway [33]. This mechanism, called "para-hormesis", keeps antioxidant enzymes at the optimal levels consistent with good health [33]. In light of this, additional research is necessary to establish how bergamot-derived extracts interact with human physiological and pathological processes and what level of consumption (metabolism/excretion) is required to achieve health benefits.

In this work, we used a cafeteria (CAF) diet-fed rats, an excellent experimental model for studying human metabolic syndrome, as it is able to induce severe obesity with insulin resistance and high plasma triglyceride levels than a traditional lard-based high-fat diet used in rodent models [32]. To test the bergamot effect, we administered BPF to CAF-diet-fed rats. We hypothesized that the increased oxidative stress in obesity models may contribute to organ damage through activation of apoptosis signaling pathways and that BPF could have beneficial antioxidant effects. In order to test this assumption, we evaluated in our experimental models (i) plasmatic pro-oxidant/antioxidant status, (ii) expression of cytoplasmic antioxidant enzymes (SOD1 and GSTP1) and (iii) extrinsic and intrinsic apoptotic pathways.

2. Materials and Methods

2.1. Animals

Five-week-old male Rcc:Han WIST rats (n = 26; Harlan Laboratories, Indianapolis, IN, USA) were housed under controlled lighting (lights on at 7:00 a.m. and lights off at 7:00 p.m.) and temperature (20 ± 2 °C) conditions. The animals had access to water and were fed ad libitum with standard chow diet (SC, Harlan Teklad) for 3 weeks before assignment to one of four experimental groups. Ethics Statement: this animal study was approved by a local animal welfare committee and by the Italian Ministry of Health (project code: 01-24/09/2013), according to Legislative Decree 116/1992, which was in force when the study was proposed (before 4 March 2014). All surgery was performed under anesthesia and all efforts were made to minimize animal suffering.

2.2. Experimental Design

Rats (n = 26), at 8 weeks of age, were randomly assigned to two basic experimental groups: CAF diet group (n = 15) and control (CTR) diet group (n = 11). These two groups were subdivided into two subgroups: one received a BPF supplement (~50 mg/kg body weight/day) in drinking water (BPF, n = 6; BPF + CAF, n = 8) and the other received drinking water without BPF (CTR, n = 5; CAF, n = 7). The experimental diets administration started after a week and lasted 91–95 days until the day of sacrifice. Food consumption and body weight gain were monitored weekly for 13 weeks.

2.3. Diets and Supplement

The CAF diet comprised cookies (sweet or briny), milk chocolate, cereals, potato chips, processed meats, condensed milk with sugar, high-fat cheese (parmesan or provolone), and so on, were provided in excess. The CAF diet (75 kcal/rat/day) was offered in addition to standard chow (SC) diet ad libitum every 2–3 days. Each time a mix of snacks (salty and sweet) was supplied to stimulate hyperphagy. The snack consumption was monitored weekly by weighing them before and after consumption (corrected for drying), in order to calculate the amounts ingested of each one in all cages. According to the product labels' information, CTR diet provided an energy value of 3.0 kcal/g, against the mean 4.2 ± 1.1 kcal/g of the items included in CAF diet [30]. BPF, as previously prepared and characterized for polyphenol content [26,30], was kindly provided by Herbal and Antioxidant Derivatives srl. (Polistena, RC, Italy). BPF contains 40% of flavonoids. The remaining part of BPF is a mixture of other polyphenols (mainly catechins, salts, fatty acids, carbohydrates and other compounds ([27] and unpublished observations). Neohesperidose-linked flavanones, such as naringin, neoeriocitrin and neohesperidin, account for over 60% of all flavonoids. BPF was provided (diluted in drinking water) daily or every 2 days in the BPF and BPF + CAF groups. We did not observe any change in pH, color and taste within 48 h. The consumption of water and BPF was monitored daily or every 2 days to calculate the daily intake of BPF. The BPF concentration in drinking water was progressively adjusted to the mean body mass in the cage to ensure a mean 50 mg/kg/rat/day dose over a 3-month period. This dose was five times higher than the previously tested dose in humans (1000 mg/100 Kg = 10 mg/Kg [28]) to account for five times higher metabolic rate in rodents according to Khan and coworkers [34].

2.4. Blood and Tissue Collection

At week 14, the animals were sacrificed under Zoletil (80 mg/kg) and Dormitor anesthesia for blood and tissue collection. The blood was collected by cardiac puncture in appropriate blood collection tubes, centrifuged (1700× g; 10 min at room temperature) and plasma were stored at −80 °C until use. The animals were perfused with 150 mM NaCl solution to remove excess of blood and to collect organs.

2.5. Blood Analysis

In the serum, the following parameters were determined: triglycerides, total and low-density lipoprotein (LDL) cholesterol, glucose, creatinine, blood urea nitrogen (BUN), alanine aminotransferase, aspartate aminotransferase, gamma glutamyltransferase (GGT), total- (T-Bil) and direct bilirubin (bilirubin-D). The analyses were performed using commercial reagents on a Dimension EXL analyzer (Siemens Healthcare Diagnostics s.r.l., Milan, Italy). Routine blood counts were performed on EDTA-treated samples on Advia 2120 blood cell counter (Siemens, Erlangen, Germany).

2.6. Measurement of Plasma Oxidative Status

Plasma and tissue oxidative status determinations were measured by using photometric measurement kits and a free radical analyzer system with a spectrophotometric device reader (FREE Carpe Diem, Diacron International, Grosseto, Italy), which are routinely used in our laboratory [35–38]. Plasma oxidative stress was assayed using a Diacron-reactive oxygen metabolite (dROM) test. Results are expressed in Carratelli Units (UC; 1 UC = 0.8 mg/L of hydrogen peroxide). Total plasma antioxidant capacity was assayed using a biological antioxidant capacity (BAP) test. Results are expressed in μmol/L of the reduced ferric ions.

2.7. Western Blot and Densitometric Analysis

Tissue samples (800 mg) were prepared in a solution containing RIPA buffer (1.6 mL) and protease inhibitor cocktail (Sigma, St Louis, MO, USA), and then centrifuged at 14,000 rpm for 20 min at 4 °C. Protein quantification was performed with Bradford reagent kit (Sigma, St Louis, MO, USA). Proteins (50 μg) were heated (five minutes) in Laemmli buffer (Sigma, St Louis, MO, USA), separated by SDS-PAGE in a Bio-Rad Mini Protean III, and electroblotted onto nitrocellulose membrane (NitroBind, Maine Manufacturing, Sanford, ME, USA) using a mini trans-blot (Bio-Rad Laboratories, Hercules, CA, USA). Membrane was blocked in TBS-T buffer (5% non-fat dry milk). Blots were incubated with primary antibodies diluted in TBS-T overnight at 4°C (SOD1, polyclonal goat antibody; GSTP1, monoclonal mouse antibody; pJNK, monoclonal mouse antibody; Caspase 8, monoclonal mouse antibody; Caspase 9, polyclonal rabbit antibody); blots were incubated with peroxidase linked secondary antibodies for 1 h at room temperature. All antibodies were supplied by Santa Cruz Biotechnology, Inc., Dallas, USA. Immunodetection was obtained by an enhanced chemiluminescence kit (ECL Plus, GE Healthcare Amersham, Buckinghamshire, UK) and X-ray Films (Hyperfilm ECL, GE Healthcare Amersham, Buckinghamshire, UK). Digitalized immunoblots were subjected to densitometric analysis by WCIF Image J based on 256 grey values (0 ¼ white; 256 ¼ black) and results were expressed as means ± SE (standard error) of five determinations for each sample.

2.8. Statistical Analysis

Data was analyzed using the GraphPad/Prism version 5.01 statistical software (SAS Institute, Abacus Concept, Inc., Berkeley, CA, USA). Statistical differences were examined using two-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test. Data are expressed as the mean ± SE.

3. Results

3.1. Effects of CAF Diet and BPF Treatment on Obesity and Blood Parameters

In laboratory rodents, obesity is defined as the achievement of a 20% increase in body mass index [39]. The CAF diet rapidly induces weight gain and obesity and after 14 weeks of treatment we found an increase in body weight of 31.97%. The BPF supplementation significantly reduced the final body weight in our obesity model indeed BPF + CAF group presents an increase in body weight of 19.46% with respect to CTR group. No significant effects of BPF on body weight were

observed in animals fed SC diet and consuming daily bergamot polyphenols (BPF group) (Table 1). Concerning plasmatic biochemical profiles after 14 weeks of treatment, CAF diet led to a significant increase in blood glucose levels (+52.17%) with respect to CTR rats. Similarly, CAF diet significantly upregulated triglycerides level (+51.4%). Interestingly, our CAF diet protocol did not alter total and HDL cholesterol levels, while we observed a significant reduction of LDL-cholesterol in all treated groups. Triglyceridemia, significantly upregulated in CAF group, was potently reduced in BPF + CAF rats (−28.3%, $p = 0.006$), in agreement with our previous observations [28]. BPF also reduced blood glucose levels in CAF animals (−11.5%, $p = 0.044$). No significant differences in any relevant hematologic parameter were observed between the CTR and BPF groups (Table 1). The analysis of creatinine levels shows no variation in any of the experimental groups, indicating that renal function is preserved (Table 1).

Table 1. Body weight and biochemical profiles of Wistar rats at 22 weeks of age, fed control (CTR) or cafeteria (CAF) diet, supplement or not with bergamot polyphenol fraction (BPF; 50 mg/kg/rat) for 14 weeks. Data are expressed as Mean ± standard deviation (SD; *,#, $p < 0.05$ and **,##, $p < 0.01$ when compared with CTR animals or CAF animals, respectively).

Body Weight and Biochemical Profiles	CTR	BPF	CAF	BPF + CAF
Body weight (g)	466.0 ± 34.1	460.4 ± 49.6	615.0 ± 29.3 **	556.7 ± 55.7 #
Glucose (mg/dL)	230.0 ± 27.6	266.5 ± 28.4	350.86 ± 30.2 **	310.4 ± 47.6 #
Triglycerides (mg/dL)	57.0 ± 18.7	55.0 ± 23.6	86.3 ± 14.6 *	61.9 ± 14.6 ##
Cholesterol, total (mg/dL)	92.8 ± 12.2	89.2 ± 12.0	90.5 ± 11.8	73 ± 11.7 #
HDL cholesterol (mg/dL)	63.6 ± 11.2	63.4 ± 12.0	61.6 ± 9.3	50.8 ± 4.9 #
LDL cholesterol (mg/dL)	25.2 ± 9.2	19 ± 7.9 *	18.1 ± 5.7 *	13.1 ± 4.7 #
Creatinin (mg/dL)	0.8 ± 0.12	0.74 ± 0.11	0.8 ± 0.08	0.8 ± 0.07

3.2. Plasmatic Oxidative Status

We analyzed the trend of both oxidative stress (d-ROMs) and antioxidant capacity efficiency (BAP) in plasma of CTR, BPF, CAF and BPF + CAF rats. The analysis of the plasmatic oxidative balance shows that the oxidative index is decreased in all treated groups compared to the control (Figure 1a) while the antioxidant capacity effectiveness is considerably decreased in the CAF group and strengthened in the animals treated with BPF (Figure 1b). These results indicate that the CAF diet induces a perturbation of the oxidative equilibrium with an antioxidant capacity depletion as overused. On the contrary, BPF treatment enhances the plasmatic ability to neutralize the oxidative insults, mainly in the case of redox disturbance due to the CAF diet (Figure 1b).

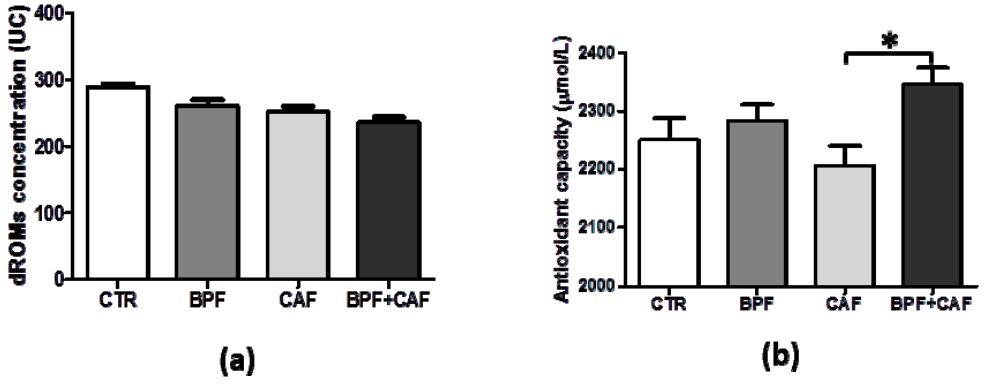

Figure 1. Plasmatic values of Diacron-reactive oxygen metabolite (dROM) (**a**) and biological antioxidant capacity (BAP) (**b**) test in control (CTR), bergamot polyphenol fraction (BPF), cafeteria (CAF) and BPF + CAF rats. Data are expressed as mean ± standard deviation (SD; $n = 12$). Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple comparisons test (* $p < 0.05$).

3.3. Antioxidant Enzymes' Expression

We examined the expression of two important cytoplasmatic antioxidant enzymes, SOD1 and GSTP1, in CTR, BPF, CAF and BPF + CAF rats. The expression of SOD1, the most important preventive antioxidants, shows no significant changes in any of the treated groups (Figure 2). Concerning GSTP1, the expression of GSTP1 monomer (23 kDa), the form with antioxidant and proliferative activity, is increased by BPF treatment while considerably and significantly decreased by the CAF diet (Figure 3). The expression of the enzymatically active dimeric form (46 kDa) does not differ significantly in the CAF diet group while it is increased in BPF group and is decreased in BPF + CAF group (Figure 4).

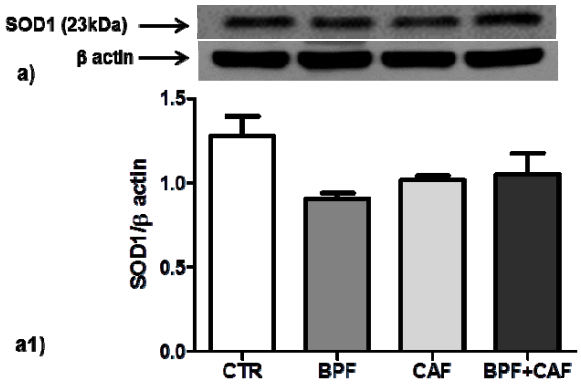

Figure 2. SOD1 expression in rat kidney. Western blotting of SOD1 (**a**) in the kidney extracts of CTR, BPF, CAF and BPF + CAF rats; (**a1**) shows the densitometric quantification of the blots. Protein loading was verified by using the anti-β actin antibody. Data are means ± standard deviation (SD) of five determinations for each animal (n = 3). Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple comparisons test.

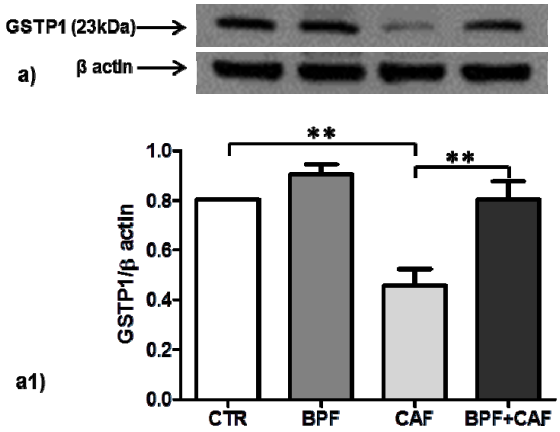

Figure 3. Monomeric GSTP1 expression in rat kidney. Western blotting of monomeric GSTP1 form (**a**) in the kidney extracts of CTR, BPF, CAF and BPF + CAF rats; (**a1**) shows the densitometric quantification of the blots. Protein loading was verified by using the anti-β actin antibody. Data are means ± SD of five determinations for each animal (n = 3). Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple comparisons test (** $p < 0.001$).

3.4. Apoptotic Pathways

In CTR, BPF, CAF and BPF + CAF rats, we analyzed apoptosis activation by evaluating the expression of pJNK (detected as double bands: pJNK1, 46 kDa; pJNK2, 54 kDa), apoptotic extrinsic pathways by evaluating the expression of caspase 8, and apoptotic intrinsic pathways by evaluating the expression of caspase 9. The expression of pJNK1, marker of apoptotic aspecific activation, is increased only in the CTR+BPF group (Figure 5a1) while the expression of pJNK2, preferentially activated by oxidative stress, is increased in all treated groups, significantly only in the BPF group (Figure 5a2).

Both Caspase 8 (marker of apoptotic extrinsic pathways) and Caspase 9 (marker of apoptotic intrinsic pathways) show the same expression profile: a significant upregulation in BPF and CAF groups while no activation in the BPF + CAF group (Figures 6 and 7). These results indicate that the CAF diet induces apoptotic injuries mitigated by BPF treatment.

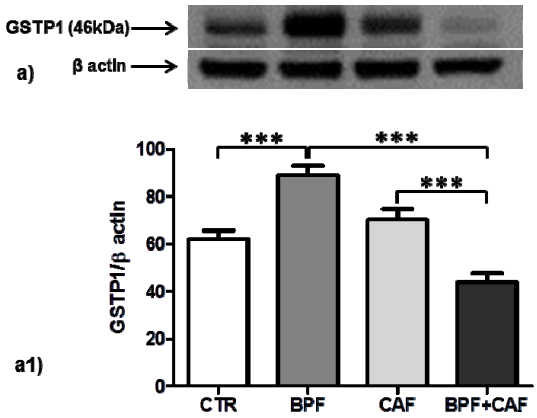

Figure 4. Dimeric GSTP1 expression in rat kidney. Western blotting of dimeric GSTP1 form (**a**) in the kidney extracts of CTR, BPF, CAF and BPF + CAF rats; (**a1**) shows the densitometric quantification of the blots. Protein loading was verified by using the anti-β actin antibody. Data are means ± SD of five determinations for each animal (n = 3). Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple comparisons test (*** $p < 0.0001$).

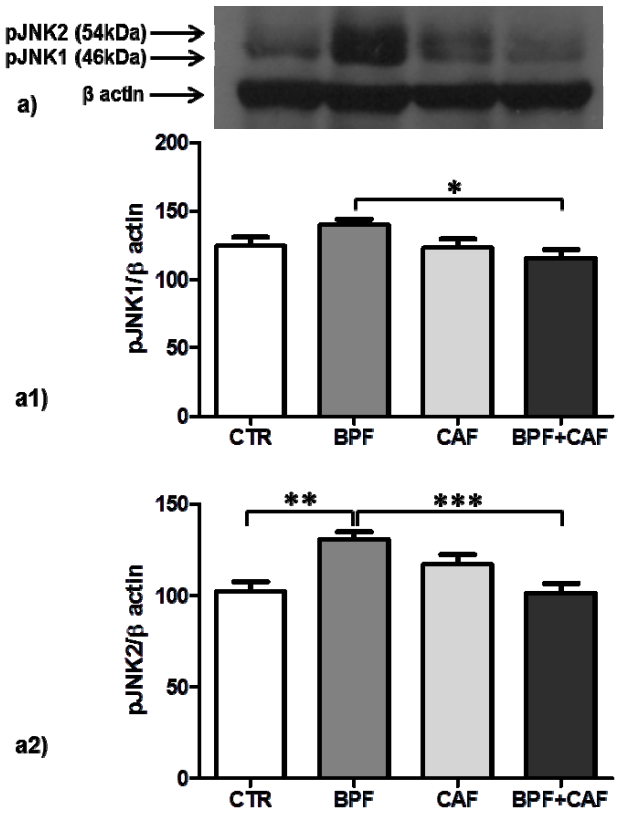

Figure 5. pJNK expression in rat kidney. Western blotting of pJNK (**a**) in the kidney extracts of CTR, CTR, BPF, CAF and BPF + CAF rats; (**a1**,**a2**) show the densitometric quantification of the blots. Protein loading was verified by using the anti-β actin antibody. Data are means ± SD of five determinations for each animal (n = 3). Statistical differences were evaluated by two-way ANOVA followed by Turkey's multiple comparisons test (* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$).

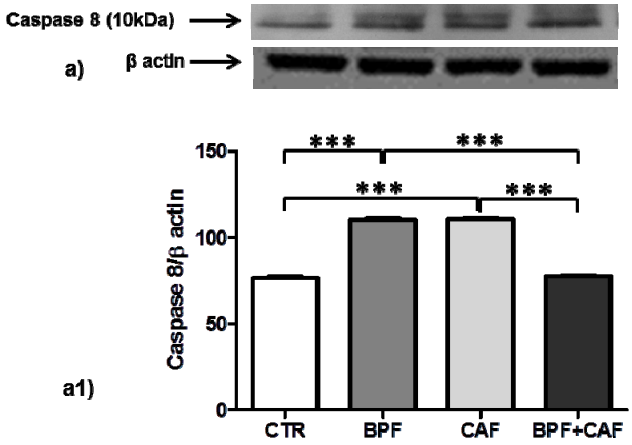

Figure 6. Caspase 8 expression in rat kidney. Western blotting of Caspase 8 active fragments cleaved (**a**) in the kidney extracts of CTR, BPF, CAF and BPF + CAF rats; (**a1**) shows the densitometric quantification of the blots. Protein loading was verified by using the anti-β actin antibody. Data are means ± SD of five determinations for each animal (n = 3). Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple comparisons test (*** $p < 0.0001$).

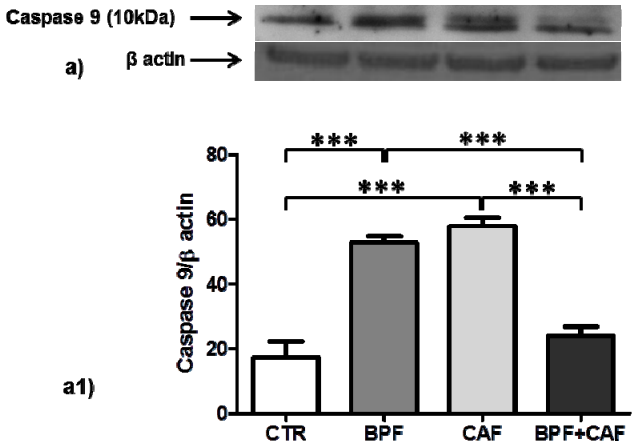

Figure 7. Caspase 9 expression in rat kidney. Western blotting of Caspase 9 active fragments cleaved (**a**) in the kidney extracts of CTR, BPF, CAF and BPF + CAF rats; (**a1**) shows the densitometric quantification of the blots. Protein loading was verified by using the anti-β actin antibody. Data are means ± SD of five determinations for each animal (n = 3). Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple comparisons test (*** $p < 0.0001$).

4. Discussion

This study was designed to evaluate if the CAF diet, a robust model of human metabolic syndrome, can affect the plasmatic oxidative balance and can influence renal tissue alterations, in terms of both oxidative damage (altered expression antioxidant enzymes) and apoptotic pathways (intrinsic/extrinsic) activation. In parallel, the antioxidant and cytoprotective effects of the Citrus flavonoids from bergamot, BPF, were evaluated. Our results showed relevant alterations in redox status in the CAF-fed rats compared to controls and revealed that BPF treatment enhances the plasmatic and cellular ability to neutralize the oxidative insults, mainly in the case of redox disturbance due to the CAF diet.

The CAF diet is considered to be the most appropriate regime to induce in rodents severe obesity, glucose intolerance, insulin resistance, high plasma triglyceride levels, and liver steatosis [30,32]. In addition, the CAF diet reflects the typical food of Western societies, thus representing an important model of diet-induced obesity in humans [40]. We used a CAF diet-fed rat, an excellent experimental model of obesity-induced organ damage particularly useful for analyzing the intricate link between

obesity and kidney damage. Most of the rodent models of diet-induced obesity were obtained by high fat (HF) diets [41,42], but our experimental model has been shown to induce hyperphagia and obesity in rodents to a greater extent than the classic HF diets [43,44]. In our study, we have verified that the CAF rats were already obese after eight weeks and presented significant changes in blood glucose and triglyceride levels after fourteen weeks. In our experimental model, steatosis induced by CAF diet and fat content of cells have been extensively analyzed in a precedent study [30]; however, markers like adipokines (leptin, adiponectin) have not yet been evaluated. Despite the state of overt obesity and the clear metabolic alterations, after 14 weeks of CAF diet, renal function was still preserved at this time point.

Obesity is characterized by complex metabolic abnormalities and acts as an important risk factor for renal diseases as highlighted by several studies in which BMI has been correlated with the risk of both development and progression of CKD [7,8], nephrolithiasis [45] and renal cancer [46–48]. Paradoxically, obesity has been consistently associated with lower mortality rates in patients with advanced CKD [49,50] and ESRD [51,52]. This apparent discrepancy makes it particularly important to study the mechanisms, still unknown, by which obesity can cause or exacerbate CKD. It is well known that the comorbidities typical of obesity, such as diabetes and hypertension, have a deleterious effect on renal function, but there are also direct effects on kidney tissue induced by the endocrine activity of the adipose tissue [6]. Indeed, some studies have highlighted a BMI-independent association between abdominal obesity and mortality in patients with ESRD [11] and kidney transplant [12], suggesting a direct role of visceral adiposity.

The link between obesity and renal damage could be represented by oxidative stress. Some authors have reported that obesity and obesity-induced insulin resistance are associated with systemic oxidative stress [53] and, in several models, the mechanism has been identified by the activation of the c-Jun N-terminal kinase pathway [54–58]. Our results indicate that the CAF diet induces a perturbation of the plasmatic oxidative equilibrium with the repairing intervention of the antioxidant capacity that is thus decreased as overutilized. It is well known that an oxidative insult determine a depletion of non-enzymatic antioxidants since the ROS species neutralization implies their consumption [59]. The occurrence that the efficacy of total plasma antioxidant capacity is significantly depleted in relation to metabolic disorders (such as diabetes, obesity, and dyslipidemia) had already been highlighted by our research group as the first detectable event of a redox disturbance in humans [37]. Further evidence of this mechanism is that BPF treatment enhances the plasmatic ability to neutralize the oxidative insults, mainly in the case of redox disturbance due to the CAF diet.

Concerning enzymatic antioxidants, regulation mechanisms are more complex: in the case of low/medium oxidative stimulation, enzymatic antioxidant activity can increase, but, if oxidative stress is persisting, or its level is very high, the damage caused to proteins becomes profound and a decreased expression/activity may occur via direct oxidative damage of the molecules and/or oxidative-altered gene expression. Alterations in the enzymatic antioxidant defense mechanisms are reported in obesity models including human, but the described scenario is very intricate [60]. In obese mice, expression levels and activities of antioxidant enzymes decreased particularly at adipose tissue level [61] while the levels of antioxidant enzymes in hamsters were not greatly modified by CAF diet-induced obesity [62]. In renal tissues of our experimental model, we examined the expression of two key cytoplasmatic antioxidant enzymes, SOD1 and GSTP1. The expression of SOD1, the most important preventive antioxidants that catalyze the dismutation reaction of superoxide anion to the more stable hydrogen peroxide [63,64], shows no significant changes in relation to CAF or BPF treatments while the expression of GSTP1 monomeric form, with antioxidant and proliferative activity, is increased by BPF treatment while considerably and significantly decreased by the CAF diet. These results corroborate the recent genomic evidence that highlights as the CAF diet induced alterations in the white adipose gene transcriptome, with important suppression of glutathione-related genes and pathways involved in mitigating oxidative stress [65]. The non-catalytic role of GSTP1

monomeric form plays a key role in stress response cellular pathways acting as a JNK-proliferative pathway activator [66] in both humans and rodents [67,68].

Several studies have shown that GSTP1 acts as a stress response protein that multimerizes through disulfide cross-links, if affected by oxidative stress, and loses its ability to bind JNK, causing an increase in JNK-apoptotic pathways [67]. In renal tissue, by analyzing the apoptotic signal, we found that CAF diet induces apoptotic injuries induced by oxidative events, mitigated by BPF treatment. In particular, the expression of pJNK1, marker of apoptotic aspecific activation, shows no significant changes in CAF diet-fed rat while the expression of pJNK2, preferentially activated by oxidative stress, is increased in all treated groups, significantly only in the CTR+BPF group. JNK is a mitogen-activated protein kinase (MAPK) and plays both physiological and pathophysiological role in cells. Several lines of evidence propose that JNK is a crucial mediator in oxidative stress-induced apoptotic cell death in obesity and insulin resistance [69,70]. Our result confirmed this finding; indeed, in our obesity model, we detected a specific and robust pJNK2 activation, which supports the hypothesis that enhanced JNK activity is a critical mechanism underlying the apoptotic response to oxidant injury in obesity. Apoptosis signaling has been widely classified into extrinsic (initiated by death receptors) and intrinsic (initiated by mitochondrial events) pathways, and pJNK plays a central role in both of these pathways [71,72]. The apoptotic extrinsic pathway activation is mediated by caspase-8 [73] and the activation of the intrinsic pathway is mediated by caspase-9 [74]. In the rat model for kidney disease, the apoptotic process is associated with both intrinsic and extrinsic pathways [75]. In the present study, by analyzing both the extrinsic and intrinsic apoptotic pathways, we found that, in kidney tissue, both caspases 8 and 9 show a significant upregulation in CAF obese group and no activation in the BPF + CAF group, confirming the apoptotic response to oxidant injury in obesity and the protective effect exerted by the BPF. Our results support the existing data on the flavonoids nephroprotective effect mainly exerted on oxidative perturbations affecting apoptotic pathways [76].

An interesting element that emerges from our results is represented by the "negative" effect exerted by the BPF in the absence of redox stimulation. Indeed, in this case, BPF induces a pro-oxidant effect as made evident by the upregulation of antioxidant enzymes. In addition, the obese rats treated with BPF show an improvement of their apoptotic profile, while the controls treated with the same BPF doses show a worsening of their apoptotic profile. This suggests that high doses of bergamot polyphenols, well-tolerated at the liver level and sufficient to balance pro-oxidative effects of CAF diet [30], might be actually slightly nephrotoxic, if applied in association with the normocaloric diet. Accordingly, such an undesirable effect of flavonoids on renal tissues has been recently highlighted. Despite its beneficial and antioxidant activity in some types of kidney disease, high doses of green tea polyphenols exerted clearly nephrotoxic effects in streptozotocin-induced diabetic mice [77] as well as in chronic renal failure by reducing the elimination of nephrocardiovascular toxins [78]. These data underscore again that the use of antioxidant (but also other) supplements should be reserved for situations of proven lack. Indeed, in humans, nutraceutical supplementation has induced improvements in blood lipid profile but not in BMI if not associated with diet [79,80] while consumption of flavonoid-containing foods was inversely associated with both obesity and markers for metabolic syndrome [81].

5. Conclusions

Data collected in the present study show that the link between obesity and renal damage could be represented by oxidative stress. Moreover, we highlighted the alteration of the plasma antioxidant capacity as the first detectable and reversible event. Our results can contribute to better understand mechanisms underlying the relationship between obesity and renal tissue damage.

6. Clinical Perspectives

Obesity is a complex, multi-factorial pathology and represents a potent risk factor for kidney disease. The aim of our study was to assess if the CAF diet, a useful model for studying human

metabolic syndrome, can induce renal tissue damage in terms of both oxidative perturbation (altered expression antioxidant enzymes) and apoptotic pathway (intrinsic/extrinsic) activation. In parallel, we evaluated the BPF cytoprotective effects. Our results showed significant alterations in redox status in the CAF-fed rats and revealed that BPF treatment enhances the plasmatic and cellular ability to neutralize the oxidative insults, mainly in the case of redox disturbance due to the CAF diet. A deeper understanding of the mechanisms underlying the relationship between obesity and progression of renal tissue damage can provide new treatment possibilities.

Author Contributions: Conceptualization, D.P. and D.L.R.; methodology, D.L.R., F.G., A.M., and M.P.; software, D.P.; formal analysis, D.P. and D.L.R.; investigation, D.L.R., F.G., A.M., and M.P.; resources, D.P. and E.J.; writing—original draft preparation, D.P.; writing—review and editing, D.P. and E.J.; funding acquisition, D.P. and E.J.

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Full Paper VII

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Bergamot Polyphenols Boost Therapeutic Effects of the Diet on Non-Alcoholic Steatohepatitis (NASH) Induced by "Junk Food": Evidence for Anti-Inflammatory Activity

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Abstract: Wrong alimentary behaviors and so-called "junk food" are a driving force for the rising incidence of non-alcoholic fatty liver disease (NAFLD) among children and adults. The "junk food" toxicity can be studied in "cafeteria" (CAF) diet animal model. Young rats exposed to CAF diet become obese and rapidly develop NAFLD. We have previously showed that bergamot (*Citrus bergamia Risso et Poiteau*) flavonoids, in the form of bergamot polyphenol fraction (BPF), effectively prevent CAF diet-induced NAFLD in rats. Here, we addressed if BPF can accelerate therapeutic effects of weight loss induced by a normocaloric standard chow (SC) diet. 21 rats fed with CAF diet for 16 weeks to induce NAFLD with inflammatory features (NASH) were divided into three groups. Two groups were switched to SC diet supplemented or not with BPF (CAF/SC±BPF), while one group continued with CAF diet (CAF/CAF) for 10 weeks. BPF had no effect on SC diet-induced weight loss, but it accelerated hepatic lipid droplets clearance and reduced blood triglycerides. Accordingly, BPF improved insulin sensitivity, but had little effect on leptin levels. Interestingly, the inflammatory parameters were still elevated in CAF/SC livers compared to CAF/CAF group after 10 weeks of dietary intervention, despite over 90% hepatic fat reduction. In contrast, BPF supplementation decreased hepatic inflammation by reducing interleukin 6 (*Il6*) mRNA expression and increasing anti-inflammatory *Il10*, which correlated with fewer Kupffer cells and lower inflammatory foci score in CAF/SC+BPF livers compared to CAF/SC group. These data indicate that BPF mediates a specific anti-inflammatory activity in livers recovering from NASH, while it boosts lipid-lowering and anti-diabetic effects of the dietary intervention.

Keywords: flavonoids; hepatic steatosis; cytokines; inflammation; nutraceutical treatment; food supplement

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder in Western countries, caused by fat and sugar-rich diet, sedentary life style and genetic predisposition [1–3]. The hallmark of NAFLD is excessive triglyceride (TGL) accumulation in the form of lipid droplets (LDs) in the cytoplasm of hepatocytes, which may be an isolated event (non-alcoholic fatty liver) or accompanied by evidence of inflammation and cell injury with or without fibrosis (non-alcoholic steatohepatitis, NASH). If untreated, NASH, the more aggressive form of NAFLD, may progress to cirrhosis and hepatocellular carcinoma [4].

NAFLD has become the leading cause of chronic liver disease among all age groups. Particularly alarming is the fast increase of adolescents and children diagnosed with NAFLD, reaching 10–20% in some studies [5–7]. Within the next 10 years, juvenile NAFLD is expected to become the most prevalent cause of liver pathology, liver failure and indication for liver transplantation in childhood and adolescence in the Western world [5–7].

The cause of rising incidence of NAFLD in children and adolescents is unclear, but it is believed that wrong alimentary behaviors, such as regular consumption of energy dense, highly refined and processed foods is a driving force for metabolic co-morbidities in young patients [8]. Such foods, rich in simple sugars, saturated and trans fats, and preservatives and poor in nutrients, prevalent in the Western diets, are commonly defined as junk food [8,9].

The effect of junk food can be modeled in experimental conditions by so-called cafeteria (CAF) diet, consisting of several favorite supermarket snacks of children such as sweet or briny cookies, milk chocolate, potato chips, processed meats, condensed milk, etc. [10,11]. Young laboratory animals exposed to CAF diet develop NAFLD as early as in two months which later degenerates into NASH [10–12]. This is associated with hyperglycemia and hypertriglyceridemia in rats.

Insulin-resistance (IR) is the central event in the pathogenesis of this disease, causing lipid overload in hepatocytes and lipid peroxidation. Since type 2 diabetes, obesity and dyslipidemia are the most prevalent associated co-morbidities, it has been proposed that NAFLD is the liver involvement of metabolic syndrome. The current FDA-approved medical recommendation for patients diagnosed with NAFLD is low-fat, fruit and vegetables-based diet and regular physical activity [4,13–15]. Indeed, dietary polyphenols, a large and heterogeneous group of phytochemicals in herbs and plant-based foods, have been associated with lower risk of NAFLD [16,17]. In particular, *Citrus* flavonoids have been shown to exert several positive health effects on glucose and lipid metabolism in experimental models [12,16,18–22] and humans [23–25].

In particular, bergamot is an endemic plant of Calabria region (Italy) belonging to the Rutaceae family [26]. Bergamot has attracted considerable attention due to its peculiar composition and high content of flavonoids, some of which are also found in other *Citrus* species [18,27]. BPF is a highly concentrated extract of glycosylated flavanones (naringin, neoeriocitrin and hesperidin) and flavones (diosmetin, apigenin and luteolin glycosides) from bergamot fruit juice [19,20,28]. BPF phyto-complex contains around 40% of flavonoids, but also sugars, salts and other natural compounds with a possible detoxifying activity [19,20]. A particular feature of certain flavonoid glycosides, abundant in bergamot juice and in BPF, such as bruteridin and melitidin, is the presence of covalently linked 3-hydroxy-3-methylglutaryl (HMG) moiety [28,29]. Computational studies have suggested that both molecules may bind to the catalytic site of HMG-CoA reductase and inhibit cholesterol synthesis by replacing the endogenous substrate HMG-CoA [29]. Such a theoretical mechanism has been proposed to explain BPF efficacy as a cholesterol-lowering food supplement in clinical trials performed on metabolic syndrome patients. These studies also demonstrated strong reduction of blood TGL levels and mild effects on glucose levels in individuals taking BPF [23,24,30]. These effects might depend on the ability of BPF flavonoids to stimulate AMPK, which should also explain proautophagic proprieties of these compounds in models of hepatic steatosis in vitro and in vivo [12,19,20]. Finally, BPF may also have anti-inflammatory activity, since bergamot juice extract have been shown to reduce inflammation in animal models of ischemia/reperfusion and inflammatory bowel disease [31,32].

In our previous work, we have validated an animal model of NAFLD, which displayed some characteristics of human NASH and have shown that the supplementation of BPF is an effective strategy to prevent NAFLD in CAF-fed rats [12]. Although prevention studies in animal models of NAFLD/NASH have significantly contributed to the understanding of this pathology, they have been less accurate in translating aspects of clinical trials. In fact, human trials address the efficacy of therapeutic interventions, in association with diet and life style modifications, which are the most effective ways to promote liver fat removal and amelioration of histological severity [1,14,33]. For this reason, the main goal of this this work was to analyze the effects of BPF supplemented to SC diet as the primary treatment on rats with advanced NAFLD/NASH. The second goal was to provide further characterization of pathological changes in livers from rats exposed to CAF diet for much longer times (26 weeks) than previously investigated (14 weeks) [12]. Although a normocaloric diet alone was sufficient to cause regression of steatosis, BPF improved its therapeutic effects, proving to be a potent anti-inflammatory and lipid-lowering food-supplement for the treatment of NAFLD/NASH, but not obesity.

2. Materials and Methods

2.1. Animal Procedures

Thirty-two male 5-week-old Rcc:Han WISTAR rats (Harlan Laboratories, Indianapolis, IN, USA) were housed individually in steel cages under controlled conditions (temperature 20 ± 2 °C, light 07:00–19:00). The animals had access to water and were fed ad libitum with standard chow (SC) diet 2016 ("SC", energy value of 3.0 kcal/g, calories from protein 24%, calories from fat 18%, calories from carbohydrates 58%, Harlan Teklad) for 3 weeks before and during the experiment. This animal study was approved by a local animal welfare committee and by the Italian Ministry of Health, according to Legislative Decree 116/1992, which was in force when the study was proposed (before 4 March 2014).

2.2. Experimental Design

Starting from Week 8 of age, 24 rats were fed with CAF diet (see below) and 8 rats with SC diet for 15 weeks. After this period, 3 representative CAF and 3 SC rats were sacrificed for RNA isolation and NASH evaluation. At Week 16 of induction phase, 21 obese rats were evenly assigned to 3 experimental groups: CAF/CAF (7 rats), CAF/SC (7 rats) and CAF/SC+BPF (7 rats). CAF/CAF group continued to be fed with CAF diet, while CAF/SC and CAF/SC+BPF groups were switched to SC diet with and without BPF, respectively, for the subsequent 10 weeks. The remaining 5 lean rats continued to be fed with SC diet (SC/SC) for additional 11 weeks. During the intervention phase, CAF/SC+BPF rats were subjected to BPF treatment, which was supplemented to drinking water at suitable concentration to ensure an average 50 mg/kg/rat daily dosage. This dose was calculated as follows: the previously tested dose in humans, 1000 mg/100 kg = 10 mg/kg, was multiplied by 5 to account for higher metabolic rate in rodents, as previously described [12,34]. After 16 + 10 weeks of treatment, all animals were sacrificed for blood and tissue collection.

2.3. Diet and Supplementation

The CAF feeding regimen contained 15% protein, 70% carbohydrates and 15% fat, in the form of cookies, milk chocolate snacks, crackers, cheese, processed meats, condensed milk, etc. [11,12]. It was provided in excess, together with normal SC diet. Caloric intake in CAF-fed rats was typically 110 kcal/day/rat, while SC diet provided 60 kcal/day/rat [12]. Nutritional composition of CAF diet components is provided in Table S1 in the Supplementary Materials.

BPF was prepared by the absorption on polystyrene resin columns and alkaline elution as described in detail in the European patent (No. EP 2 364 158 B1) and characterized for polyphenol content by high-pressure liquid chromatography [12,18,20]. It was kindly provided by Herbal and Antioxidant Derivatives S.r.l. (Bianco, RC, Italy). BPF diluted in drinking water was provided daily or every 2 days and during this time it remained stable, as it did not change color and taste. The

amount of water and BPF was monitored to calculate the daily intake of BPF. The concentration of BPF added to drinking water varied from 1 to 2 mg/mL and was adjusted to rat body mass and daily water consumption to ensure a mean 50 mg/kg/rat/day dose over a 10-week period.

2.4. Blood and Tissue Collection

After the intervention phase, food was removed for 4 h before sacrifice under Zoletil 80 mg/kg and Dormitor anesthesia for blood and tissue collection. The blood was collected by cardiac puncture with heparinized 21G needle and divided for plasma (2 mL) and serum preparations (3–4 mL) in appropriate blood collection tubes. For blood tests on Week 4 of experiment, orbital sinus blood sampling was performed by an experienced veterinarian after 5% isoflurane anesthesia (Merial, Toluse, France), as described previously [12]. Before tissue collection, animals were perfused with 150 mM NaCl solution to remove blood and then collected. All the organs were weighed and divided for histological processing and shock frozen in liquid nitrogen for biochemical analysis.

2.5. Blood Analysis

Hematochemical parameters, such as TGL and glucose, were determined in the serum. The analyses were performed using commercial reagents on a Dimension EXL analyzer (Siemens Healthcare Diagnostics s.r.l., Milan, Italy). Routine blood counts were performed on EDTA-treated samples on Advia 2120 blood cell counter (Siemens).

The concentrations of insulin and leptin were measured using ELISA kits (Rat/Mouse Insulin ELISA Kit; Mouse Leptin ELISA Kit, EMD Millipore Corporation, Darmstadt, Germany) according to the manufacturers' instructions. Approximate insulin resistance (IR) was calculated using the homeostasis model assessment (HOMA)-IR using the following formula: (glucose (mmol/L) × insulin (μU/mL))/22.5 [35].

The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were determined using commercial kits (Siemens Healthcare Diagnostics s.r.l., Milan, Italy) and automated biochemistry analyzer (Dimension EXL, Siemens Healthcare Diagnostics s.r.l.).

2.6. Histochemistry and Digital Image Analysis of LDs in Rat Liver Sections

The procedure for preparing liver tissue for cryosectioning and analysis of LDs was described previously [12]. Briefly, 10-μm liver sections were obtained by sectioning, using a cryostat at −20 °C (Leica Biosystems Inc., Buffalo Grove, IL, USA), and then histochemistry of LDs in perfused rat liver sections was performed by Oil Red O (ORO, Sigma-Aldrich, St. Louis, MO, USA) staining according to the previously described procedure [12].

Equivalent ORO plus hematoxylin-stained liver sections of each experimental group were first examined in bright-field with Leica Microscope DM4000B (Leica Microsystems GmbH, Wetzlar, Germany) equipped with 10×, 40× and 100× objective lenses. For each liver section, at least three independent images, from equivalent central lobe areas were captured at 10×, 40× and 100× magnification. For quantitative and qualitative LDs analysis, images of ORO-stained sections were processed and analyzed using a semi-automatic procedure implemented in Image J2x software package (National Institute of Health, Bethesda, MD, USA) and analyzed for percentage of total LDs area and the number of LDs.

2.7. Total Liver Lipid Assay

Total liver lipids were extracted according to the modified procedure of Folch et al. [12]. Briefly, frozen liver tissue (~400 mg) was thoroughly homogenized with 800 μL of deionized water (15 strokes). Four milliliters of chloroform/methanol (2:1 vol/vol) mixture (Sigma-Aldrich) was then added to the mix to generate a distinct organic and aqueous phase and extract lipids according to the previously described procedure [12].

2.8. Histology and Histological Examination

2.8.1. Toluidine Blue Staining

Liver were quickly excised cut into small pieces, and fixed by direct immersion in 4% glutaraldehyde in phosphate-buffered saline (PBS 0.1 M, pH 7.2) for 48 h at 4 °C. After post-fixation for 2 h with osmium tetroxide (1% in the same buffer), samples were dehydration in an increasing series of ethanol and then embedded in epoxy resin (Araldite 502/Embed 812, Electron Microscopy Sciences, Hatfield, PA, USA). Semi-thin sections (1 μm) were stained with toluidine blue, observed and photographed by a LM LEITZ Dialux EB 20 (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera.

2.8.2. Silver Impregnation (SI)

Silver staining (SI) detects collagen III and B (argyrophilic reticulin) fibers, which are highly increased in liver fibrotic tissue [36]. A standard histochemical protocol of silver impregnation (SI) kit (Bio-Optica s.r.l., Milan, Italy) was applied, according to manufacturer guidelines. In brief, 4 μm-thick serial sections were obtained from a representative block of formalin-fixed, paraffin-embedded tissue, mounted on coated glass slides, and heated at 60 °C for 60 min. Hematoxylin/eosin (HE) staining was performed on the first section to observe the tissue morphology. The second serial section was used for the SI staining.

2.8.3. Morphological Evaluation

Double-blind evaluation of all sections stained with TB, SI and HE as well as NAFLD activity scoring (NAS) were performed by two expert pathologists independently. NAS was performed on HE and TB stained sections for steatosis (score 0–3), lobular inflammation (score 0–3) and hepatocellular ballooning (score 0–2), using the NASH Clinical Research Network (CRN) scoring system as described previously [37]. Five animals (n = 3 sections for each animal) were scored for every experimental group.

2.9. Analysis of Cytokine Gene Expression

Small pieces (0.4–1 g) from the central part of the main lobe of rat livers were shock-frozen in liquid nitrogen and stored until needed at −80 °C. This choice of tissue portion was intended to minimize possible differences due to tissue heterogeneity within the lobe. Frozen tissue was fragmented and 50–100 mg samples were homogenized with glass douncer on ice with 1 mL of TRIzol Reagent (Life Sciences, Invitrogen, Carlsbad, CA, USA). Total RNA (totRNA) was extracted using the TRIzol reagent method followed by DNase treatment (Qiagen, Germantown, MD, USA). Total RNA was carefully quantified and its integrity was verified on 0.8% denaturing agarose gel. The expression of cytokines was analyzed by RT^2 Profiler PCR array plates (Qiagen, SABiosciences, Frederick, MD, USA) for rats before the treatment (15 weeks) or by a standard reverse transcription followed by quantitative PCR (RT-qPCR) based on SYBR Green detection for rats after 10-week diet treatment.

For array analysis, total RNAs from three representative rats of the same experimental group were pooled (3 × 5 μg) and cDNA was synthesized from 500 ng of pooled RNA with Qiagen One Step RT-PCR kit according to the instructions (Qiagen). The relative gene expression was assayed by using PARN-157Z and PARN-084Z RT^2 Profiler PCR arrays (SABiosciences) and, for the purpose of this article, only cytokine and housekeeping (HK) genes data were used. qPCR was performed on an iQ5 Real Time PCR (BioRad Lab., Hercules, CA, USA) using SYBR Green (cycling conditions: 95 °C, 10 min; 95 °C, 15 s; and 60 °C, 1 min; repeated for 40 cycles) and subsequent analyses were carried out according to the manufacturer's recommended protocol. Each pooled cDNA was analyzed on four independent plates. The data from each CAF plate were compared to four SC plates. The data analysis was performed with dedicated software available at the GeneGlobe Data Analysis Center (http://www.qiagen.com/it/shop/genes-and-pathways/data-analysis-center-overview-page/). The

same normalization method was used for all analyzed array plates i.e., manually selected HK genes. LDH was excluded from 6 standard HK genes.

For standard RT-qPCR analysis, totRNA was isolated as described above from 5–6 rats for each experimental group and RNA representing one rat was processed separately. cDNA was synthesized from 5 μg of totRNA with TransScript® II First-Strand cDNA Synthesis SuperMix (Transbionovo, Carlo Erba Reagents, Cornareado (MI), Italy, cat. FC40AH30102) according to manufacturer instructions. RT-qPCR was performed on QuantStudio 3 Real Time PCR Detection System (Applied Biosystems Europe, Monza (MI), Italy), using TransStart Top Green qPCR SuperMix (Carlo Erba, cat. AQ131-01) with addition of a copy of rat cytokine-specific primers. These primers were previously tested for good performance in SYBR green-based detection of very low gene expression of liver cytokines [38] (see Table 1). The applied cycling conditions were: 95 °C, 10 min; 95 °C, 15 s; and 61 °C, 1 min; repeated for 40 cycles. Samples were analyzed in triplicate with hypoxanthine phosphoribosyl transferase 1 (Hrpt1) as a HK control. Only results with the amplification of a single product, as verified by melting curve analysis, were considered. Relative gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method using the SC liver tissue cDNA (15 weeks on SC diet) as a starting point control.

2.10. Data Analysis and Statistical Procedures

Animal experiment was performed one time and the final mean values were based on at least 5 animals for each experimental group, except for leptin Elisa assay and for mRNA profiling experiment, where RNA pools from 3 rats were assayed. The data for one animal were a mean of at least three triplicate measurements (except for blood test) with standard deviation (SD). For statistical analysis, Prism 7.0 GraphPad software (GraphPad Inc., San Diego, CA, USA) was used or Excel Office 365. Nonparametric Mann-Whitney U test (comparisons between groups with no assumption about the scatter of the data) was performed for majority of datasets. Otherwise, the statistical method is indicated in the figure legend.

Table 1. The primers sequences used in RT-qPCR.

Gene		Sequences (5′-3′)
Il1b	Forward	CACCTCTCAAGCAGAGCACAG
	Reverse	GGGTTCCATGGTGAAGTCAAC
Il6	Forward	TCCTACCCCAACTTCCAATGCTC
	Reverse	TTGGATGGTCTTGGTCCTTAGCC
Il10	Forward	GTTGCCAAGCCTTGTCAGAAA
	Reverse	TTTCTGGGCCATGGTTCTCT
Tnfa	Forward	AAATGGGCTCCCTCTCATCAGTTC
	Reverse	TCTGCTTGGTGGTTTGCTACGAC
Hrpt	Forward	CTCATGGACTGATTATGGACAGGAC
	Reverse	GCAGGTCAGCAAAGAACTTATAGCC

3. Results

3.1. Supplementation of SC Diet with BPF Has a Powerful Effect on Levels of Blood TGL

To assess the effect of BPF supplementation to the low-sugar/fat diet regime as a therapeutic treatment for advanced NAFLD/NASH, we induced NAFLD with features of NASH in 21 Wistar male rats by feeding them with CAF diet for 16 weeks (induction phase), exactly as described previously [12]. These animals were then divided into three groups for the intervention phase of the experiment. 14 animals were subjected to SC diet with (CAF/SC+BPF) and without BPF (CAF/SC), while 7 rats continued with CAF diet (CAF/CAF) for further 10 weeks, according to the scheme in Figure 1. A small group of 5 rats was maintained on normocaloric SC diet during induction and intervention phases of the experiment as a basic control group (SC/SC) (Figure 1). After the induction

phase, there was a statistical difference in the body weight between SC control and CAF animals (Figure 2A). As expected, the diet change during the intervention phase led to a significant reduction of the mean body weight in CAF/SC rats as compared to CAF/CAF group (Figure 2B). No significant effects of BPF on the body weight were observed in animals fed SC diet and consuming bergamot polyphenols with respect to CAF/SC rats.

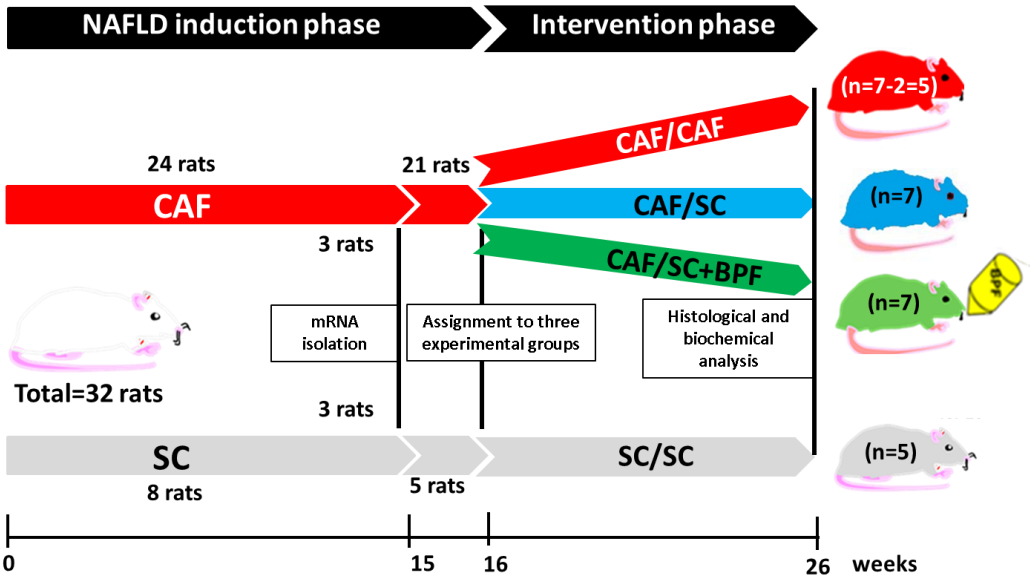

Figure 1. The experimental design and timeline of the study presented in this work: NAFLD/NASH induction phase for 16 weeks plus the intervention phase for 10 weeks. Black lines and boxes indicate the time of sample collection.

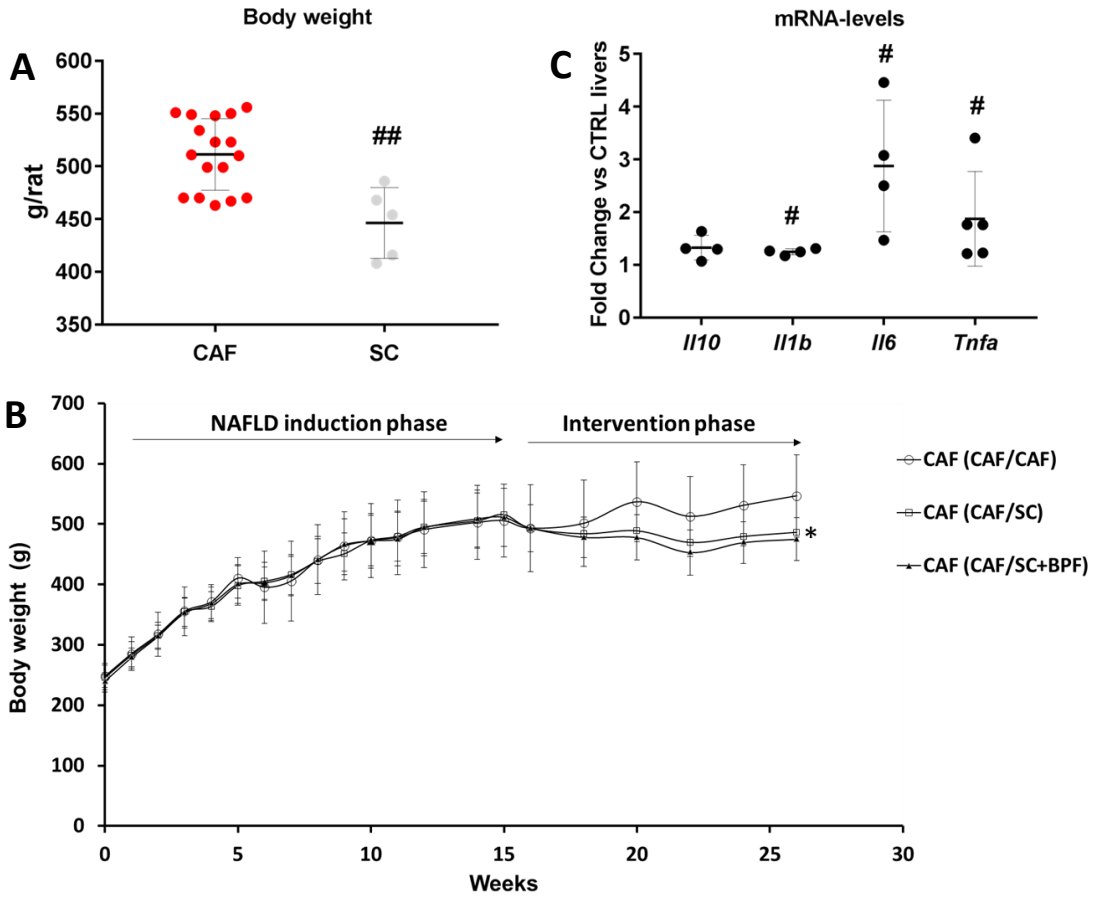

Figure 2. Starting conditions after the induction phase (16 weeks of CAF diet). (**A**) Distribution of body weight in rats fed with CAF diet (n = 21) or control SC diet (n = 5) after NAFLD induction phase (16 weeks). ## Statistically significant difference at $p < 0.003$ when compared to SC control group. (**B**) Body weight changes over the entire period of the study. Data are represented as means ± SD (n = 7), * $p < 0.05$ indicates significant differences between CAF/CAF and CAF/SC groups. (**C**) Significant upregulation of the gene expression of inflammatory cytokines in rat livers at week 15 of CAF diet, before the intervention phase with SC diet. The graph shows the results of cytokine-related mRNA expression profiling performed on 96-well RT² PCR array plates in three pooled RNA liver samples from three rats. The fold change of relative mRNA levels in CAF livers compared SC and normalized to five HK genes, is presented. Each dot shows the fold change value from one RT² array plate (three CAF livers vs. SC livers), and the line shows the mean of presented data. Statistical significance at $p \leq 0.02$ (#), when compared with control SC livers is reported according to GeneGlobe software (Qiagen), which calculates the *p* values based on a Student's *t*-test of the replicate $2^{-\Delta\Delta CT}$ values for each gene in the control and treatment groups.

Furthermore, CAF/CAF rats had developed severe NAFLD, characterized by a massive accumulation of LD lipid droplets and infiltration of Kupffer cells, according to previous observations in livers exposed to CAF diet for 14 weeks [12], indicating a histological severity compatible with NASH. To evaluate the presence of NASH, we performed gene expression profiling of pro- and anti-inflammatory cytokines, by RT-qPCR in 15-week CAF-fed rat livers. The results indicated a clear up-regulation of proinflammatory cytokines in CAF-fed livers when compared to control (SC) livers at Week 15. In particular, we observed a statistically significant increase of Interleukin 1β (*Il1b*), Interleukin 6 (*Il6*) and Tumor necrosis factor α (*Tnfa*) and no significant change in Interleukin 10 (*Il10*) gene expression (Figure 2C). These experiments yielded further information on Interferon gamma (*Ifng*) and Transforming growth factor beta (*Tgfb*) expression in 15-week CAF livers, but it was below a detection limit in case of Ifnγ or unchanged with respect to 15-week control livers in case of *Tgfb* (data not shown). Taking together, these data indicate CAF diet in Wistar rats induces NASH.

We observed a significant decrease of final body weight in CAF/SC and CAF/SC+BPF groups compared to CAF/CAF group, but BPF treatment did not cause any further reduction of body weight compared to CAF/SC group (Figure 3A). Among the CAF/CAF group rats, one died prematurely due to heart attack and another one could not feed due to a dentition defect, so it was excluded from statistical analysis.

Next, we tested whether the switch to SC diet and BPF treatment reduced blood parameters which are elevated in CAF/CAF diet-induced metabolic syndrome. We observed that triglyceridemia was reduced in CAF/SC rats after both 4 and 10 weeks of SC diet (Figure 3A,B). However, the concomitant administration of BPF resulted in a further reduction of blood TGL with significant differences between CAF/SC and CAF/SC+BPF groups at Week 10. Importantly, no significant differences in body weight were observed between CAF/SC and CAF/SC+BPF and SC/SC groups at Week 10 (Figure 3C). Similarly, no significant differences in blood glucose were found between the same experimental groups (Figure 3D).

These data suggest that supplementation of CAF/SC diet with BPF has strong effects on blood TGL, but modest effects on obesity and diabetes.

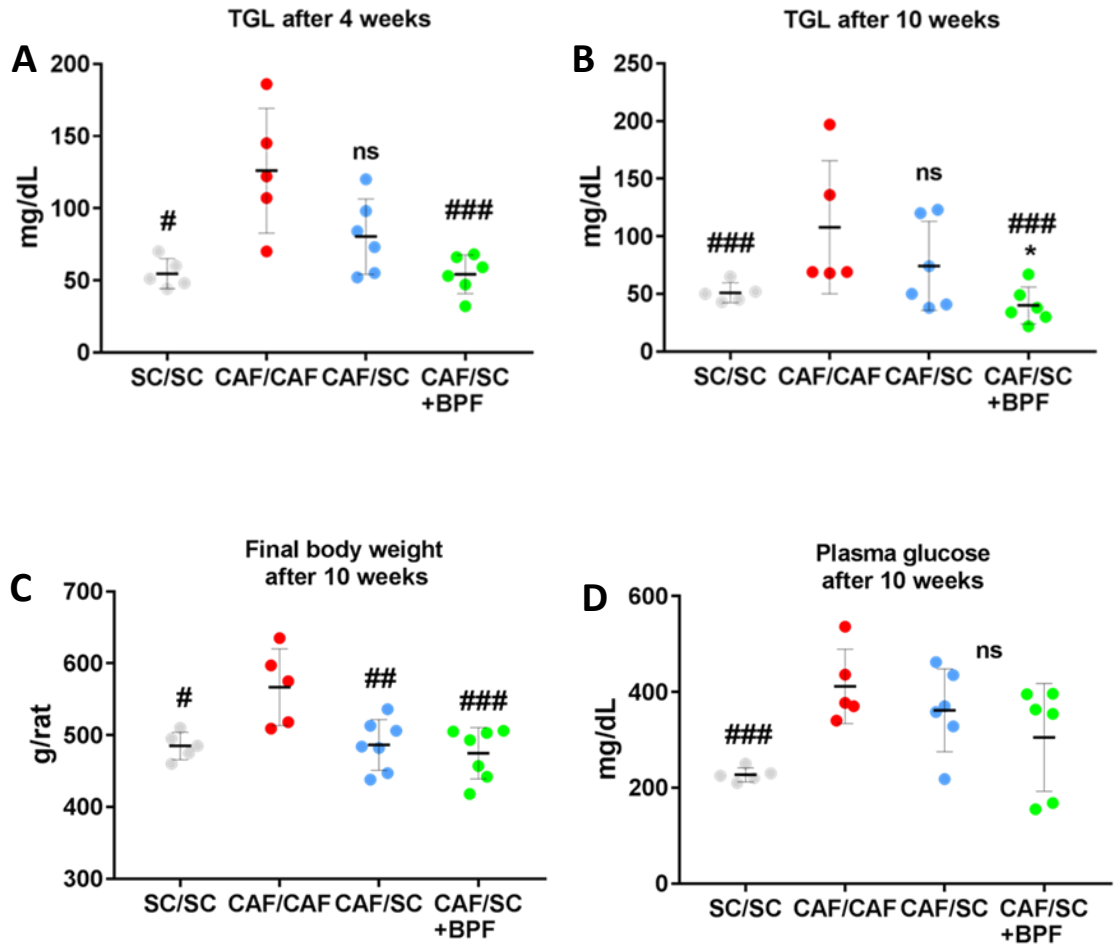

Figure 3. Supplementation of SC diet with BPF causes a reduction of blood TGL, but BPF effects on blood glucose and body weight loss are not significant. Serum TGL (**A**,**B**) and glucose (**D**) were measured after 4–5 h fasting in CAF rats treated for 4 or 10 weeks with SC diet ± BPF. #, ###, statistically significant difference vs. CAF/CAF group at $p \leq 0.05$ and $p \leq 0.006$, respectively. *, CAF/SC vs. CAF/SC+BPF at $p \leq 0.5$. (**C**) Distribution of final body weight after 10 weeks of the intervention phase. ##, ###, statistically significant difference vs. CAF/CAF at $p \leq 0.02$ and 0.002, respectively.

3.2. BPF Augments LDs Loss and Reduces Hepatic Inflammation When Supplemented to SC Diet during Recovery from NASH

To address the effect of SC diet and BPF treatment on CAF diet-induced fat accumulation, serial sections of livers from each diet group were evaluated using ORO histochemistry. Hematoxylin and ORO staining of liver sections showed accumulation of numerous, big and giant LDs in hepatocytes of CAF-fed rats (Figure 4B,F,L). This phenotype was greatly attenuated in CAF/SC (Figure 4C,G,M) and CAF/SC+BPF (Figure 4D,H,N) groups.

The above findings were confirmed by total lipid content analysis (Figure 5A). A significant reduction in total lipids was observed in CAF/SC livers compared to CAF/CAF livers. Furthermore, the supplementation of SC diet with BPF resulted in a substantial reduction in total lipids content in CAF/SC+BPF group compared to CAF/SC group.

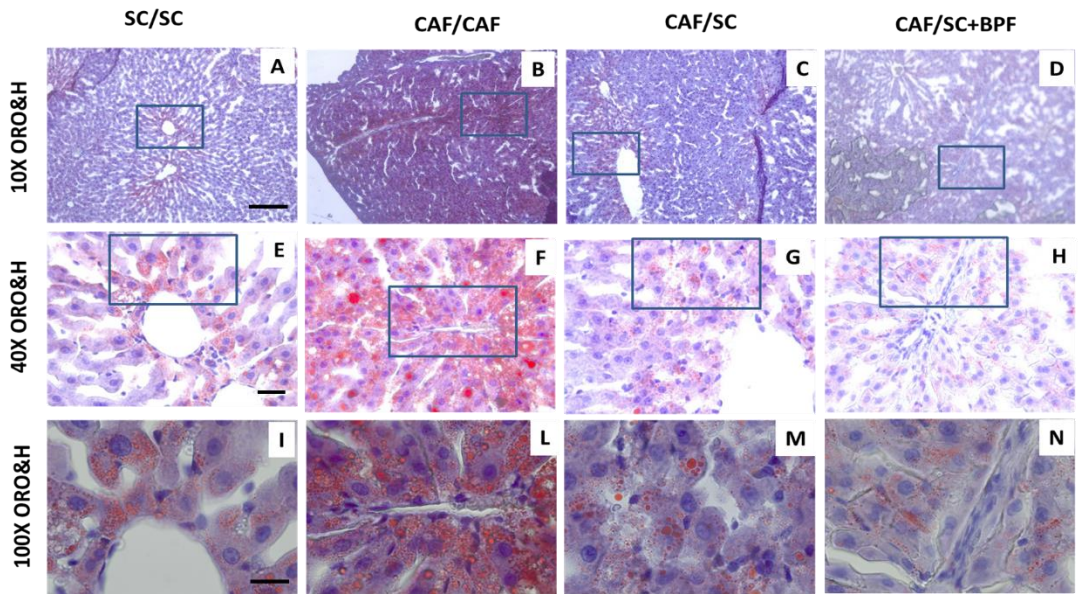

Figure 4. Strong LDs reduction in CAF/CAF livers after 10 weeks of treatment with standard chow (CAF/SC) diet or SC diet supplemented with BPF (CAF/SC+BPF). ORO and Hematoxylin (ORO&H) staining of representative liver sections from four treatment groups: SC/SC (26 weeks) (**A**,**E**,**I**); CAF/CAF (16 + 10 weeks) (**B**,**F**,**L**); CAF/SC (**C**,**G**,**M**); and CAF/SC+BPF diet (**D**,**H**,**N**). ORO&H staining allows visualization of unsaturated fatty acids and neutral fats (red, ORO) and nuclei (blue stained). Scale bars are as follows: (A–D) 200 μm ; (E–H) 40 μm; and (I–N) 20 μm. Boxes indicate magnified regions. 10×, 40× and 100× indicate objective magnifications.

Next, we evaluated morphometric parameters of ORO-stained LDs in liver sections from the four experimental groups (Figure 5B,C). In livers from CAF/CAF rats, LDs covered a big area of the digitalized image, whereas a relatively small area was occupied by LDs in livers from SC/SC, CAF/SC and CAF/SC+BPF rats, respectively (Figure 5B,C). Moreover, the total number of LDs was reduced by six-fold in CAF/SC livers compared with CAF/CAF livers. A significant difference in LDs numbers between the BPF-treated CAF/SC and CAF/SC groups was also reported (Figure 5B).

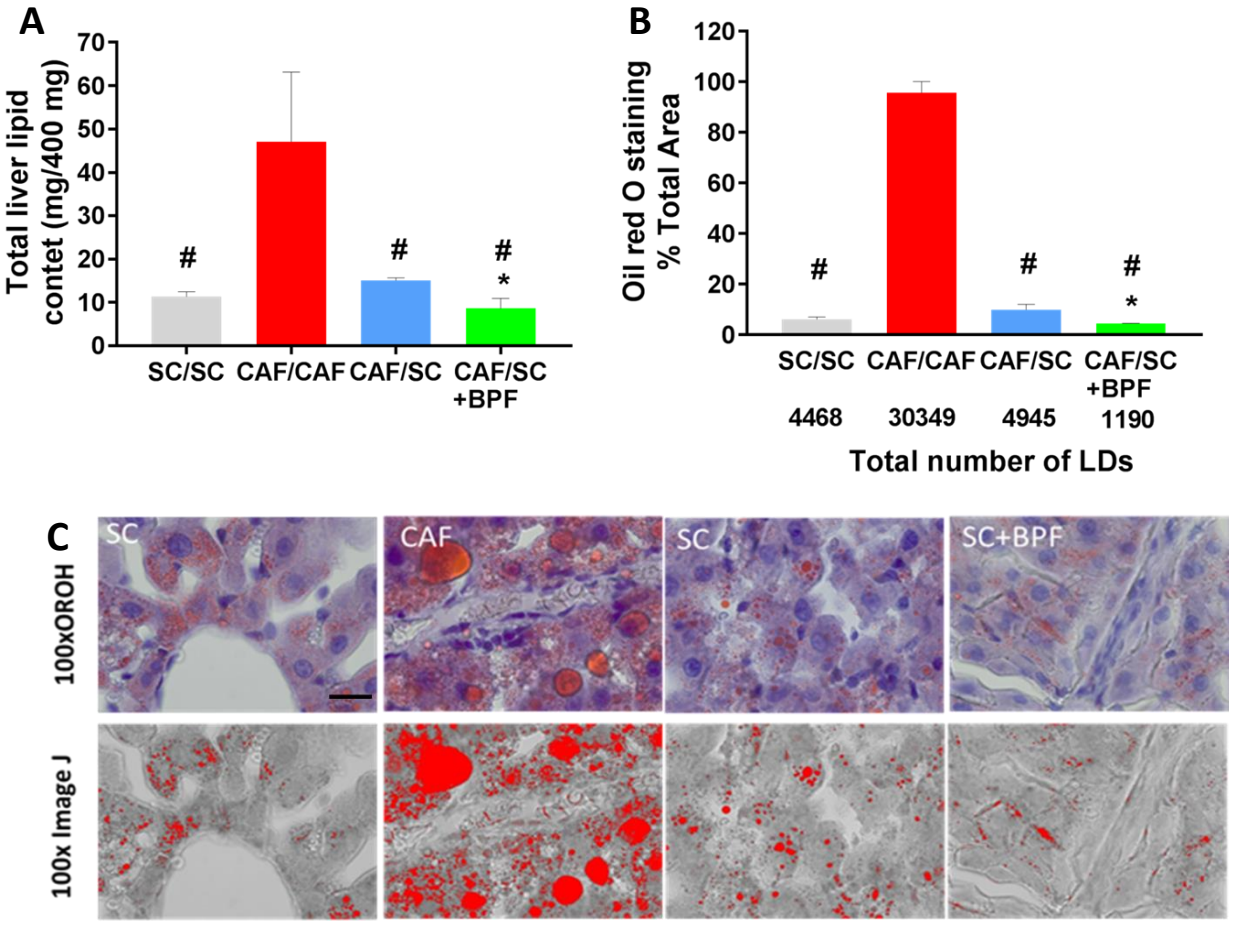

Figure 5. BPF supplementation to SC diet causes stronger reduction of total liver lipid content, size and numbers of LDs compared to SC diet alone for 10 weeks. (**A**) Total lipids were extracted from 400 mg of liver tissue by Folch method and gravimetrically determined. (**B**) Quantification of the total area occupied by LDs in ORO-stained sections revealed a significant increase in CAF/CAF vs. other groups (# $p \leq 0.02$), while BPF reduced the extent of intrahepatic fat accumulation, when compared to normocaloric diet (* $p \leq 0.02$ vs. CAF/SC). Each bar represents the median of four animals ± SD. (**C**) Binary transformation of ORO staining using ImageJ. 100x, objective magnification; scale bar = 20 µm.

Moreover, different sections of livers from each diet group were also evaluated using toluidine-blue staining. Microscopic examination of toluidine-stained semi-thin sections of resin-embedded livers from CAF/CAF, CAF/SC and CAF/SC+BPF rats revealed important changes in CAF/SC and CAF/SC+BPF liver histology with respect to CAF/CAF group (Figure 6 and Figure S1). CAF/SC and CAF/SC+BPF liver parenchyma appeared homogeneous with a regular distribution of hepatocytes. The hepatocytes showed uniform size with large rounded nuclei usually located in the center of the cells and cytoplasmic glycogen granules, but no LDs. In contrast, CAF/CAF livers presented large areas of steatotic tissue with a completely disorganized parenchyma lacking the natural arrangement of hepatocytes. The cytoplasm of hepatocytes appeared highly vacuolated and with a large amount of glycogen granules and LDs. Ballooning hepatocytes, considered a histological hallmark of NASH [39], were also found, more frequently at the portal region (Figure 6A and Figure S1).

Moreover, the sinusoid organization was irregular in CAF/CAF livers compared to CAF/SC and CAF/SC+BPF livers. Within the lumen of sinusoids, large populations of Kupffer cells were observed and some more rounded lymphocytes. Importantly, Kupffer cells and lymphocytes were also present in livers recovering from NASH, but fewer inflammatory cells were observed in CAF/SC+BPF group when compared to SC group (Figure 6B and Figure S1). Quantification of lobular inflammatory foci, by NAS scoring (Figure 6A,B), further confirmed that BPF supplementation has important anti-

inflammatory effects. To detect fibrosis, we evaluated reticular fibers, which are thick and form bundles in connective tissue. Reticular fibers in normal liver tissue are thin and less abundant. They are usually not visible in histological sections stained with hematoxylin/eosin or toluidine blue, but can be demonstrated by using silver impregnation (SI). SI staining revealed many regions of thick fibers networks, mainly in the proximity of blood vessels in CAF/CAF liver samples. On the contrary, fewer and only thin reticular fibers were present in CAF/SC and CAF/SC BPF livers.

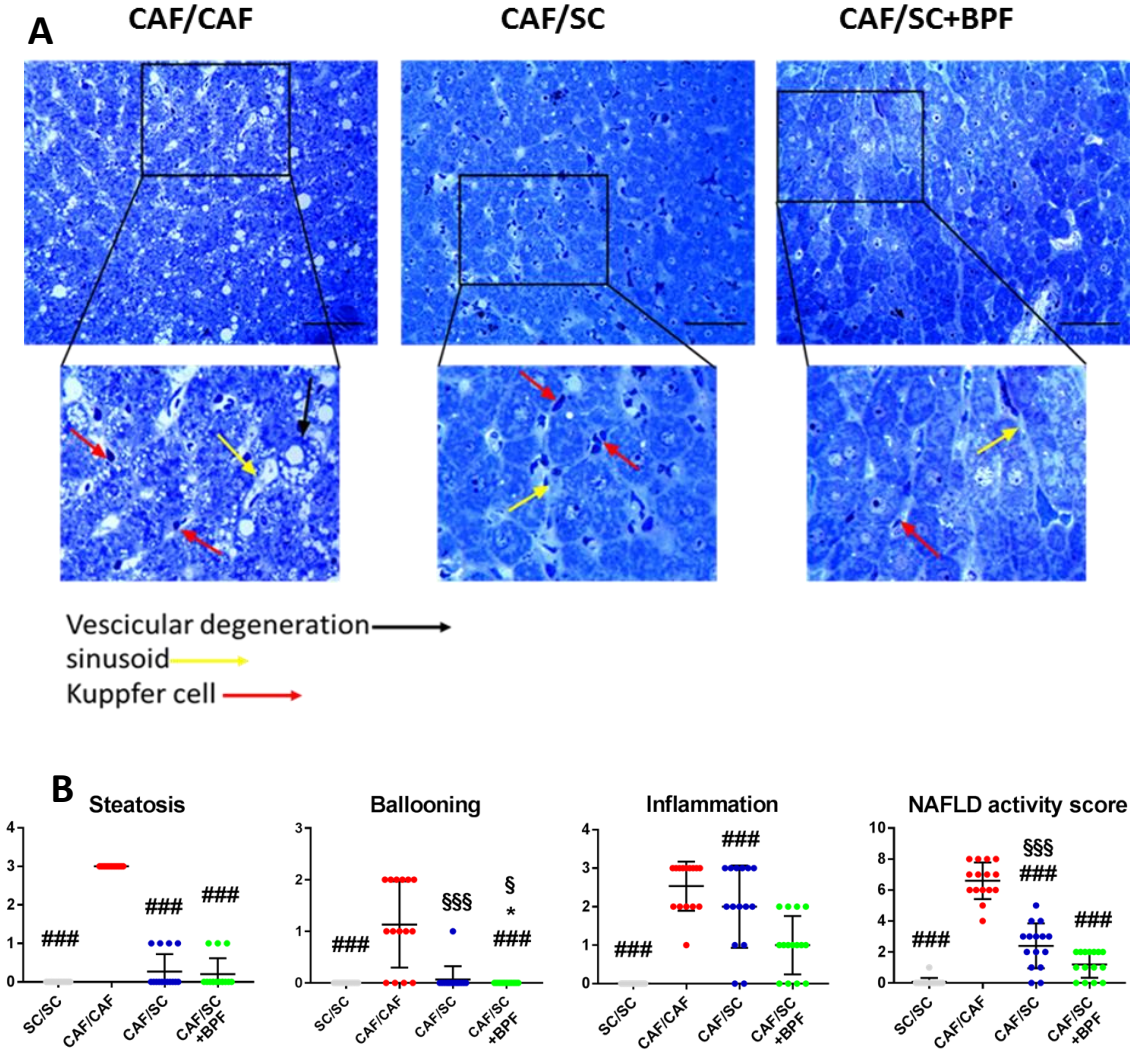

Figure 6. Histological evaluation of NASH. (**A**) Toluidine-blue staining of representative liver sections from three different treatment groups CAF/CAF (16 + 10 weeks), CAF/SC, and CAF/SC+BPF diet. Scale bar 50 µm. (**B**) Quantification of NAFLD Activity Score (NAS) parameters such as steatosis, ballooning and inflammation. Data are represented as means ± SD (n = 15 (five animals and three independent sections for each animal)). * $p \leq 0.01$ denotes differences statistically significant CAF/SC vs. CAF/SC+BPF; ### $p \leq 0.0007$ CAF/CAF vs. SC/SC, CAF/SC, CAF/SC+BPF; § $p \leq 0.01$ SC/SC vs. CAF/SC+BPF; §§§ $p \leq 0.01$ SC/SC vs. CAF/SC. For statistical analysis, Kruskal-Wallis test was performed.

3.3. Analysis of Plasmatic Levels of Insulin and Leptin in SC/SC, CAF/CAF, CAF/SC and CAF/SC+BPF Diet Fed Rats

The dysregulation of lipid metabolism in NAFLD can have devastating consequences on glucose homeostasis and lead to insulin resistance (IR) and type 2 diabetes. On the other hand, IR plays a primary role in triggering a series of reactions that lead to hepatic steatosis [40].

To test whether BPF supplementation in association with healthy diet could improve insulin sensitivity, we analyzed plasma levels of insulin in the four experimental groups. As expected, CAF/CAF rats showed much higher insulin levels than SC/SC rats (Figure 7A), suggesting IR.

Importantly, the fasting plasma insulin concentrations of the rats fed with CAF/SC diet supplemented with BPF were significantly lower than those of the rats fed with CAF/SC diet (Figure 7A). In addition, the HOMA-IR index was also significantly lower in CAF/SC+BPF group when compared with CAF/SC group (Figure 7B). HOMA-IR index has been shown to have a strong and direct correlation with the insulin tolerance tests in Wistar rats, and can be used as a surrogate marker of IR in rats [35,41,42]. Thus, BPF supplementation significantly improved the effect of the diet on HOMA-IR values.

The IR is often strongly associated with leptin-resistance in obese subjects [26]. Leptin is a protein hormone secreted by adipose cells that plays a role in helping the body to balance food intake with energy expenditure. The serum leptin levels were significantly elevated in rats fed with CAF/CAF diet compared with those fed with the SC/SC diet; however, CAF/SC diet, along BPF supplementation, did not cause any further significant decrease in serum leptin levels (Figure 7C).

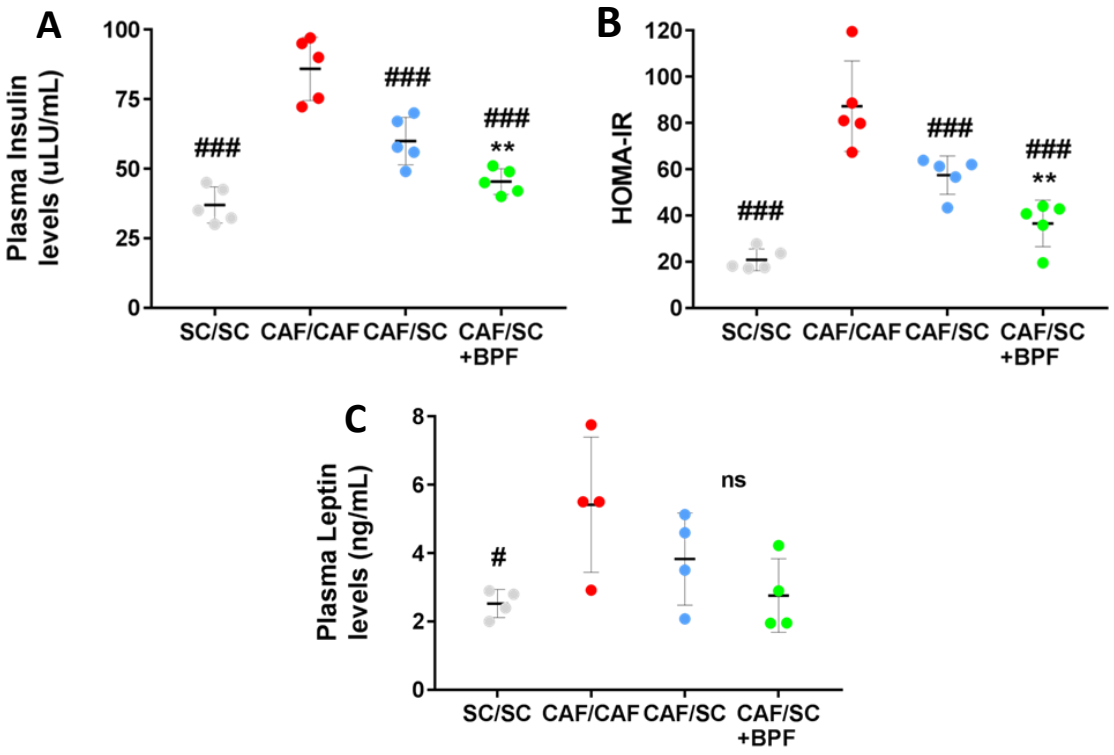

Figure 7. Fasting plasma levels of insulin and leptin and insulin resistance after treatment with SC diet ± BPF for 10 weeks. (**A**) Insulin; and (**C**) leptin were assessed by ELISA in blood samples collected at the end of treatments after 4–5 h fasting. Insulin and leptin levels are elevated in CAF-fed rats when compared to SC/SC ((### $p \leq 0.008$ and # $p \leq 0.02$, insulin and leptin, respectively, CAF/CAF vs. SC/SC). (**B**) Insulin sensitivity was measured by HOMA-IR index (** $p \leq 0.01$ CAF/SC+BPF vs. CAF/SC). (**A**–**C**) Each horizontal line and vertical bar represent the median ± SD, respectively, of n = 4–5 rats.

3.4. Anti-Inflammatory Effect of BPF in CAF/SC Diet Treated Livers

Following histological analysis, we evaluated liver injury parameters such as ALT, AST and LDH. However, ALT and AST values were not significantly altered in CAF/CAF group with respect to SC/SC group (Supplementary Materials, Figure S2A,B), suggesting that these biochemical tests do not correlate well with liver inflammation in our rat NASH model. Conversely, we found that measuring LDH level might be helpful, as it is significantly elevated in CAF/CAF group compared to all other groups. In addition, we found that BPF supplementation ameliorated the LDH values in the diet intervention groups (Supplementary Materials, Figure S2C).

Finally, we addressed if inflammatory parameters could be improved by SC diet and BPF supplementation. RT-qPCR analysis indicated that, although the CAF/SC diet did not affect gene expression levels of pro-inflammatory cytokines 1β, *Il-6* and *Tnfa* (Figure 8A,C,D), BPF treatment resulted in a significant reduction in the mRNA levels of *Il-6* (Figure 8A).

Furthermore, the mRNA levels of anti-inflammatory cytokines *Il-10* were significantly elevated in rats fed with the CAF/SC compared with those fed with the CAF/CAF diet and BPF supplementation caused a further increase of mRNA levels of *Il-10* (Figure 8B) suggesting that BPF supplementation exerts anti-inflammatory effects on steatohepatitis.

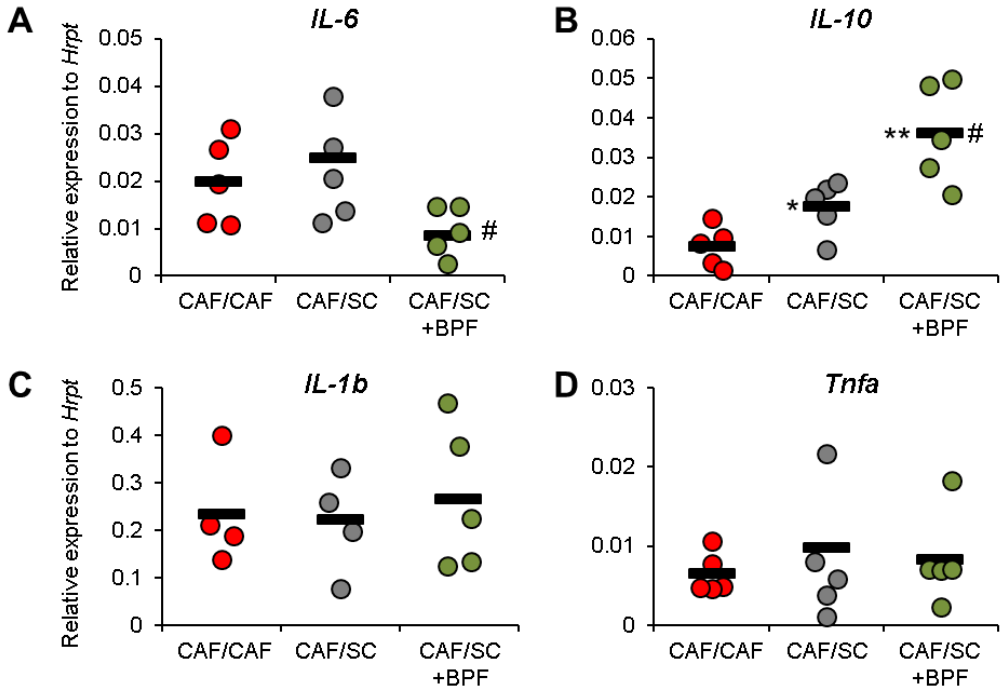

Figure 8. Effect on pro-inflammatory cytokines and anti-inflammatory *Il-10* upon BPF supplementation to CAF/SC diet in rat livers after the intervention phase (10 weeks). RT-qPCR analysis of mRNA levels of inflammatory cytokines *Il1b*, *Il6*, and *Tnfa* and anti-inflammatory cytokine *Il10*. The graphs show relative gene expression versus HK gene *Hrpt*. Each dot represents the average relative expression for one rat analyzed at least in triplicate by standard methods, and lines are the mean values of presented dots. Statistical analysis was performed by unpaired, two-tailed *T* test. *, #, Statistically significant difference at $p < 0.05$ when compared with CAF/CAF rats or when compared with CAF/SC rats, respectively. **, Statistically significant difference as above at $p < 0.01$.

4. Discussion

Our previous work showed that the supplementation of hypercaloric diet with an extract of natural *Citrus* polyphenols from bergamot (BPF) prevents NAFLD through the stimulation of autophagy in the liver [12], which was further confirmed by in vitro studies [19,20]. The main aim of the present study was to examine the effect of BPF supplementation to normocaloric diet on CAF diet-induced advanced NAFLD in therapeutic regime. Most of the studies on animal models of

NAFLD or NASH, including our previous work, address the preventive effect of a treatment [43–47]. Conversely, in this paper, we used a therapeutic approach which is more appropriate for translational interpretation of the results, since it has been designed as a typical clinical study on sick patients, in which a dietary intervention is associated or not with a pharmacological or nutraceutical treatment [4,5,15].

As expected, 10-week SC diet intervention phase caused weight loss (~15%) associated with a dramatic decrease of total liver lipid content (~70%) and ORO staining (~85%). Furthermore, we observed a powerful effect of SC diet on total liver lipid content, number and size of LDs. Indeed, in our model, abundant and large LDs accumulated in the cytoplasm of hepatocytes of rats fed with CAF diet, occupying an area about 10-fold greater than that in SC-fed group. Upon administration of normocaloric SC diet, the LDs total area as well as LDs total number were significantly reduced (Figure 5A–C). Importantly, the concomitant administration of BPF during the intervention phase significantly restrained liver fat content and number and size of LDs with respect to SC group.

In line with this observation, BPF had important effects on blood TGL, leading to a significant reduction of TGL compared to CAF/SC group, after only four weeks of BPF supplementation to SC diet. On the other hand, BPF effects on blood glucose were modest with respect to CAF/SC group even after 10 weeks, although they could likely become statistically significant, if the tested groups were bigger.

Similarly, BPF did not affect body weight, but we cannot exclude a weak effect demonstrable by increasing the number of tested rats. Human NAFLD is very sensitive to the diet and an intensive lifestyle intervention focused on diet with a goal of 7–10% weight reduction leads to significant improvement in liver histology in patients with NASH [33,48]. Weight loss improves steatosis, reduces hepatic inflammation and hepatocellular injury, including fibrosis, suggesting that body weight and liver fat accumulation are strictly correlated [1]. However, our data indicate that liver and body fat might be regulated by distinct mechanisms. It seems that liver fat accumulation is sensitive to pleiotropic effects of flavonoids, while body fat is less affected. Our observations are in line with a recent study, that shows a potent effect of Polyphenol-Rich Rutgers Scarlet Lettuce extract on NAFLD induced with high-fat diet and treated with low-fat diet and no effect on body weight [49]. In humans, isocaloric diet modifications also improve NAFLD, but has little effect on body weight [14]. In contrast, most of the studies in the literature performed using different polyphenol-rich extracts as a preventive measure in animal models of NAFLD, show concomitant reduction of body weight gain and liver lipid content [43,47,50–52]. This difference arises probably from the fact that other nutraceutical approaches are less liver-specific compared to BPF and they are assayed in NAFLD prevention studies.

Our data regarding leptin modulation support this hypothesis. In fact, BPF improved insulin sensitivity compared to SC diet treatment alone, but had no additional effect on leptin levels (Figure 7A,C). CAF rats as well as being obese showed higher plasma levels of insulin and leptin. Leptin is an adipokine that is primarily secreted from adipose tissue and has a critical role in the regulation of body weight and fat mass. Circulating leptin is strongly associated with both subcutaneous and visceral fat, and different studies demonstrate that obesity might induce a state of leptin resistance, when, despite high plasma levels of leptin, the biological activity of this hormone is very low. Several studies indicate that leptin expression is stimulated by insulin [53,54], whereas other studies suggest that high leptin concentrations may contribute to IR [2,55]. Importantly, we found that, although BPF supplementation improved insulin resistance determining a significant decrease in insulin levels and HOMA-IR index, it had no significant effects on body fat and leptin levels. These results suggest a specific action of BPF on liver fat accumulation and glucose metabolism rather than on body weight loss.

The most important finding of this paper is that BPF shows a significant anti-inflammatory effect on NASH livers, with respect to 10-week SC diet treatment, suggesting that SC diet alone has limited effects on inflammation at this timepoint, while BPF supplementation accelerates recovery during the intervention phase. CAF-treated livers were characterized by the presence of ballooning hepatocytes and increased numbers of Kupffer cells and some lobular inflammatory loci. This correlated with

increased expression of all pro-inflammatory cytokines in CAF livers, with respect to SC livers, that could be detected already after 15 weeks of CAF-induction phase. In particular, we observed strongly increased levels of *Tnfα* and *Il6* mRNAs and moderately increased *Il1b* gene expression (Figure 2C), which are the main pro-inflammatory cytokines typically elevated in NASH. This was associated with a moderate increase of anti-inflammatory *Il10* mRNA, likely as a compensatory mechanism. Interestingly, 10-week SC diet intervention did not lead to significant changes in pro-inflammatory cytokine gene expression in CAF/SC livers (Figure 8), even though we found much less steatosis and improved NAS with respect to CAF/CAF livers. This may be related to still ongoing remodeling and recovery process in CAF/SC livers at this time point. However, low gene expression levels as well as an intrinsic expression variability of cytokines in liver samples, makes it difficult to detect subtle changes. This might be true for *Tnfa* and *Il6*, but not for *Il1b*, which has relatively high hepatic expression and we detected no significant differences between groups. However, we could show that BPF flavonoids induced suppression of pro-inflammatory *Il6* and potently boosted the gene expression of anti-inflammatory *Il10*, which was moderately up-regulated also in CAF/SC livers (Figure 8). In addition, we did not observe detectable changes in other cytokines in CAF/SC+BPF livers with respect to CAF/CAF and CAF/SC livers. This suggests that, during diet-induced recovery from NASH, some inflammatory features persist, while BPF reduces inflammation by acting on *Il6* and *Il10*. In fact, we found increased numbers of immune effector cells, mainly Kupffer and some lymphocytes, infiltrating CAF/SC livers while much fewer immune cells in CAF/SC+BPF at week 10 of dietary intervention supplemented with bergamot polyphenols. To our knowledge, this is the first time that some persistent inflammation is described in animal models of NASH after dietary intervention, despite a prominent reduction in LDs. This may be explained by still ongoing phagocytosis of apoptotic cells and debris mediated by Kupffer cells, accompanying hepatic tissue regeneration and healing. Considering that CAF diet livers were badly compromised by 16-week CAF induction phase, it is likely that 10-week SC-intervention phase is not sufficient to obtain a full regression of NASH. We propose that this recovery process is strongly accelerated by antioxidant and anti-inflammatory supplements such as BPF.

The anti-inflammatory activity of *Citrus* flavonoids is well-documented in the scientific literature and it has been studied both for flavanone and flavone glycosides as well as for their aglycones [56–58]. This is true for naringin and hesperidin [27], which are abundant in BPF as well as for diosmetin, apigenin, and luteolin glycosides, which are less abundant components of BPF [19,20]. The anti-inflammatory properties of neoeriocitrin, the most abundant flavanone-7-O-neohesperidoside in bergamot fruits and BPF, have not been formally demonstrated, because it has been poorly investigated being a rare flavonoid in other *Citrus* plants [18,28,30]. However, it been shown that its aglycone, eriodictyol, restrained the elevation of plasma IL-6 and C-reactive protein (hs-CRP) in mice fed high fat diet, suggesting that other eriodictyol glycosides, such as neoeriocitrin, are also active [59]. In fact, glycosides undergo deglycosylation by gut bacteria and usually are adsorbed by enterocytes as aglycones or their metabolites [18,27,60], therefore the in vivo studies on flavonoid aglycones and glycosides should generally yield overlapping results. In fact, the intestinal absorption of naringenin glycosides is comparable to the aglycone [60]. However, their bioavailability depends on the mode of administration and it is much higher, over 30% for naringenin-O-glucoside, if it is given to rats as a food supplement [60], as in our study. Considering high amount and relatively good bioavailability of naringenin, hesperetin and eriodictyol glycosides [61], it is likely that the main BPF flavanones are responsible for majority of immunomodulatory activity present in BPF. Future comparative studies addressing the anti-inflammatory efficacy of individual bergamot flavonoids should answer this question. Nevertheless, by analogy to the study on proautophagic activity of *Citrus* flavonoids [19,20], we expect that the combinatorial effect of the BPF phytocomplex may be quite different and likely superior to the effects of individual polyphenols.

The second goal of our study was to characterize rat livers after longer NAFLD/NASH induction with CAF diet. In our hands, Wistar rats feeding with CAF diet for 26 weeks induced, beside inflammation, different other NASH features, such as ballooning and portal fibrosis, documented here by SI staining of liver sections (Figure S3). This approach can be successfully used to visualize

areas of liver fibrosis [62,63], as it detects collagen III and B fibers, which are highly increased in liver fibrotic tissue [36]. Thus, our data suggest that CAF diet is fibrogenic, even in Wistar rats, typically resistant to fibrosis in response to high-fat diet [64]. The presence or absence of fibrosis in response to CAF diet likely depends on the animal strain. In fact, CAF diet induced portal fibrosis in livers and severe fibrotic changes in heart and kidneys of BALB/c mice treated for 15 weeks [65], while fibrosis can be easily induced in certain mouse and rat strains even if the animals are treated with less effective high-fat diet [37]. To our knowledge, this the first report documenting the presence of liver fibrosis in CAF-diet treated rats.

5. Conclusions

For a long time, NAFLD has been underestimated as a condition of poor clinical relevance. However, the rising incidence of NASH in the Western world, also among children, suggests an urgent need to develop strategies of effective pharmacological or nutraceutical treatment.

In light of the promising evidence presented in this paper, the supplementation of BPF to normocaloric diet may represent a useful anti-inflammatory approach to accelerate patients recovery from advanced NAFLD/NASH.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Toluidine-blue staining of representative liver sections from 3 different treatment groups: CAF/CAF (16 + 10 weeks), CAF/SC, and CAF/SC+BPF, Figure S2: Evaluation of serum biochemical parameters of liver injury, Figure S3: Histological analysis of liver fibrosis in Wistar rats exposed to CAF diet for 26 weeks (CAF/CAF) or exposed to CAF diet for 16 weeks and then to SC or SC+BPF diet for 10 weeks, Table S1: List of the food items used to assemble Cafeteria (CAF) diets.

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Full Paper VIII

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Data Article

Qualitative and quantitative analysis of the proautophagic activity of Citrus flavonoids from Bergamot Polyphenol Fraction

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ABSTRACT

Bergamot Polyphenol Fraction (BPF®) is a natural mixture of *Citrus* flavonoids extracted from processed bergamot fruits. It has been shown to counteract cardiovascular risk factors and to prevent liver steatosis in rats and patients. Hepatic effects of BPF correlate with its ability to stimulate liver autophagy. Six aglyconic flavonoids have been identified in the proautophagic fraction of the hydrolysis product of BPF (A-BPF): naringenin, hesperetin, eridictyol, diosmetin, apigenin and luteolin. We report here the output parameters of high resolution mass spectrometry analysis of these flavonoids and chemical structures of their parent compounds. The second set of data shows the proautophagic activity of BPF flavonoids in a hepatic cell line HepG2 analyzed by a flow cytometry approach. The method is based on the red to green fluorescence intensity ratio analysis of DsRed -LC3- GFP, which is stably expressed in HepG2 cells. Proportional analysis of ATG indexes allowed us to address a relative contribution of individual compounds to the proautophagic activity of the A-BPF mixture and evaluate if the effect was additive. Qualitative analysis of ATG indexes compared the effects of flavonoids at equal concentrations in the presence and absence of palmitic acid and chloroquine. The

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Excel files reporting the analysis of flow cytometry data are available in the public repository.

Specifications Table

Subject area	Pharmacology and cell biology
More specific subject area	Flavonoid pharmacology and autophagy
Type of data	Tables, text file, graphs, dot-plots
How data was acquired	liquid chromatography- high resolution mass spectrometry (LC-HRMS) Q-ExactiveTM (Thermo Scientific) and flow cytometry (FACS Canto II, BD Biosciences)
Data format	Raw and analyzed
Experimental factors	Flavonoid aglycons and their mixtures, treatments for 6 h, chloroquine 2 h, palmitic acid added for 22 h and then withdrawn
Experimental features	GR-LC3-HepG2 cells (HepG2 cells expressing DsRed -LC3- GFP)
Data source location	Campus Germaneto, Catanzaro, Italy
Related research article	Lascala et al. *Analysis of proautophagic activities of Citrus flavonoids in liver cells reveals the superiority of a natural polyphenol mixture over pure flavones* [1]. https://doi.org/10.1016/j.jnutbio.2018.04.005

Value of the Data

- We provide output LC-HRMS parameters for naringenin, hesperetin, eriodictyol, diosmetin, apigenin and luteolin and the list of parent flavonoid glycosides found in BPF.
- A fast flow cytometry method to analyze autophagy is illustrated in a detailed manner and supported by row data, so it can be easily reproduced by other researchers.
- We describe here a “proportional” approach to the analysis of the proautophagic activities in a mix of compounds that can be applied to other mixtures.
- The autophagy index (ATG index) data for six typical flavonoid aglycones reported here, can be used as a reference for other cell lines and compounds.

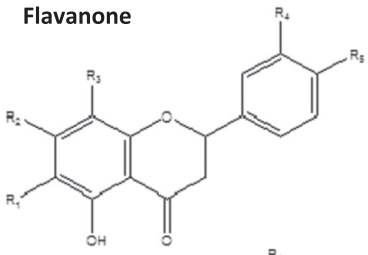
1. Data

Bergamot Polyphenol Fraction (BPF®) is a natural mixture of *Citrus* flavonoids extracted from processed bergamot fruits [2,3]. It has been shown to counteract cardiovascular risk factors and to prevent liver steatosis in rats and patients [4–10]. Protective effects of BPF correlate with its ability to stimulate autophagy in livers of rats fed cafeteria diet [9]. The proautophagic activity of BPF is mediated by a hydrophobic fraction of hydrolysed BPF (A-BPF) containing mainly flavonoid aglycones [1]. Table 1 reports the LC-HRMS output parameters for six major flavonoid aglycones identified in A-BPF, such as retention times (RT), theoretical and measured mass to charge ratio (m/z), signal intensity as well as a calculated relative and absolute abundance of each flavonoid in the mix (see material and methods). The aglycones listed in Table 1 (column A) originate from several known and unknown parent compounds (i.e. flavonoid glycosides), shown in Fig. 1, that have been previously identified among bergamot polyphenols [3].

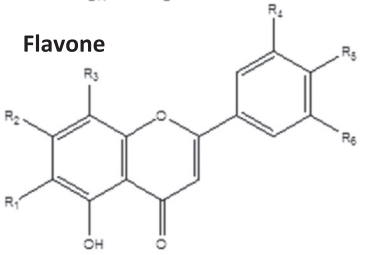
The exposure of hepatocytes to palmitic acid (PA) causes an accumulation of intracellular lipid droplets and models non-alcoholic fatty liver disease (NAFLD) *in vitro* [1]. We used this approach to

Table 1
Output parameters of LC-HRMS analysis of flavonoid aglycones identified in A-BPF and their expected parent compounds. Column G shows a theoretical quantitative representation of aglycones in A-BPF, while the H column the amount (in μg) of aglycones in 60 μg of A-BPF, as calculated based on data in G.

A Name	B RT (MIN)	C Molecular formula	D m/z $[M-H]^-$ (measured)	E *m/z* $[M-H]^-$ (theoretical)	F Signal intensity (NL) x E6	G % Total signal	H in 60 μg	I Parent compounds
ERIODICTYOL	21.09	$C_{15}H_{12}O_6$	287.0564	287.0561	34.6	20.51	12.3	NEOERIOCITRIN, ERIODICTYOL-7-O-NEOHESPERIDOSIDE-6″-O-HMG (PERIPOLINA)
NARINGENIN	23.61	$C_{15}H_{12}O_5$	261.0615	261.0612	55.9	33.4	19.9	MELITIDIN, NARINGIN
HESPERETIN	24.38	$C_{16}H_{14}O_6$	301.0722	301.0718	51.8	30.7	18.4	BRUTERIDIN, HESPERETIN-7-O-GLUCOSIDE, NEOHESPERIDIN
LUTEOLIN	24.46	$C_{15}H_{10}O_6$	285.0408	285.0405	3.99	2.37	1.4	LUTEOLIN-7-O-NEOHESPERIDOSIDE
APIGENIN	26.47	$C_{15}H_{10}O_5$	269.0459	269.0455	7.01	4.16	2.5	RHOIFOLIN, APIGENIN-7-O-NEO-HESPERIDIOSIDE-6″-O-HMG
DIOSMETIN	26.70	$C_{16}H_{12}O_6$	299.0564	299.0561	15.4	9.13	5.5	DIOSMIN, NEODIOSMIN, DIOS-METIN-7-O-GLUCOSIDE, DIOS-METIN-7-O-NEOHESPERIDO-SIDE-6″-O-HMG



FLAVANONE	R1	R2	R3	R4	R5
HESPERETIN 7-O-GLUCOSIDE	H	O-GLU	H	OH	OCH_3
NEOHESPERIDIN	H	O-NEO	H	OH	OCH_3
NARINGIN	H	O-NEO	H	H	OH
NEOERIOCITRIN	H	O-NEO	H	OH	OH
PERIPOLIN		O-NEO-HMG	H	OH	OH
BRUTERIDIN	H	O-NEO-HMG	H	OH	OCH_3
MELITIDIN	H	O-NEO-HMG	H	H	OH
HESPERETIN-7-O-GLUCOSIDE-6′-O-HMG	H	O-NEO-HMG	H	OH	OCH_3
NARINGENIN-7-O-GLUCOSIDE-6′-O-HMG	H	O-NEO-HMG	H	H	OH



FLAVONE	R1	R2	R3	R4	R5
DIOSMIN	H	O-GLU	H	OH	OCH_3
DIOSMETIN -7-O-GLUCOSIDE	H	O-GLU	H	OH	OCH_3
LUTEOLIN-7-O-NEOHESPERIDOSIDE	H	O-NEO	H	H	OH
RHOIFOLIN	H	O-NEO	H	OH	OH
NEODIOSMIN	H	O-NEO	H	OH	OH
DIOSMETIN -7-O-NEOHESPERIDOSIDE -6′-O-HMG	H	O-NEO-HMG	H	OH	OCH_3
APIGENIN-7-O-NEOHESPERIDOSIDE -6′-O-HMG	H	O-NEO-HMG	H	H	OH

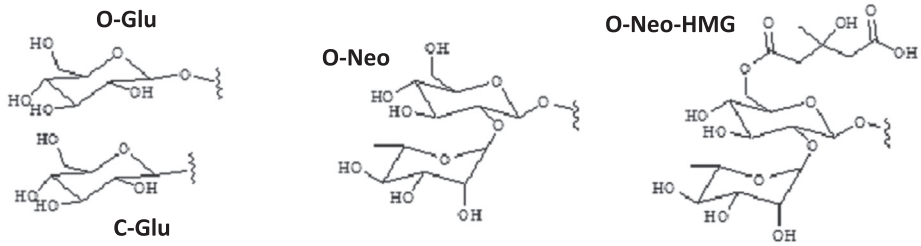


Fig. 1. Structures of parent compounds of 6 main aglycones present in A-BPF and their sugar moieties: O-Glu, O-glucoside; C-glucose, C-glucoside; O-Neo, O-Neohesperidoside; O-Neo-HMG, 3-hydroxy-3-methyl-glutaryl-neohesperidoside.

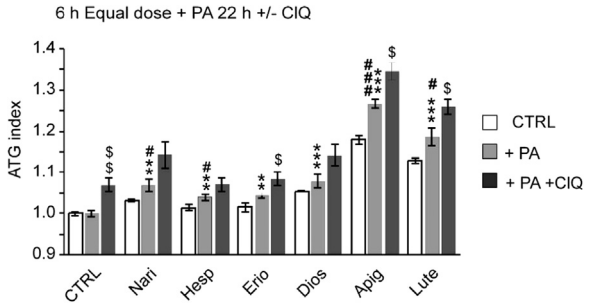
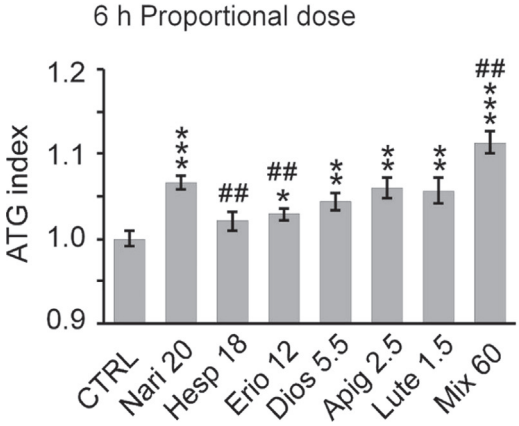


Fig. 2. Proautophagic activities of flavonoid aglycones in hepatic cells with high lipid content. ClQ co-treatments further increase ATG index. (A) GR-LC3-HepG2 cells were treated with PA 0,3 mM or vehicle (ethanol, EtOH) for 22 h. Subsequently, the medium was exchanged and aglycones (30 μg/mL) were added for 6 h. 2 h before analysis ClQ (50 μM) or vehicle (H_2O) was added to PA-treated cells. Red and green fluorescence was recorded by flow cytometry. The graph shows the mean ATG index +/− SEM, from four independent experiments performed each time with triplicate independent samples. Naringenin (Nari), hesperetin (Hesp) and eriodictyol (Erio), followed by diosmetin(Dios), apigenin (Apig) and luteolin (Lute). Red/green ratio data are normalized to controls. Statistical analysis: student *T*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, when compared to vehicle-treated controls; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ when compared to the relative PA-untreated, the same flavonoid-treated cells or $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, when compared to respective PA-treated, ClQ-untreated cells. See S1 data set (Supplementary data set S1) with row data supporting this figure.

A



B

NAME	ATG Index ± SEM	% ATG Index increase
Control (vehicle)	1000 ± 0,004	0
NARINGENIN	1,066 ± 0,008	6,6
HESPERETIN	1,021 ±0,011	2,1
ERIODICTYOL	1,03 ±0,007	3,0
DIOSMETIN	1,044 ±0,010	4,4
APIGENIN	1,060 ±0,013	6,0
LUTEOLIN	1,057 ±0,015	5,7
Total increase		27,8 ±6,4
Mix60	1,113 ±0,013	11,3±1,3

Fig. 3. Proportional analysis of proautophagic activities of aglycones present in A-BPF. (A) GR-LC3-HepG2 cells were treated for 6 h with the doses of pure aglycones corresponding to those present in 60 μg A-BPF (reported as numbers of μg/mL after compound abbreviation) or with the mix of these compounds (Mix60) and analysed for ATG index in six independent samples +/− SEM. Statistical analysis: two-tailed, unpaired T-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to control (CTRL), vehicle-treated cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ when compared to naringenin. (B) Table showing the analysis of additive effects of six flavonoid aglycones on ATG index. See Supplementary data set S2 (Multimedia Component 3) with row data supporting this figure.

measure the proautophagic activity of six main flavonoid aglycones present in A-BPF in the presence and absence of lipotoxic stress (Fig. 2). This was done by the qualitative analysis, i.e. equal concentrations of aglycones were used to induce autophagy in HepG2 cells expressing DsRed -LC3- GFP, which turns red when autophagy is induced or LC3-II accumulates [1,11]. GR-LC3-HepG2 cells were treated with PA (0.3 mM) to cause intracellular lipid overload, and 22 h later exposed to flavonoid aglycons +/− chloroquine (ClQ) to address the autophagic flux modulation. These data were then compared with ATG index (red/green ratio) induced in the absence of PA by six polyphenols in independent experiments performed otherwise under identical conditions (Fig. 2 and Supplementary data set S1).

Next, we addressed the quantitative contribution of each aglycone present in A-BPF to the proautophagic activity of A-BPF, which we defined here as “proportional analysis” of proautophagic activity as opposed to “qualitative analysis”, shown in Fig. 2, where equal doses of compounds are compared for their activity. We calculated the *proportional* amounts of six aglycones, as described in Experimental design. Then we treated GR-LC3-HepG2 cells with calculated amounts of standards and analyzed autophagy by flow cytometry 6 h post-treatment. In the same experiment, we also measured the ATG index induced by the mix of all six compounds, used at proportional concentrations to reconstitute 60 μg/mL of A-BPF (Mix60) (Table 1). The ATG index data for six tested aglycones and their Mix are reported in Fig. 3A. The data in Fig. 3A are supported by Supplementary data set S2 presenting the row data used for this analysis. The Excel file S2 contains tables with mean fluorescence intensities values recorded in 54 independent cell samples in two independent experiments. This Excel file also shows how the mean ATG index is calculated and normalized and performs statistical analysis of the data. Examples of flow cytometry dot-plots with raw data and relevant gates used for analysis are also attached to Supplemetary data set S2.

Next, we tried to evaluate if six polyphenols contributed in additive or synergistic fashion to the overall autophagic activity of BPF. The data in Fig. 3B are a numeric representation of data presented in Fig. 3A and they show that the sum of mean ATG index increases caused by individual compounds is higher than the ATG index induced by the Mix of aglycones. These data would rather suggest additive as well competitive effects, rather than synergistic effects of tested flavonoids on autophagy.

2. Experimental design, materials and methods

2.1. Cell culture

GR-LC3-HepG2 cells were descried previously in Lascala et al. [1]. They were cultured in DMEM complete medium (4.5 g/L glucose) as described for the HepG2 cells [1]. For experiments, the cells were seeded at the density 4×10^4 cm^{-2} and the last medium change was performed 24 h before the end of the experiment.

2.2. Reagents

Naringenin, hesperetin, eriodictyol, diosmetin, apigenin and luteolin were purchased from Extrasynthese (Geney Cedex, France) as $\geq$ 99% pure (HPLC-grade) powders, which were solubilized in EtOH 100% to stock solutions 5 mg/mL, except for diosmetin and apigenin diluted as 2 mg/mL stock due to their low solubility. BPF® was provided by *Herbal and Antioxidant Derivatives* srl (H&AD srl), Bianco, Italy. A-BPF was prepared by acid hydrolysis of BPF and isolation of hydrophobic phases from crude hydrolysate, according to the procedure described in Lascala et al. [1] "Mix60", or "Mix", was prepared by mixing ethanol solutions of six aglycones (as above) in natural proportions as found in A-BPF, according to the data presented in Table 1, column G and H. ChlQ (25 mM stock in PBS), PA (0.1 to 1 M stock in EtOH) were from Sigma-Aldrich. These compounds were kept in aliquots at −80 °C or −20 °C (shorter storage) and thawed shortly before each treatment.

2.3. Production of retroviruses coding for DsRed-LC3-GFP

To produce GR-LC3-HepG2 cells stably expressing DsRed-LC3-GFP reporter, recombinant retroviruses coding were generated and used to infect HepG2 cells. To produce viral stocks, HEK 293 T cells (one 100 mm plate at 90–95% confluence) were transfected with pUMVC (10 μg), pCMV-VSV- G (4 μg) and pQCXI-Puro-DsRed-LC3-GFP (10 μg) from Addgene (Cambridge, MA, USA), using Lipofectamine 2000 (Life Tech., Invitrogen, 11668027) according to manufacturer's instructions. After an overnight incubation, cell medium was replaced with 5 mL of RPMI medium (Life Tech., Invitrogen, 11875093), supplemented with 2% FBS. 48 h post-transfection, cell supernatant was collected, filtered through a 0.45 μm filter membrane, and supplemented with FBS (10% final concentration). Prior to viral transduction, HepG2 cells were seeded at a concentration of 4×10^5/well in six-well plates, and 5 mL of viral supernatant was collected and used to infect cells by spinoculation in 6-well plate sealed with parafilm, at 720 g, $T = 32$ °C, in presence of polybrene (8 μg/mL, Sigma-Aldrich, 107689) for 50 min. After 4 h of incubation at 37 °C, 5% CO_2, cells were washed with PBS and switched to standard medium. At 48 h post-infection, puromycin (2 μg/mL, Sigma-Aldrich, P8833) was added for 8 days. The transduction efficiency was evaluated by FACS analysis as the EGFP-positive cell fraction. To maintain high expression of DsRed-LC3-GFP the cells were cultivated in DMEM complete supplemented with puromycin (1 μg/mL). For experiments cells were plated without puromycin.

2.4. Flow cytometry analysis of autophagy

GR-LC3-HepG2 cells were seeded on 24-well plates and cultured as described in *Cell culture* section above. After 3 days pre-treatments with PA (Sigma-Aldrich, 0,3 mM final, 150 mM stock in EtOH) were performed 22–24 h before medium change and addition of flavonoid aglycones or other substances 6 h before cell harvesting. ClQ (50 μM in H2O) was added 2 h before cell harvesting. For the treatments all wells were treated with the same volume of EtOH (usually 3 μL), DMSO and water, which were used as vehicles. Each treatment series were performed in triplicate, but at different times. 6 h later the cells were washed once in PBS and collected by trypsinization as described before [12]. Briefly, pelleted cells were resuspended in 0.45 mL PBS containing 1% FBS and 0.1 mM EDTA (Sigma-Aldrich, E5134). To exclude dead cells in analysis they were treated with 75 μl of trypan blue (TB) solution (0.008% in PBS), added 60 s before flow cytometry recording. This was not necessary for experiments with low cell mortality (below 5% in all samples). However, the addition of TB did not

influence significantly the ATG index. Cells were acquired in 502 nm (FITC, green), 556 nm (PE, red) and 655 (PerCP-Cy5, blue) channels by FACSCanto II (BD Biosciences, Erenbodgem, Belgium). Populations of interest were identified: single cell population (or P1), viable single cells (or P6) and GFP and Ds-Red/GFP positive population (indicated as Q2). See row data sets S1 and S2. Mean fluorescence intensity (MFI) for red and green channels was determined in the populations of interest by BD FACSDiva software. The autophagy index (ATG index) was calculated as the ratio of red to green channel MFI in triplicate samples for each experimental point for a populations of interest (P6 or Q2). The data were normalized to the mean ATG index of three control samples. For further details see examples of ATG index analysis in Excel files provided as in Supplementary data sets S1 and S2.

2.5. A-BPF preparation and analysis

A-BPF was obtained from BPF® by acid hydrolysis, as reported in Lascala et al. [1]. Subsequently, 2 mg of A-BPF were used to prepare a sample for LC-HRMS analysis according to the procedures described in the companion paper [1]. BPF® is a kind gift of the owner of the BPF trademark, Herbal and Antioxidant Derivatives S.r.l. (H&AD S.r.l.), Bianco (RC), Italy.

2.6. Mass spectrometry

Q-ExactiveTM (Thermo Scientific) mass spectrometer was operated using electrospray with negative polarities at 35,000 resolving power (defined as FWHM at m/z 200), IT 150 ms, and ACG target = 1,000,000, in full scan analysis (mass range 140–900 amu). Source conditions were: spray voltage 2.9 KV, sheath gas: 30, arbitrary units, Auxiliary gas: 10, probe heater temperature: 280 °C; capillary temperature: 320 °C; S-Lens RF Level: 50. The instrument was calibrated by Thermo calibration solutions prior to the beginning the analysis.

2.7. Proportional analysis of activity of individual compounds present A-BPF phytocomplex

To perform a *proportional* analysis, we estimated the amounts of six major flavonoids present in 60 μg/mL of A-BPF based on LC–mass spectrometry data described in Table 1. To this end, we assumed that the ion current signal intensity (SI) is proportional to the relative quantity of each flavonoid, which is well applicable to structurally similar compounds, according to our previous observations [13]. For sake of simplicity, we assumed that the total quantity of six identified polyphenols corresponds to 100% of A-BPF and 100% of total ion current signal intensity (TSI). SI for each flavonoid was divided by TSI and multiplied by 60 μg/mL to calculated the *proportional* amounts flavonoids contributing to A-BPF phytocomplex.

Acknowledgments

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Transparency document. Supporting information

Transparency data associated with this article can be found in the online version at https://doi.org/10.1016/j.dib.2018.05.139.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.dib.2018.05.139.

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