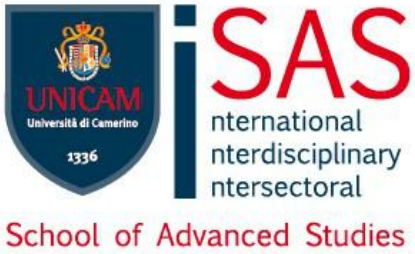

UNIVERSITÀ DEGLI STUDI DI CAMERINO

School of Advanced Studies

DOCTORAL COURSE IN

Chemical and Pharmaceutical Sciences and Biotechnology

XXXVII cycle

Preclinical studies on innovative approaches for targeting diet-related disorders

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Preclinical studies on innovative approaches for targeting diet-related disorders

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ABSTRACT

Lifestyle-associated disorders are nowadays severe concerns for their impact on social and healthcare systems. Over the past decades, the investigation of the multi-faceted molecular mechanisms beneath these disturbances has unveiled the detrimental role played by the low-grade chronic inflammation (metaflammation) and suggested the targeting of its components of as one the promising approaches for defining new strategies for counteracting its delirious consequences. The objective of this doctoral thesis was to explore and assess the involvement of two of these detrimental mediators, namely the Advanced Glycation End Products (AGEs) and casein kinase II (CK2) in the onset of chronic inflammation associated with a 12-week murine models of high-fat high-sugar diet consumption (HFHS). We evinced, for the first time, the innovative antiglycation property of Arthrospira Platensis enriched in zinc (Zn-SP) and its beneficial effects in ameliorating the systemic inflammation and glycaemic abnormalities linked to the HFHS chronic intake. Our findings suggest a new mechanism of action of Zn-SP, responsible of a reduction in AGEs endogenous formation and consequent activation of AGE/RAGE axis alongside to NFκB/NLRP3 cascades, and of boosting AGEs detoxifying defences. On the second set of experiments, we evaluated the effect of pharmacological inhibition of casein kinase II (CK2), a pleiotropic enzyme involved in the onset of different pathological contexts, with 4,5,6,7 tetrabromobenzotriazole (TBB), recording the overactivation of this kinase in the context of diet-related disturbances and the protective effects of its pharmacological inhibition in blunting the simultaneous activation of NFκB/NLRP3 cascades at hepatic level, in parallel to the systemic amelioration of both inflammatory and metabolic profiles. Overall, the experimental results presented in this doctoral thesis showed two different approaches for counteracting metabolic disturbances, strengthening the key role of metaflammation in diet-induced disorders, and propose innovative

compounds, the Zn-SP and the CK2 inhibitor, as fascinating approaches to halt metabolic inflammation.

Keywords: metaflammation, metabolic disorders, Advanced Glycation End Products (AGEs), casein kinase II (CK2).

This doctoral thesis is based on the results reported in the following articles:

1. Aimaretti E*, **Porchietto E***, Mantegazza G, Gargari G, Collotta D, Einaudi G, Alves GF, Marzani E, Algeri A, Dal Bello F, Aragno M, Cifani C, Guglielmetti S, Mastrocola R, Collino M. **"Anti-Glycation Properties of Zinc-Enriched Arthrospira platensis (Spirulina) Contribute to Prevention of Metaflammation in a Diet-Induced Obese Mouse Model".** Nutrients. 2024; 16(4):552. doi.org/10.3390/nu16040552

**These authors contributed equally to the work*

2. Unpublished study unravelling the effect of pharmacological targeting of casein kinase II(CK2) in a murine model of hypercaloric diet consumption (manuscript in preparation/submitted)

List of published articles not included in the doctoral thesis

1. Alves, GF, Aimaretti E, Einaudi G, Mastrocola R, de Oliveira JG, Collotta D, **Porchietto E**, Aragno M, Cifani C, Sordi R, Thiemermann C, Fernandes D, Collino M. **Pharmacological Inhibition of FAK-Pyk2 Pathway Protects Against Organ Damage and Prolongs the Survival of Septic Mice.** Front Immunol. 2022 Feb 1;13:837180. doi: 10.3389/fimmu.2022.837180.

2. Alves GF, Stoppa I, Aimaretti E, Monge C, Mastrocola R, **Porchietto E**, Einaudi G, Collotta D, Bertocchi I, Boggio E, Gigliotti CL, Clemente N, Aragno M, Fernandes D, Cifani C, Thiemermann C, Dianzani C, Dianzani U, Collino M. **ICOS-Fc as innovative immunomodulatory approach to counteract inflammation and organ injury in sepsis**. Front Immunol. 2022 Sep 2;13:992614. doi: 10.3389/fimmu.2022.992614.

3. Alves GF, Aimaretti E, da Silveira Hahmeyer ML, Einaudi G, **Porchietto E**, Rubeo C, Marzani E, Aragno M, da Silva-Santos JE, Cifani C, Fernandes D, CollinoM. "**Pharmacological inhibition of CK2 by silmitasertib mitigates sepsis-induced circulatory collapse, thus improving septic outcomes in mice".** Biomedicine & Pharmacotherapy 178 (2024) 117191, https://doi.org/10.1016/j.biopha.2024.117191.

CONTRIBUTION REPORT

Article 1: investigation, formal analysis, visualisation, validation, methodology, writing- original draft preparation.

Article 2: conceptualisation, formal analysis, investigation, visualisation, validation, methodology, writing - original draft, and writing - review & editing.

ABBREVIATIONS

AGEs Advanced Glycation End Products

ALT Alanine aminotransferase

AST Aspartate aminotransferase

CEL N"-(1-carboxyethyl) lysine

CK2 Casein kinase II

CLS Crown-like-structure

CML N"-(carboxymethyl) lysine

DAB 3,3'-diaminobenzidine

DAMP Damage-associated molecular pattern

DHA Docosahexaenoic acid

DIO Diet-induced obesity

DTT Dithiothreitol

ECM Extracellular matrix components

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme-linked immunosorbent assay

EPA Omega-3 eicosapentaenoic acid

ERK1/2 Extracellular-signal-regulated kinases

FASN Fatty acid nuclear synthase

FBP1 Fructose-1,6-biphosphatase

FDA Food and drug administration

Glo-1 Glyoxalase 1

GSDM-D Gasdermin D

H&E Haematoxylin and eosin

HDACs Class I histone deacetylases

HFHS High-fat-high sugar

HPLC High Performance Liquid Chromatography

IκB IkappaB kinase

INF-γ Interferon gamma

IKKβ Inhibitor κB kinase β

IL-1β Interleukin 1β

IL-6 Interleukin 6

IR Insulin resistance

IRS-1 Insulin receptor Substrate 1

JAK/STAT Janus kinase/signal transducers and activators of transcription

JNK c-Jun-amino-terminal kinase

LPIN1 Phosphatidic acid phosphatase lipin 1

LPS Lipopolysaccharide

MAMPs Metabolism-associated molecular patterns

MCP-1 Monocyte chemoattractant protein 1

MD Mediterranean diet

MGO Methylglyoxal

MPO Myeloperoxidase

mTORC1 Mammalian target of rapamycin complex 1

Nox NADPH oxidase

NCDs Non-communicable diseases

Nfr2 Nuclear factor erythroid 2

NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells

NLRP3 Nucleotide-Binding Domain, Leucine-Rich–Containing Family, Pyrin Domain–Containing-3

oGTT Oral glucose tolerance test

ORO Oil red o staining

PAMP Pathogen-associated molecular pattern

PI3K/AKT Phosphoinositide 3-kinase (PI3K)/protein kinase B

protein kinase B(AKT)

PRRs Pattern recognition receptors

PUFAs Polyunsaturated essential fatty acids

RAGE Receptor for Advanced Glycation End Product

ROS Reactive oxygen species

SDS-PAGE Dodecyl sulphate polyacrylamide gel electrophoresis

SEM Standard error of mean

SMKI Small kinase inhibitor

SREBP1/2 Sterol regulatory element-binding proteins

SSBs Sugar-sweetened beverages

T2D Type 2 diabetes

TBB 4,5,6,7 tetrabromobenzotriazole

TLR4 Toll like receptor 4

TNFR Receptor of TNFα

TNFα Tumor necrosis factor α

vAT Visceral adipose tissue

Zn-SP Zinc enriched Spirulina

INDEX

1. INTRODUCTION

1.1. The role of metaflammation in metabolic disorders

Lifespan increase over the past sixty years has highlighted the critical need for finding effective therapies for counteracting the impact on healthcare system of non-communicable diseases (NCDs) and of their major risk factors [1].Among these latter, the epidemic proportion assumed by life-style associate disorders, such as obesity, hyper-glycaemia and insulinemia represent a relevant clinical and social burden to handle. Nowadays, the higher rate of metabolic conditions is ascribed to the establishment of an unbalanced lifestyle, characterized by increased consumption of refined foods, enriched in fats and simple sugars (termed as Western-type diets), with high caloric intake and glycaemic indexes, paralleled to reduced physical inactivity [1–4]. Indeed, long-term consumption of these products can influence whole body homeostasis and elicit patho-mechanisms that result in the promotion of a systemic low-grade sterile inflammatory status, defined as *metaflammation.* This definition was introduced, for the first time, by Hotamisligil in 2006 as the result of nutritional and energetic overload, that at a subacute level, ends in immune cell's infiltration and consequent overactivation of several inflammatory pathways [5]. Following almost two decades of research, the scientific community has converged upon this intriguing hypothesis and further unravelled the role of inflammation in the pathogenesis of metabolic disorders. For instance, several treatments targeting inflammatory mediators have been proved to be effective in counteracting metaflammation and, hence, encouraging a better understanding of the complex mechanism(s) prompt by unbalanced nutrient intake [6–8].

Chronic immune activation evoked by western diet consumption has been documented to be detrimental in several organs, starting from the adipose tissue, which in response to its homeostatic alteration, generates a dysfunctional response that further exacerbates diet-induced challenges at systemic levels. This abnormal response could be defined as a pathological "trained immunity" which potentiates the harmful effects of the nutrient overload and results in a feed-forward loop that fuel metaflammation [9, 10].

In details, the visceral adipose tissue (vAT) is the major storage site of the nutrient excesses introduced exogenously. vAT is characterised by a high plasticity and heterogeneity, represented by adipocytes themselves, resident immune cells, extracellular matrix components, each of them deeply affected by dietary challenges [11]. For instance, vAT undergoes a structural and phenotypical changes: the positive energetic balance is converted in triglycerides and as a consequence, the tissue encounters a hypertrophic enlargement, ended in the macroscopic feature of obesity, the weight gain. On the other hand, the immune cells' activation elicits a shift in the macrophages polarisation from M2 (anti-inflammatory) to M1 (pro-inflammatory), which are qualitatively and quantitatively detectable as the crown-like-structure (CLS), and besides, resulting in the production and release of pro-inflammatory mediators such as tumor necrosis factor α(TNFα), interleukin 1β (IL-1β) and interleukin (IL-6), that further exacerbates diet-induced derangements, promoting the activation of pro-inflammatory signaling cascades [2, 12].

It has to be pointed out that the immunologic recruitment and activation is specular in liver and skeletal muscle, in which hepatocytes and myocytes are responsible for boosting inflammation throughout the production of detrimental mediators i.e. cytokines and chemokines [12].

The activation of pro-inflammatory signaling cascades is tightly regulated by the recognition of several diet-derived harmful triggers, such as saturated fatty acids (palmitate, stearate, laurate) in metabolic tissues *i.e.* skeletal muscle, liver, adipose tissue[13, 14]. Indeed, these exogenous dietary sources interact with inflammatory receptors, such as toll like receptor 4 (TLR4), whose activation has been widely documented to be altered in obese condition, promoting an abnormal stimulation of TLR4 axis. This sophisticated interaction between lipids component and TLR4 is explained by their mimicking action on TLR4 of lipopolysaccharide (LPS), a key constituent of Gram-negative bacteria cell walls, natural agonist of the receptor [15, 16].

Additional dietary derivatives that dramatically dampen metaflammation are simple sugars. In westernized-diets, their main source as added sugar is

represented by sugar-sweetened beverages (SSBs), that promote weight gain by supplementing additional "liquid calories", with consequent a high rate of glucose absorption. Furthermore, SSBs can be highly rewarding and may trigger addictive-like behaviours, which could lead to their overconsumption [17, 18]. Sugars-driven hyperglycaemia is a fuelling status boosting inflammation and its vicious circle, detrimental to glucose handling capability itself: for instance, it has been proven the triggering effect of high levels of blood glucose as a damage-associated molecular pattern (DAMP) molecule in immune cells and further activation of TLR4 axis [19, 20].

In summary, the endogenous danger signal molecules introduced with westernized diets could be defined as metabolism-associated molecular patterns (MAMPs), boosting inflammation in a pattern recognition receptors (PRRs) mediated-manner and driving the production of proinflammatory mediators [21, 22].

Once the inflammation is prompted by MAMPs, the release from tissues of pro-inflammatory mediators, namely cytokines and chemokines, such as TNFα, IL-1β, monocyte chemoattractant protein 1 (MCP-1), induces a systemic perturbation and a promotes an interorgan crosstalk that affect metabolic processes, *i.e.* insulin resistance (IR), lipid storage and trafficking[23, 24] . IR is a complex and multi-faced condition referring to a decreased responsiveness to insulin, resulted from an impaired interaction of the hormone and its down-stream signaling cascades in metabolic active organs. It is widely recognized how cytokines such as TNFα and IL1β can interfere with insulin cascade, disrupting its functionality and ending in a reduced glucose intracellular uptake [25].

Unveiling the roles of signalling proteins that potentially affect the activation of the insulin cascade, or the functionality of its mediators will be helpful in identifying promising targets for insulin resistance treatment[26]. For instance, it is widely recognized that several kinases, such as c-Jun-amino-terminal kinase (JNK) and inhibitor κB kinase β (IKKβ) are crucial for IRS-1 (Insulin receptor Substrate 1) functionality: both JNK and IKKβ mediated phosphorylation on Ser^{307} uncoupled IRS-1 from the receptor, disrupting insulin cascade [27, 28].

On the other hand, nutrient overload and consequent adipocytes hypertrophy evoked alterations in the fatty acids storage and synthesis, being detrimental to the balanced processes of hepatic lipogenesis/lipolysis, resulting in systemic dyslipidaemia and ectopic lipid deposition. These pathological outcomes are further impaired by sterile inflammation itself that can potentiate the oxidation of low-density lipoproteins and consequent activation of inflammatory cascades [29]

Therefore, in our experimental model of diet- induced obesity, the chronic exposure to a high-fat-high sugar diet (HFHS) mimics in mice the harmful effects of western-diets on human [30].

In the next sections (1.2.1. and 1.2.2.) will be discussed, in a deep-in-focus, the role of two key class of mediators, the advanced glycation end products (AGEs) and kinases, in the pathogenesis of metaflammation.

1.2. Unravelling the cross-talk of inflammatory mediators involved in metaflammation

Metabolic inflammation is a multifaceted event triggered by a plethora of mediators. Exploring their interconnection and identifying key new hub of communication is a current challenge of the scientific community and potentially represents an interesting approach for counteracting metabolic disorders.

1.2.1. The crucial role of Advanced Glycation End Products (AGEs) as significant triggers of metabolic inflammation

Among the molecules recognized as MAMPs a relevant and peculiar role is played by the Advanced Glycation End Products (AGEs). AGEs are the results of a non-enzymatic reaction (*Maillard Reaction*) between the amine group of a macromolecule (protein, DNA, or lipid) and a carbonylic group of a reducing sugar [31].

Initially, labile and transient products (Schiff base adducts) are formed upon the condensation the C=O of the sugar aldehydes and the -NH_2 free terminal of the amine residue. Schiff bases, also known as aldimine, are further chemically re-arranged in a Amadori products, which are defined as the early

glycation products. Indeed, Amadori products are then subjected to irreversible reactions leading to the formation of AGEs [31–33]. According to the late-stage modification of the Amadori products, different pathways are responsible for their formation. Hence, one of the most important is their auto-oxidation, resulting from the Hodge pathway. On the other hand, Wolff pathway forms carbonilyc compounds through the metal-catalysed auto-oxidation and dehydration of monosaccharides such as glucose, while the Namiki pathway promotes the cleavage of dicarbonyl compounds from unstable Schiff bases [34]. Despite of these mechanisms, dicarbonilyc compounds can also be the results of physiological *in vivo* reactions of the polyol and glycolytic pathways and lipid peroxidation, that provide reactive intermediates further convertible into AGEs by the condensation with amine groups [35, 36]. The detailed phases of AGEs formation are described in Figure 1.

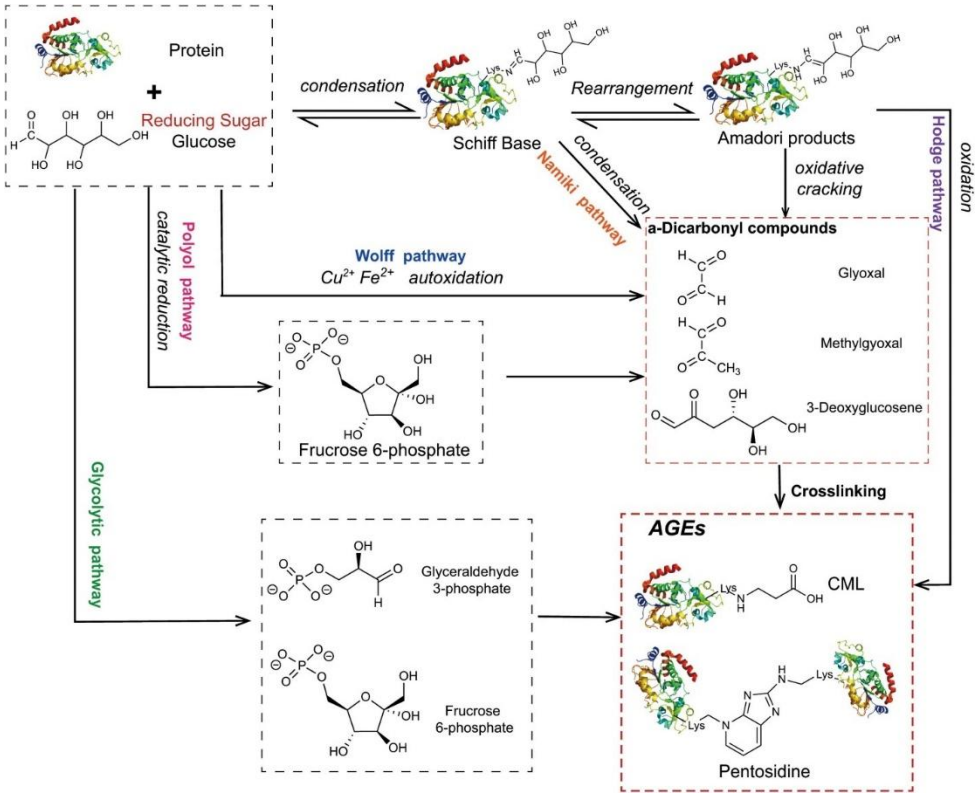

Figure 1. AGEs formation according to the multiple pathways (Hodge, Namiki, Wolff, polyol and glycolytic routes). Chen C-y et al., 2022, Front. Med. 9:837222. doi: 10.3389/fmed.2022.837222 [35].

AGEs can be classified with respect to different parameters:

- *chemical structure*: AGEs present different structural moieties of the sugars and amines involved in the reaction, alongside with the different chemical modifications occurred at the Amadori products (oxidations, reductions, and hydrations). The most abundant (and studied) AGEs are N"-(carboxymethyl) lysine (CML), N"-(1-carboxyethyl) lysine (CEL).
- *origins*: AGEs can derive from an exogenous source, such as the diet, or can be endogenous products. Exogenous AGEs are the result of food processing techniques that use high temperature, long-time cooking, low level of hydration and high pH[37, 38]. For this reason, the lifestyle changes that increased the consumption of junk food and ready-to-eat products further exacerbate the *per se* impact of the AGEs. On the other hand, the *in vivo* AGEs synthesis is a physiological process during the lifespan, driving to their accumulation; nevertheless, under hyperglycaemic and oxidative stress conditions, this process is accelerated due to the greater availability of glucose-derived reactive compounds, *i.e*. dicarbonyls or oxoaldehydes [39, 40].

The pathological consequences of AGEs are ascribable to their capacity to induce oxidative stress and inflammation. This occurs through their crosslinking with proteins, thereby modifying their structure and function or by the interaction with specific receptors [40]. In the first case, the crosslinking interaction prolonged the lifespan of the proteins, thus reducing their degradation and turnover. Several proteins can be affected by AGEs, the most specific are structural proteins of extracellular matrix components (ECM) such as collagen, vitronectin, and laminin [41, 42].

On the other hand, different receptors have been recognized to bind AGEs. The most abundant and studied is the Receptor for Advanced Glycation End Product (**RAGE**). RAGE is expressed in different cell types and tissues, such as hepatocytes, cardiomyocytes, endothelium, and macrophages, etc [43, 44]. RAGE is a member of the immunoglobulin composed of three structural portions (Figure 2):

-an extracellular domain, consisting in a V-type and two C-type domains. The V-type region is the binding site of AGEs and other DAMPs, such as HMGB1 (High Mobility Group Box 1), calcium-binding protein S A8/A9 (S100A8/A9) [45];

-transmembrane portion, with the structural function of securing RAGE anchorage to the membrane;

-intracellular tail, responsible for engaging cytosolic signal transduction;

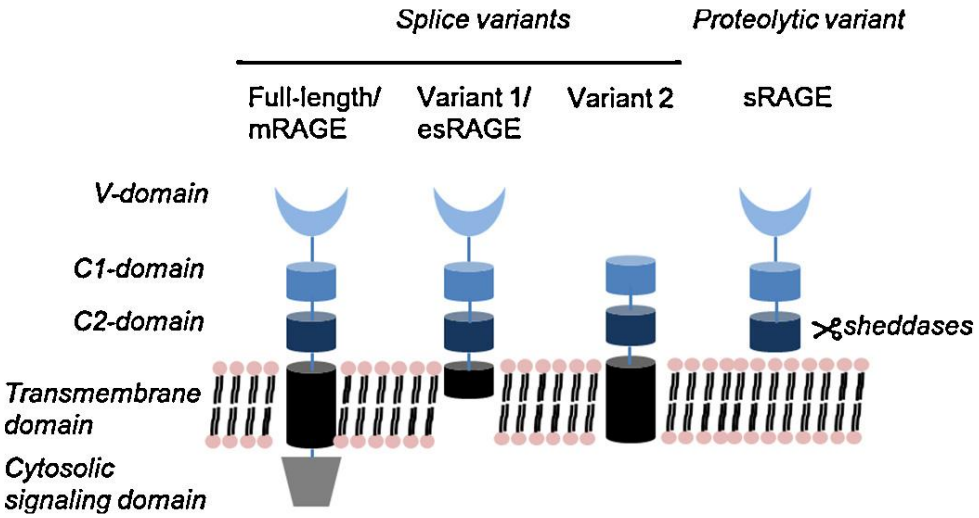

Figure 2. Structure of the Receptor for Advanced Glycation End Product (RAGE). Reynaert NL et al., *Int J Biochem Cell Biol*. 2016;81(Pt B):403-418. doi:10.1016/j.biocel.2016.06.016 [39].

Other splice variants con be originated from RAGE full-length structure, which can be lacking the cytosolic domain, unable to convey intracellular communication (esRAGE), or incapable of binding molecules (variant 2). On the other hand, an interesting truncated form of RAGE is the soluble RAGE (sRAGE), which undergoes a proteolytic cleavage. Despite this modification, sRAGE has binding capacity, and acts as a scavenger receptor, competing with RAGE for its ligands. Numerous findings as reported the inverse correlation of plasma sRAGE concentration and metabolic biomarkers associated with metaflammation [46–48].

Scientific community agrees on the strict and causal correlation of the AGE-RAGE axis activation in diet-induced models of obesity and different genetic

or pharmacological approaches have been proposed: either knockout model of RAGE deletion or its pharmacological inhibition suggest the beneficial impact of dampening AGE-RAGE pathways. Indeed, when $Ager^{-/-}$ mice (mice with a global deletion of RAGE) were fed with a high fat diet, these animals were protected against diet induced obesity, and insulin sensitivity was improved [49]. On the other hand, when high fat fed mice were subjected to a diet switch, enriched with the RAGE inhibitor RAGE229, they displayed a reduced body weight and improve glucose handling capability [50].

Moreover, several antiglycation molecules has been tested in model of hypercaloric diet consumption, mainly derived from natural products, for their high content in phytochemicals[51]. For instance, we and other have assessed the potential beneficial role of pyridoxamine in counteracting the harmful effects of high fat diet consumption in mice, such as glucose intolerance and AGEs accumulation [52, 53].

Once the AGEs-RAGE axis is triggered, oxidative stress and several signalling cascades are activated, leading to a pro-inflammatory environment that culminate with a transcriptional activation the production of cytokines, adhesion molecules and RAGE itself [54, 55]. In details, RAGE mediated the activation of NADPH oxidase (Nox) [56, 57], which in turn increases the reactive oxygen species (ROS) production, and upregulation of Nox protein expression, generating a pro-oxidative status that further exacerbates AGEs endogenous production and affects antioxidant defences [58–60]. The positive feed forward loop elicited by ROS production triggers the activation of several downstream effectors, such as p38 mitogen-activated protein kinase, extracellular-signal-regulated kinases (ERK1/2) and phosphoinositide 3-kinase (PI3K)/protein kinase B (PI3K/AKT) pro-survival pathway, overall leading to the activation of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway [61–63]. NFκB activation potentially promotes the priming signal for NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome activation and the production of several inflammatory pro-inflammatory mediators, boosting aberrant cellular processes of inflammation, apoptosis, and proliferation [31]. Another signalling axis that could interact with AGE is the

Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, hinting at the pleiotropic role of these molecules in interfering with some of the most important cascade involved in inflammatory conditions[64]. A schematic representation of the AGE-RAGE axis is represented in Figure 3.

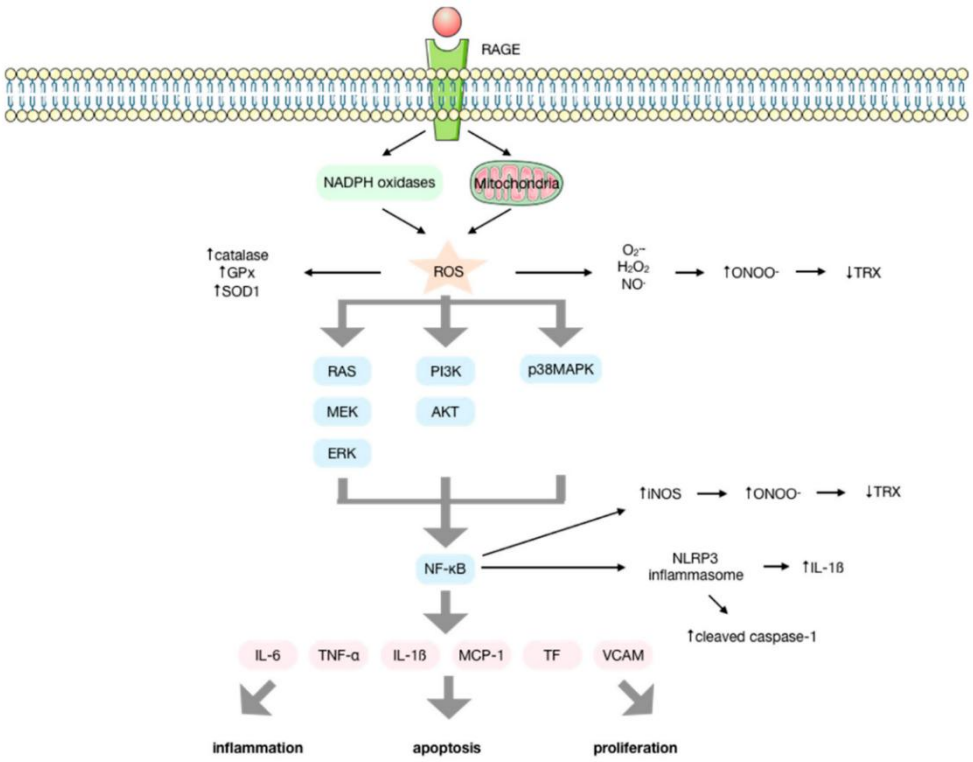

Figure 3. AGE-RAGE axis and its downstream molecular signalling. Cepas V., et al. 2020, Antioxidants 2020, 9, 142; doi:10.3390/antiox9020142 [31].

The AGEs mediated activation of the aforementioned pro-inflammatory pathways has been widely documented in diet-induced obesity (DIO) rodents' models [65, 66]. For instance, we have documented how high fat diet chronic feeding resulted in the increased nuclear NF-κB translocation in AGEs-mediated pathway [52]. Another key player involved in the onset of metaflammation is the NLRP3 inflammasome. *Nlrp3* gene transcription is regulated by NF-κB hinting for instance at the interconnection between these two-master regulators of inflammation. Intriguingly, the NLRP3 activation signal could be triggered by ROS: for this reason, the oxidative environment generated by AGEs accumulation is a trigger stimulus that enhances NLRP3

assembly with its other molecular moieties the adaptor protein ASC and caspase-1 [67, 68].

Altogether, the blockade of AGE-RAGE pathway is an effective strategy for counteracting diet-induced derangements: identifying new therapeutic agents blunting its activation could be an effective approach for halting metabolic inflammation.

AGEs detoxifying system

To unravel the potential mechanism of action of an antiglycation product, it is extremely important to assess its capability to act as a booster of the AGEs detoxifying systems. Specifically, the antioxidant enzyme that plays a role in this context is **glyoxalase (Glo-1).** Glo-1 is the limiting enzyme that catalyses the glutathione (GSH)-dependent conversion of methylglyoxal into S-D-lactoylglutathione, which is then further converted in D-lactate and GSH by glyoxalase 2 (Glo-2). Glo-1 is further regulated at transcriptional level by a key antioxidant mediator, the nuclear factor erythroid 2 (Nfr2), that encodes for antioxidant enzymes such as Glo-1, catalase and superoxide dismutase. For instance, Nrf2 reduced nuclear translocation could be detrimental for Glo-1 enzymatic action[69–72]. Moreover, Nrf2 is important for promoting the recycling of GSH, key co-factor in the Glo-1 detoxifying reaction [73]. Figure 4 is representative of the enzymatic reaction mediated by Glo-1 and its Nrf2-mediated transcription. Identifying molecules that can both reduce AGEs accumulation and potentiate antioxidant defences could be a double-sword mechanism for counteracting AGEs-RAGE axis.

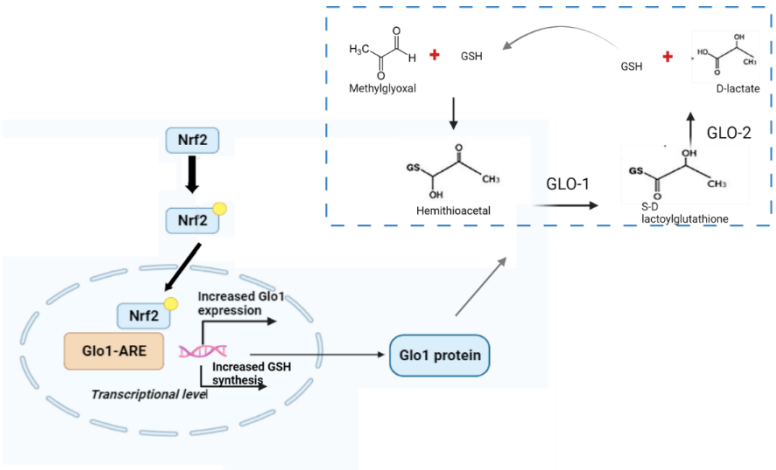

Figure 4. Nrf2 and Glo-1 detoxifying systems in mediating AGEs clearance. Image adapted from Alhujaily, M., Life 2024, 14, 263. https://doi.org/10.3390/life14020263 [74] on Biorender software.

1.2.2. Exploring the kinome: a subcellular map for new mediators with an intriguing feedforward loop of communication

The concept of "kinome" referred to the whole protein kinases detectable in a human organism. Nowadays, kinome comprises around 560 members, divided in 8 different categories, according to different criteria, such as the aminoacidic residue affected by the phosphorylation or the category of disease in which they are involved [75]. Despite the successful efforts of identifying their structure in the past years, the complexity and their mutual correlations is still a going concern in the scientific community.

The central role of kinases in the onset of several pathological conditions, such as immune disorders, cancers and neurological syndromes have been demonstrated over the past years. Protein phosphorylation is a crucial regulatory mechanism in cells concerning the activation status of many cellular elements and this machinery is tightly regulated by kinases, alongside with its opposites components (phosphatases) [76]. The biological results of kinases' activation are very different, according to the mediators affected by the phosphorylation: the plethora of mechanisms elicited by kinases underlined the importance of these intracellular components in the regulation of the homeostatic cellular processes. For instance, it has been proven how kinases are involved in the orchestration of the cell's activity, controlling processes such as apoptosis, subcellular translocation and inflammation [77]. Specifically for this latter, growing body of evidence have consistently documented that kinases play a pivotal role in the pathogenesis of inflammatory disorders, such as rheumatoid arthritis, atopic dermatitis, etc, and how their pharmacological inhibition is effective strategy for the amelioration of the disease [75]. Concerning this, several kinase inhibitors have been synthesized and tested in a wide range of pathology and nowadays 80 therapeutic agents have been approved by FDA and around 180 protein kinase inhibitors are in clinical trials, pointing at their promising

switch from “bench to bedside” [78]. For this reason, the remarkable role of kinases in plenty of diseases and the consequent development of therapeutic strategies have introduced the strategic concept of drug repurposing, which can be defined as the use of already approved and available drugs in a different pathological condition distinct from their original therapeutical approval [79].

In conclusion, not only kinases are key emerging regulator of whole-body homeostasis, but several drugs are available and could be repositioned in different diseases with common pathogenic origins.

Kinases & metabolic inflammation

Being widely accepted the central role played by the pro-inflammatory mediators evoked by the consumption of westernized diet and their intracellular cascades, the identification of mediators such as kinases that can affect the activation status of these effectors is an interesting strategy also in the context metabolic inflammation. Among the intracellular cascades activated by nutritional challenges, a pivotal role is played by the NF-κB and the JAK/STAT pathways[80, 81]: kinases that can affect their activation could be a double-hedge sword for counteracting simultaneously their detrimental effects.

For instance, the cytokines’ effects on the receptor of TNFα (TNFR) and TLR4 axes triggers the downstream activation of inhibitor κB kinases, whose catalytic activity is mainly played by the isoforms α and β (IKKα/β); this stimulus elicits the IKK-mediated phosphorylation of two critical aminoacidic residues (Ser 32/36) of the N-terminal regulatory domain of IκBα kinase. The phosphorylation of these amino acids is critical for determining the ubiquitination IκBα: this post-transcriptional modification is the key event that enable the NFκB release from IκBα-mediated cytosolic retention [82]. NFκB is composed of two moieties the p65, responsible of the NFκB canonical pathway and p50, that instead promotes the non-canonical signalling [83]. NFκB presents multiple phosphorylation sites that can further boost its nuclear translocation, such as the Ser on 529 [84]. Once that NFκB has reached the nucleus, it mediates the transcription of pro-

inflammatory genes and adhesion molecules. A schematic representation is shown in Figure 5.

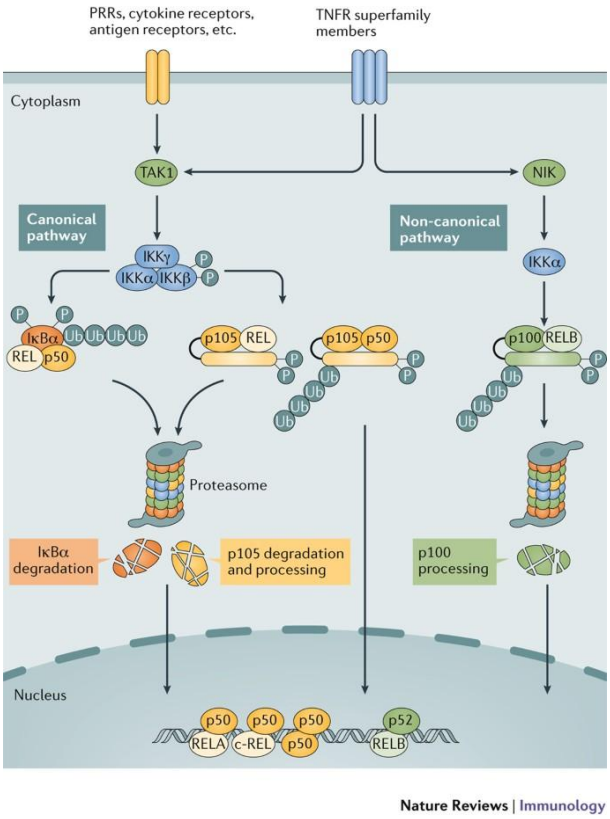

Figure 5. NFκB canonical and non-canonical pathways. Sun, SC. The non-canonical NF-κB pathway in immunity and inflammation. Nat Rev Immunol 17, 545–558 (2017). https://doi.org/10.1038/nri.2017.52 [83].

As previously mentioned in the section 1.2.1 the NLRP3 inflammasome cascade is another signalling pathway activated in metaflammation and whose promotion is highly dependent on NFκB, being *Nlrp3* one of its target genes. Once the priming signal is achieved, the secondary necessary step, the activation, could be mediated by different stimuli such as PAMPs or DAMPs molecules and ROS[85]. This step is necessary for the NLRP3 inflammasome assembly with its adaptor protein ASC and the effector protein, caspase 1. In details, after its stimulation, NLRP3 undergoes oligomerization step that facilitate ASC recruitment. Several ASC molecules further combine into a single molecule (ASC speck) that finally recruits caspase 1 self-cleavage. Cleaved caspase-1 will further be responsible for the activation of IL-1β and IL-18 in their active forms, eliciting their pro-inflammatory potential [86]. Another detrimental process NLRP3-driven is

the pyroptosis. Pyroptosis is a programmed cell death mechanism in which the cell undergoes a membrane disruption by the formation of pores through the action of gasdermin D(GSDM-D). After this mechanical damage, inflammatory cytokines and cellular contents are released from the cell resulting to the release of cells' content and increased inflammatory response [87]. Most notably, GSDM-D action is regulated by NLRP3 activation, which through the cleavage action of caspase 1 initiates the cleavage of GSDM-D C-terminal domain that enables the N-portion to engage the pore formation [88]. Figure 6 summarizes the activation mechanisms of NLRP3.

The detrimental effects of the NFκB-NLRP3 axis are well documented in scientific literature as well as its involvement in metaflammation. Specifically, our research group evinced the key role of NLRP3 in a murine model of hypercaloric diet consumption, proving the harmful consequences of NLRP3 activation in disrupting glucose handling capability, body weight maintenance, etc while *Nlrp3* knock out or its pharmacological inhibition with BAY-117082 ameliorates the deleterious repercussions of HFD chronic exposure [6].

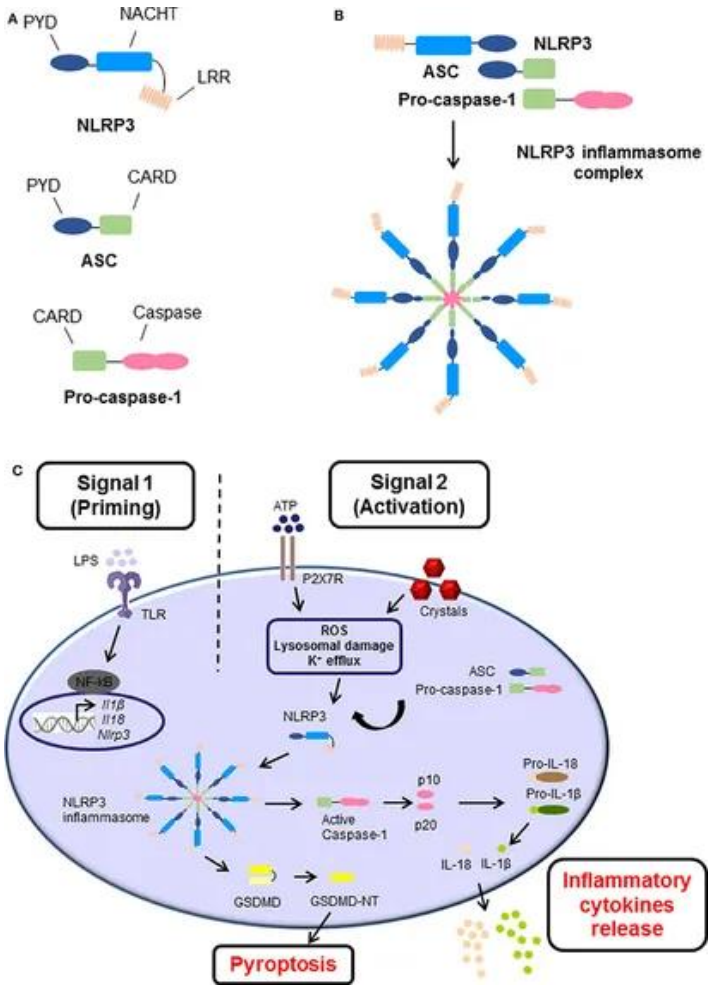

Figure 6. NLRP3 inflammasome structure and assembly, with the priming and activation signals. Hamarsheh S., et al., Front. Immunol., 08 July 2020, https://doi.org/10.3389/fimmu.2020.01444 [89]

On the other hand, JAK/STAT pathway is prompted by the interaction between cytokines, such as IL-6, and their cytokines receptors [90]. After this initial step, JAK kinase undergoes dimerization evoking a reciprocal auto-phosphorylation of the two JAK molecules and a phosphorylation of the cytoplasmic tail of the receptor, necessary for the STAT-anchorage. This modification enables its tyrosine-activity and provoked the phosphorylation of its downstream mediator STAT on its C-terminal situs. Once phosphorylated, STAT dimerizes and translocate into the nucleus, prompting the transcription of pro-inflammatory genes [91]. Different isoforms of JAK and STAT are expressed in different tissues and results in different biological events, that are represented in Figure 7. Among them, the JAK2 isoform has to be highlighted for unveiling the role of

JAK/STAT in the metabolic context as it has been associated with a cross-talk with the insulin signalling cascade, specifically binding to protein kinase B(PKB or AKT) and reducing its activity, resulting in an impairment of glucose uptake in muscle cells [92]. Once JAK2 is activated, different STATs can be affected: one of the most studied is STAT3 due to its transcriptional promotion of pro-inflammatory target genes and moreover for its intercommunication with other inflammatory cascades such as NFκB and NLRP3. Indeed, STAT3 is involved in the acetylation of p65 NF-kB subunit and consequent persistent NF-kB nuclear activity[93]. Furthermore, activation of JAK2/STAT3 cascade has been proven to affect NLRP3 expression, promoting its expression and activity in different inflammatory models, highlighting the pivotal role of JAK/STAT in orchestrating intracellular signalling cascades [94, 95].

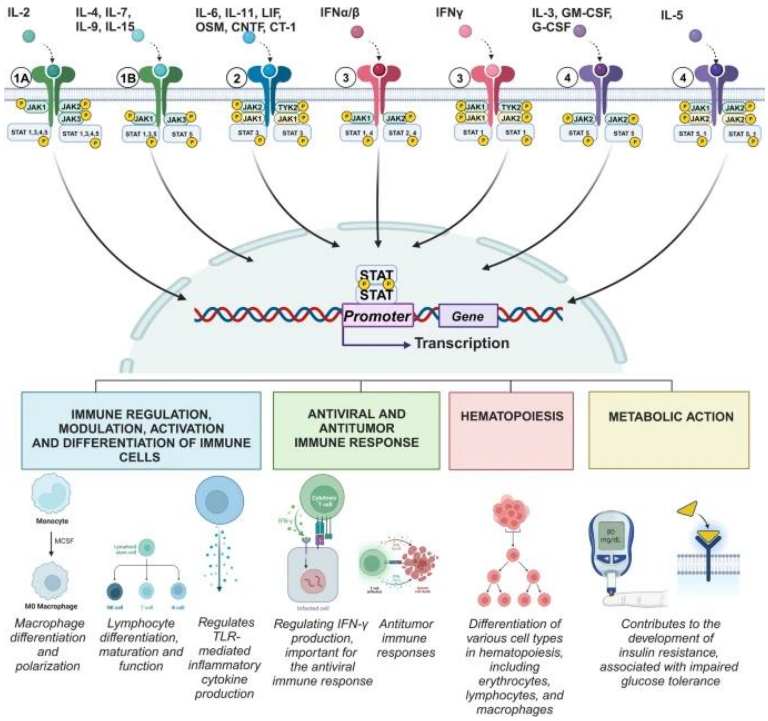

Figure 7. JAK-STAT pathways and their pleiotropic action in cell fate. Sarapultsev, A., Gusev, E., Komelkova, M. et al. JAK-STAT signaling in inflammation and stress-related diseases: implications for therapeutic interventions. Mol Biomed 4, 40 (2023). https://doi.org/10.1186/s43556-023-00151-1 [96].

Most notably, we have already proven the effectiveness of JAK/STAT inhibition in three different experimental models (sepsis, haemorrhagic shock, metaflammation), highlighting the central role of this pathway in governing inflammation-driven effects [97–99]. For instance, the administration of Baricitinib, a JAK1/2 inhibitor, in a murine model of high fat diet consumption, efficiently counteracted diet-induced metabolic abnormalities, affecting both metabolic and inflammatory profiles at local and system levels.

Based on these solid scientific outcomes and relevant prospectives to have a shift from bench to bedside of small kinases inhibitors, the identification of key kinases that simultaneously can affect the activation of multiple intracellular mediators could be a challenging approach for counteracting *metaflammation.* The main reason relies on the fact that the pharmacological intervention on one single mediator has shown poor clinical relevance because of the several cascades responsible for the onset of metaflammation, additionally to the plethora of immune cells affected by the dietary challenges.

1.3. Innovative strategies for counteracting metaflammation

Nowadays, several treatments are available for counteracting metabolic diseases *i.e.* obesity, type 2 diabetes (T2D) and dyslipidaemia. Despite of this, the current global prevalence of these disorders alongside with their role in increasing cardiovascular risk has enlightened the need for introducing new interventions to halt metabolic disturbances. The first intervention to be implemented is a change in dietary lifestyle, with the reduction of the consumption of highly processed foods and an increased intake of fruits and vegetables and foods enriched in fibres. For instance, different clinical studies have reported the beneficial effects of dietary interventions in the amelioration of glycaemic control and systemic inflammation in T2D patients [100] as well as in weight reduction in obese patients on Mediterranean diet (MD) [101], which is a well-recognized for its high nutritional quality [102]. Nevertheless, the nutritional shift towards a more balanced diet must be accompanied by a sustained and regular physical activity, with the aim of counteracting the sedentary behaviour associated the technological advancements over the past decades [103].

On the other hand, new therapeutic options for dampening metabolic abnormalities are under investigation. For instance, the bioprospecting approach of the scientific community over the past decades has highlighted the potentiality of the natural world to provide useful tools for the prevention of metabolic disturbances [104]. Indeed, their potentiality is strongly supported also for the greater patients' adherence to the treatment as well as their minimal toxic effects [105, 106]. Furthermore, the great abundance of bioactive molecules, known as nutraceuticals, in fruits and vegetables and in the so-called "novel food" represents a valid approach for counteracting metabolic disturbances without using medicaments. Nevertheless, drug intervention is necessary for treating severe cases of metabolic derangements and an extension of the therapeutic options is auspicial. With regard to this, not merely identifying new targets but also "druggable" ones is a point that need to be considered in basic research, considering also the aforementioned concept of drug repurposing that is become an attractive perspective for its cost-effectiveness and timesaving concepts[79].

1.3.1. The promising role of green-blue algae for alleviating diet-induced abnormalities

Natural products have been generally acknowledged as a potential source of antiglycative compounds. Specifically, their mechanisms of action are attributed to their multi-faceted components that are recognized for different protective effects, such as polyphenols, carotenoids, vitamins [107]. The variety of phytochemicals already recognized as antiglycative relies mainly on their antioxidant properties as ROS scavengers. As an example, several polyphenols such as catechins (epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate) have reported to reduce *in vitro* glycation process not only throughout an antioxidant activity, but also by trapping of methylglyoxal (MGO), which is one of the most dangerous dicarbonyl precursors for AGEs formation, alongside with the inhibition of key enzymes in the carbohydrates' metabolism and proteins' integrity [108]. Moreover, we and other have consolidated the protective effects of pyridoxamine, analogue of B6 vitamin, as an efficient antiglycative product capable of reducing local and systemic AGEs accumulation in high fructose fed mice, as well as reducing diet-induced

impairments[53, 109]. Its beneficial effects are attributable to its scavenger activity and action as a potent quencher of the dicarbonyl compounds [110, 111].

Based on these assumptions, the investigation of new natural sources of compounds is an attractive field of research in the metabolic context. A new fascinating realm emerged in the natural library screening are the marine products, and specifically the microalgae [112]. Interestingly, several members that have been investigated in metabolic disturbances, hinted at their beneficial role in counteracting metabolic abnormalities [113–117].

Among these pleiotropic organisms, ones of the most intriguing and potentially interesting molecules are the so-called "green-blue algae", which are now classified as part of the Cyanobacteria phylum, due to their morphological and structural peculiarities [118]. Indeed, Cyanobacteria produce several bioactive compounds, counting 40% lipopeptides, 5.6% amino acids, 4.2% fatty acids, 4.2% macrolides and 9% amides [119, 120]. Specifically, some of the most attractive products reside in the carotenoids class, embracing molecules such as *β*-carotene and lycopene that exert their antioxidant activity as ROS scavengers [120]. Furthermore, phycobiliproteins are interesting macromolecules that beyond being responsible for the typical colour of the algae, are recognized for their multi-faceted actions as ROS scavengers as well as boosters of the expression of antioxidants enzymes and GSH glutathione, essential cofactor of the detoxifying reactions [121].

As a result of their massive antioxidant capacity, Cyanobacteria could represent an interesting source of antiglycative compounds: for instance, the antiglycation properties of some Cyanobacteria's derivatives have already been confirmed in *in vitro*. The compounds saclipin A and B, oxylipins derived from the alga *Aphanothece sacrum,* have been proved to reduce the glycation of structural proteins such as elastin and collagen, alongside with a radical scavenging activity in human fibroblasts [122], while the fermented extract of *Aphanizomenon flos-aquae* alga presented likewise an antiglycation and antioxidant effects on RAW264.7 macrophages [123]. Moreover, another derivate from *Aphanothece halophytica*, the mycosporine-like amino acids (MAAs) has been proven to inhibit glycation-dependent protein cross-linking [124], hinting at the beneficial role of the cyanobacteria-derived compounds.

However, the assessment of cyanobacteria's effectiveness in affecting AGEs accumulation in *vivo* needs to be investigated to confirm the promising *in vitro* data.

For instance, encouraging finding from Husain and colleagues depicted a promising effect of a phycobiliprotein, C-phycocyanin(C-PC), derived for *Plectonema* species, in streptozotocin induced diabetic rats where C-PC ameliorated hyperglycaemia and lipid profile, alongside with improvement in oxidative and glycation markers [125]. However, this study only investigated systemic early markers of the glycation process, such as ketoamine and dicarbonyl contents, in addition to the generic evaluation of lipid peroxidation, urging to better elucidate the direct effect of C-PC on the formation of AGEs and the activation of AGE/RAGE cascades.

On the other hand, the effectiveness of a singular cyanobacterium as an antiglycation compound *in vivo*, embracing its multifaceted composition, has not been yet investigated. Currently, only *Aphanothece sacrum* has been proven to prevent AGEs accumulation in lens of diabetic rats suffering of cataract [126], limiting the beneficial effects to a very specific pathology, although AGEs accumulation is known for its detrimental role in the onset of cataract [127]. Intriguingly, one of the most abundant and studied green blue alga, *Arthrospira platensis,* commonly known as Spirulina, has not been yet investigated as a potential tool for reducing AGEs accumulation and the consequent metabolic disturbances.

1.3.1.1.Harnessing the bioactive potential of Arthrospira platensis (Spirulina): a focus on its possible effects in managing metabolic dysfunctions

Arthospira Platensis is one of the filamentous microalgae belonging to Cyanobacteria which has gained attention over the past years, thanks to its nutritional components that enables Spirulina to be included into the novel foods and its traditional use in different Indian tribes [128]. The composition of Spirulina is dependent on several factors, *i.e.* source, cultivation condition, manufacturing's process and seasonality [129, 130]. Despite of this, the most abundant categories and peculiar components are widely recognized.

Specifically, Spirulina composition comprises great abundance of proteins, representing the 60% of its dried mass, with high nutritional quality and ratings similar the ones found in vegetable sources [131]; in addition to the conventional proteins, Spirulina presents elevated content of phycobiliproteins, such as phycoerythrin, allophycocyanin and phycocyanin, known for their antioxidant properties [130]. Moreover, Spirulina-derived lipids are also polyunsaturated essential fatty acids (PUFAs) such as omega-3 eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and also omega-6 (arachidonic acid and γ-linoleic acid), well-known lipid components necessary for the whole-body homeostasis [132–134]. On the other hand, polysaccharides represent another adjuvant source of intriguing antioxidant molecules [123, 135], displaying around the 10% of Spirulina dried mass [136]. Spirulina is recognized as well for its high content of micronutrients that comprises a large variety of vitamins and essential elements. For instance, Spirulina is a valid source of vitamins B, especially of B12 vitamin, being documented the presence also of its pseudo-derivative named corrinoid [137], alongside with riboflavin (B2), and folic acid (B9) [138–140]. In addition, Spirulina contains significant concentrations of vitamin K[141] and β-carotene, this latter being the precursor to vitamin A [142]. Most notably, Spirulina is an excellent source of almost all the essential elements, such as potassium, calcium, copper, iron, magnesium, manganese, phosphorous, selenium, and zinc [143–145]. This important abundance of microelements relies on the peculiar characteristic of this green-blue alga to accumulate metal during growth [146]. Among the microelements in which Spirulina is enriched, **zinc (Zn)** is one with the highest percentage of accumulation [147]. Zinc is recognized for its pivotal role in immune cells' homeostasis: in details, it is an essential co-factor in enzymatic reactions, as well as it is required for Zn-finger transcription factors activity [148], and as antioxidant agent [149, 150]. Zinc deficiency has been associated with aging [151], but also with pathological conditions such as metabolic disturbances that have shown in patients a correlation between lower Zn levels and negative outcomes [152, 153]. Most notably, Zn deficiency has been observed in obese patients, reinforcing the central role of this trace element in governing metabolic function [154, 155].

Based on this assumptions, the effect of Spirulina in different experimental condition has already been investigated, proving its beneficial role in counteracting oxidative stress, dampening inflammation consequent of dietary challenge [156, 157] and as a hypolipidemic agent [158]. Despite being recognized its remarkable effects in the prevention of metabolic disturbances, the potential effects of Spirulina as an antiglycative compound have not been explored yet. Filling this gap in research could partially explained the Spirulina mechanism(s) of action exerted by the plethora of phytocomplexes that reside its composition, exceeding the peculiar effects of its one-to-one components. Moreover, the additional enrichment in zinc of the formulation could boost the efficacy of this green-blue alga in counteracting AGEs effects in a murine model of high fat diet consumption. Overall, Spirulina-mediated effects on human on glucose handling capability and inflammation in humans are to some extent unsettled [145] highlighting a more in-depth analysis of the molecular insights of *Arthrospira Platensis*.

1.3.2. The raising function of casein kinase II (CK2) in the human kinome and its clinical relevance as a drug target.

Casein kinase II (CK2) is a serine/threonine kinase with a tetrameric form, organized in two catalytic subunits (α and α') and two regulatory moieties (β), represented in Figure 8 [159]. The catalytic subunits are encoded by two different genes, *CSNK2A1* and *CSNK2A2,* while both the β by *CSNK2B [160]*. CK2 is ubiquitously expressed and a constitutively active holoenzyme. Being constitutively active, CK2 acts an "horizontal" player in the cells [161]; on the other hand, CK2 aberrant activation could be achieved by external stimuli that alter its phosphorylation activity, thus pointing at CK2 as stress-responsive kinase [162, 163].

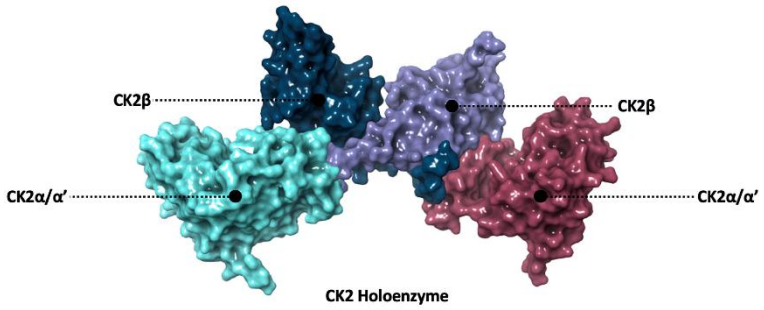

Figure 8. CK2 tetrameric structure; Day-Railey S et al., Kinases Phosphatases 2024, 2(2), 110-135; https://doi.org/10.3390/kinasesphosphatases2020007 [160].

CK2 was one of the first identified kinase (1954, by Burnett and Kennedy [164]) and for instance it has been widely and comprehensively studied: more than 200 substrates and almost 300 phosphorylation sites have been identified, pointing at its involvement different biological processes [165]. Among them, CK2 is recognized for its pivotal phosphorylation activity on cell differentiation, cell survival, apoptosis and in mediating signal transduction [166]. In this perspective, growing interest sparked around CK2 and its potential role in the pathogenesis of different disturbances. Nowadays, CK2 involvement has been recognized in a plethora of diseases such as cancer, neurodegenerative pathologies, glomerulonephritis, colitis, etc [161]. Interestingly, the aetiology of these pathologies shares the involvement of immune cells activation and of inflammatory mechanisms. This tight correlation between inflammation and CK2 could be explained by the direct effects that the kinase has in affecting the activation of multiple inflammatory cascades and in the end in the regulation of their inter-communication.

Indeed, CK2 has been documented to foster the activity NFκB pathway. In details, CK2 targets IκBα on different aminoacidic residues, resulting in the higher promotion of its degradation and, as a consequence, a reduced cytosolic retention of NFκB. In details, CK2 promotes the phosphorylation of IκBα on the C-terminal proline/glutamic acid/serine/threonine-rich (PEST) domain, which in different proteins has been associated with the orchestration of the rapid protein turnover [167–169]. Moreover, CK2 interacts with IκBα on the serine 32, which is well known residue that promotes TNF-alpha mediated NFκB pathway [170]. Intriguingly, the activity of CK2 is directly associated with NFκB-p65 subunit, on the

phosphorylation site of serine 529, which results in an increased p65 nuclear translocation[171, 172]. Another predominant cascade interacting with CK2 is the JAK/STAT pathway. For instance, CK2 interacts with JAK2, boosting its activity. Indeed, it has been proved how the pharmacological inhibition of CK2 with 4,5,6,7 tetrabromobenzotriazole (TBB) elicited a reduction in the phosphorylation status of two key amino acids Tyr1007/1008 and consequent activity [173]. In addition, STAT3, could be targeted by CK2 on different aminoacidic sites, such Ser 727 [172] and Tyr 702 [173], eliciting a further amplification of JAK2/STAT3 mediated intracellular cascade [163, 172, 174]. Moreover, STAT3 could bind to the CK2 promoter region, increasing its transcription, thus eliciting a feed forward loop of communication [173]. Figure 9 shows the CK2 interconnection with NFκB and JAK/STAT pathways.

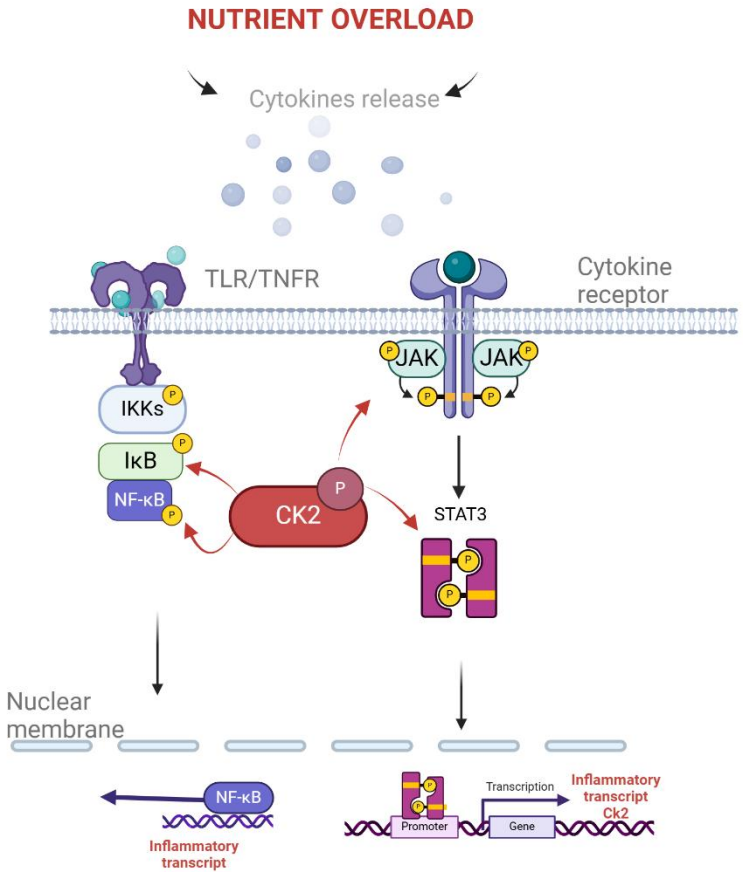

Figure 9. Representative image of CK2 interconnection with NFκB and JAK-STAT pathways. Images created with Biorender.

The impact of CK2 aberrant activity in inflammatory-associated disease has been recently investigated by our research group in a septic murine model.

Indeed, septic condition evoked by caecal ligation and puncture (CLP) procedure dramatically induced the renal overactivation of CK2 catalytic subunit in mice, boosted NFκB cascade, alongside with vascular hyporesponsiveness and systemic cytokine storm: these derangements overall ended in an increased mortality in mice, compared to sham operated ones. Intriguingly, pharmacological inhibition of CK2 with Silmitasertib, a selective CK2 inhibitor, counteracted CK2α abnormal activity and the downstream activation of NFκB axis. As a consequence of Silmitasertib administration, a reduced cytokine's production, as well as a restored vascular abnormalities were documented, leading to a prolonged survival of septic mice [175], hinting at CK2 as a key player in sepsis-induced inflammation.

Being the hypercaloric diet enriched in simple sugar and fats a chronic and detrimental stimulus eliciting the development of latent inflammatory status, exploiting the role of CK2 in the metaflammation could be a challenging approach to unveil the effect of this pleiotropic kinase in another inflammatory-driven pathology.

1.3.2.1. Unravelling the pharmacological targeting of CK2 for mitigating metabolic inflammation evoked by hypercaloric diet consumption

In support of the importance of investigating the potential involvement of CK2 in metaflammation on the strength of its role in the orchestration of different inflammatory cascades, recent evidence suggests that CK2 aberrant activity has been observed in metabolic associated disorders. For instance, Lan and colleagues reported that the hepatic expression of *CSNK2A1*, gene encoding for CK2 catalytic subunit α, and the protein itself were increased in diabetic mice: these data was further supported by the increased CK2 levels in the serum of diabetic patients, compared to controls[176]. In addition, in adipose tissue CK2 is an important modulator of adipocytes' glucose homeostasis[177]; this interconnection was strengthened by the results on patients affected by multiple symmetric lipomatosis (MSL), where they manifested a local CK2 upregulation and adipose tissue expansion[178]. Moreover, CK2 is recognized as a negative regulator of energy expenditure,

that can affect adipocytes' thermogenesis, throughout its effect on class I histone deacetylases (HDACs)[179].

Taken all together, CK2 is a potential target to investigate in the pathological context of metaflammation: targeting CK2 kinase in the context of diet-induced obesity could expand and enlighten the central role of this pleotropic kinase as a central regulator of metabolic processes. Nowadays, several CK2 inhibitors have been synthesized and are widely used in basic research, pointing at a good ratio between risks and benefits following their applications in both *in vitro* and *in vivo* models [161, 180]. There are different categories of drugs according to their mechanisms of action, which are reported in Table 1. The ATP-binding compounds are the most investigated class due to the reduced dimension of CK2 ATP pocket site, which is located between N-terminal and C-terminal domains of the catalytic subunits, compared to other kinases, which enable to small molecules an efficient CK2 binding [160, 161]. Among them, the benzotriazole subclass includes a broad lists of molecules that have similar structural bones and present different chemical moieties that have been included for improving their efficacy and affinity for CK2 [181].

CK2 INHIBITORS		
Inhibitor class	**Subclass**	**Representative compounds**
ATP-competitive inhibitors	Polyhalogenated Benzimidazol and benzotriazoles	**TBB** TDB DMAT
	Flavonoids	Quercitin
	Coumarines	Ellagic acid
	Anthraquinones	Quinalizarin
	Carboxyl acid derivates	TBCA CX-4945
	Triazol bromoguaicol derivatives	GO289
Peptide-competitive inhibitors	-	CIGB-300
Allosteric inhibitors	α/β interface targeting	Pc POMs ARCs K137-E4
Multi-target inhibitors	CK2 and HDAC targeting	CZ-4945 + CisPt

Table 1. CK2 inhibitors, classified with respect to their class, subclass and representative compounds. Table adapted from Borgo, C., D'Amore, C., Sarno, S. et al. Protein kinase CK2: a potential therapeutic target for diverse human diseases. Sig Transduct Target Ther 6, 183 (2021). https://doi.org/10.1038/s41392-021-00567-7 [161]

4,5,6,7-tetrabromobenzotriazole (TBB) belongs to the benzotriazole derivates and its chemical structure has been designed for binding with high rate to the hydrophobic region of CK2 ATP pocket, with a K_i= 0.4 μM. [182] (Figure 10). The peculiarity of TBB relies on its ability to discriminate between the other protein belonging to the kinase family, named CK1 and Golgi casein kinase (G-CK), allowing an intraclass selectivity [183]. On the other hand, an initial screening test on 30 kinases panel has confirmed the drug's selectivity[182, 184] however, a more recent update on TBB selectivity revealed that it can affect the activation of some other kinases [181].

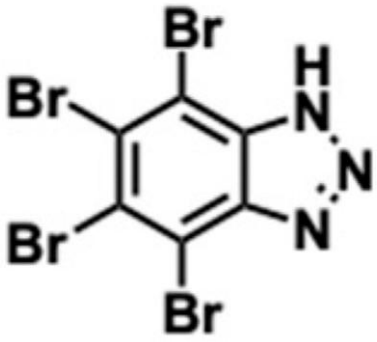

Figure 10. Chemical structure of 4,5,6,7-tetrabromobenzotriazole (TBB). The molecule presents a heterocycle, named triazole, coplanar with a benzene ring, which has four atoms of bromine as substituents. Day-Railey S et al., Kinases Phosphatases 2024, 2(2), 110-135; https://doi.org/10.3390/kinasesphosphatases2020007 [160]

Despite of this, the effectiveness of TBB as a selective and potent CK2 inhibitor (IC_{50} = 0.14 μM, [160]) is recognised and this drug is a strong candidate for both *in vitro* and *in vivo* models, hinting at its effectiveness in counteracting CK2 aberrant activation alongside with good tolerability. Moreover, TBB has also been assessed in rodents' chronic experimental models, suggesting that its prolonged administration is well tolerated, and no specific side effects have been yet documented [185, 186].

Most notably, TBB has been tested also in inflammatory experimental conditions, such as diabetic renal fibrosis [185] and ischemia and reperfusion injury [186], where TBB administration ameliorated local inflammation, blunting the activation of NFκB cascade, and improved BUN (Blood Urea Nitrogen) and creatinine levels, well-known markers of renal injury, and plasmatic concentrations of pro-inflammatory mediators such as TNFα and MCP-1.

Taken together, TBB potentially could represents a useful pharmacological tool for the treatment of metabolic disturbances associated with chronic and latent inflammatory status.

2. AIMS

The aim of this doctoral thesis is to investigate innovative strategies for counteracting metaflammation in murine models of hypercaloric diet consumption. To reach this goal, two different approaches were assessed to target specific components involved in the multifaceted patho-mechanisms of metaflammation, namely the advanced glycation end products (AGEs) and casein kinase II (CK2).

Diet supplementation with *Arthrospira Platensis*, whose use is recognized safe and valuable for its high nutritional rate, could represent a valid approach for disrupting the diet-induced AGEs accumulation. For instance, this green-blue alga, further enriched in zinc, Zn-SP, includes in its composition multiple components well-known for their antioxidant activity that could act synergistically for halting the formation of endogenous AGEs and consequent detrimental activation of AGE/RAGE cascade. We thus here investigated whether Zn-SP can interfere with diet-induced AGEs accumulation and, thus, exerting positive effects for alleviating the detrimental consequences of an impaired nutrient overload. Moreover, Zn-SP could display an important tool for ensuring a daily intake of high quality and balanced macro and micronutrients, that, alongside with a correct dietary lifestyle and physical activity could lead towards healthier metabolic functions.

On the other hand, the identification of a novel target that plays a role in the onset of metaflammation is necessary to develop effective pharmacological treatments for metabolic disorders. Here we studied the effects of the pharmacological modulation of the dysregulated activity of casein kinase II (CK2), which has already been proven in different pathological conditions. Most importantly, CK2 orchestrates the activation of multiple inflammatory cascade, representing a fascinating mediator that affects simultaneously different pathways. Several drugs that inhibit selectively CK2 has been developed and among them, 4,5,6,7 tetrabromobenzotriazole (TBB) represents a valid pharmacological strategy to test the potential beneficial role of CK2 inhibition in diet-induced alterations.

3. MATERIALS AND METHODS

3.1. Ethical statements

The experimental procedures were approved by the local Animal Use and Care Committee and the Ministry of Health (approval no. 855/PR Ministry of Health, Rome, Italy) in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes as well as the Guide for the Care and Use of Laboratory Animals.

3.2. *In vivo* animal model

3 weeks old C57BL/6JOlaHsd male mice (Envigo RMS Srl, San Pietro al Natisone, UD, Italy) were used for assessing the impact of dietary manipulation and the potential effect of Zn-Spirulina supplementation or the pharmacological intervention with TBB.

In details, mice were maintained in conventional housing conditions (n=5 cage) in a controlled environment (25 ± 2 ◦C), 12/12 h light/dark cycle, with *ad libitum* access to food and water. Body weight and food intake were monitored weekly with a precision balance (VWR International Srl, Milan, Italy). After one week of acclimatation, mice were randomly assigned into one of the following dietary regimens:

- *Standard diet (SD):* caloric intake of 3.85 kcal/g, providing 10% kcal from fat and 70% from complex carbohydrates and 20% from proteins (n = 22, where n=10 was considered for the Article 1 and n=12 for the Article 2 respectively).
- *High-Fat High-Sugar diet (HFHS)*: caloric intake of 5.56 kcal/g, providing 58 % of fat, mainly from hydrogenated coconut oil, 26% of simple sugars (sucrose) and maltodextrin, and 16% of protein. (n = 44, where n=20 was considered for the Article 1 and n=24 for the Article 2 respectively).

Both diets were purchased from ssniff® company (Soest, D-59494, Germany), identified with the codes D12450K and D12331 respectively, and the detailed formulas are reported in the table below (Table 2)

Categories	***STANDARD DIET***	***HIGH FAT HIGH SUGAR DIET***
PROTEIN	**20 % kcal** Casein, 30 Mesh (800 kcal) L-Cystin (12 kcal)	**16 % kcal** Casein, 30 Mesh (912 kcal) DL-Methionine (0 kcal)
CARBOHYDRATE	**70 % kcal** Corn starch (2200 kcal) Maltodestrin 10 (600 kcal) Sucrose (0 kcal)	**26 % kcal** Maltodestrin (680 kcal) Corn Starch (0 kcal) Sucrose (700 kcal)
FAT	**10 % kcal** Soybean Oil (225 kcal) Lard (180 kcal)	**58 % kcal** Soybean Oil (225 kcal) Coconut Oil, hydrogenated (3001,5 kcal)

Table 2. SD and HFHS formulas expressed in macronutrients categories. Diets D12450K and D12331 were purchased from ssniff® company. Food consumption was monitored weekly with an analytic balance.

Both formulas are well-known gold standard diet used in diet-induced model of metabolic derangements [187–189]. The kinetic of dietary manipulation is a key determinant for a successful experimental design. Scientific evidence supports that a 12-weeks protocol is of effective extent for eliciting the development of obesity, insulin resistance and dysmetabolism in mice, with the onset of a disturbance in body weight gain and an impaired glucose handling capability after 4/5 weeks of hypercaloric diet intervention [190–192].

For instance, following 5 weeks of HFHS feeding for the article 1 and after 4 weeks in the article 2, a subgroup of the HFHS received, until the end of the experimental procedures respectively:

- *Article 1*: Zn-Spirulina (Zn-SP) at the dose of 350 mg/kg *p.o.* trice a week (n=10) by oral gavage. The Spirulina dose was selected according to *in vivo* studies on rodents, whereas the daily intake of zinc was lower than the minimum toxic dose reported to evoke murine toxicity [193–195]. Zn-SP was obtained from Unione Spirulina Biologica Italiana USBI

(Guidizzolo, Italy). Detailed description of Zn-SP detailed composition and manufacturing are presented in the section 2.9.

- *Article 2*: 4,5,6,7-tetrabromobenzotriazole (TBB, CliniSciences, Nanterre, France) at the dosage of 2.5 mg/kg/p.o. (HFHS+TBB, *n*=12). TBB was included into the HFHS diet to achieve a dose of 2,5 mg/kg/die, on the basis of a daily intake of 2.5 g of diet for each mouse. The dose and the extent of the treatment were determined based on previous *in vivo* study in mice, whereas the drug was reported to be effective without side effects [185, 196].

A schematic representation of the in-vivo protocols is represented in Figure 11A (article 1) and B (article 2).

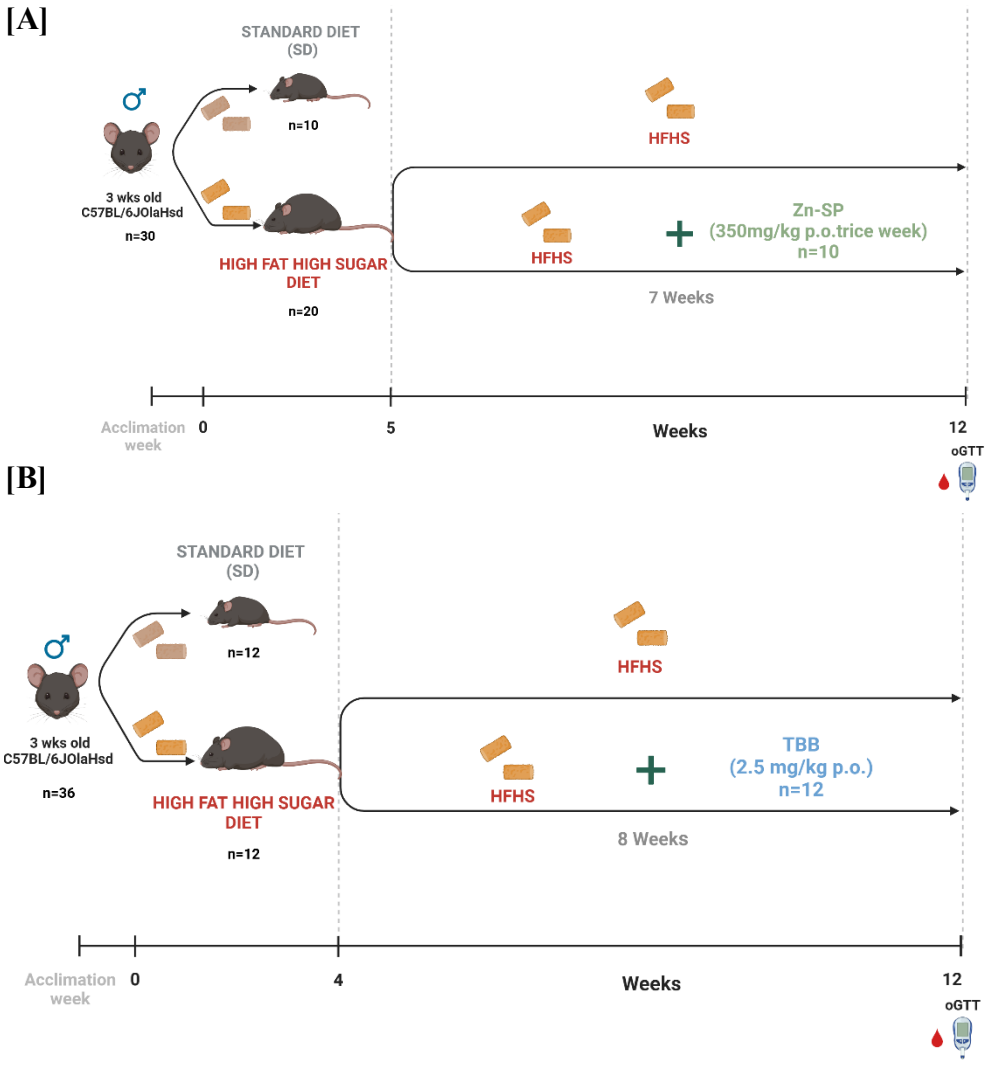

Figure 11. *In vivo* experimental protocols of Article 1(A) and Article 2 (B).

At the end of the experimental procedure, mice were individually allocated into metabolic cages for 12 hours in fasting conditions. Faeces were then collected for the microbiota analyses and mice were anesthetized with isoflurane (3%, IsoFlo, Abbott Laboratories, North Chicago, IL, USA), delivered in oxygen (0.4 L/min) and euthanized by cardiac exsanguination. Blood was collected into microtubes containing 5% ethylene-diamine-tetraacetic acid (EDTA,) and then centrifuged at 10,000× g at room temperature for 10 minutes to obtain plasma which was directly snap-frozen.

Liver and ileum content were harvested in tubes, snap frozen in liquid nitrogen and saved in the -80°C for the subsequent *ex-vivo* analyses. Sections of the hepatic tissue were collected in tubes embedded with a cryoprotective compound, the Optimal Cutting Temperature compound (OTC), frozen in liquid nitrogen and stored at -80°C for immunohistochemistry analysis.

3.2.1. Oral Glucose Tolerance Test

After an overnight fasting, but with free access to tap water, an oral glucose tolerance test (oGTT) was performed. In details, mice were administered orally a 30% D-glucose solution (2 g/kg). Blood was collected from the saphenous vein prior to glucose administration and at different timepoints (15, 30, 60, 120 min); and glucose concentration was measured with Accu-check Aviva glucometer (Roche Diabetes Care Italy S.p.A, Monza, Italy), which provides suitable approximation of plasma glucose levels. A schematic overview of the procedure is reported in Figure 12.

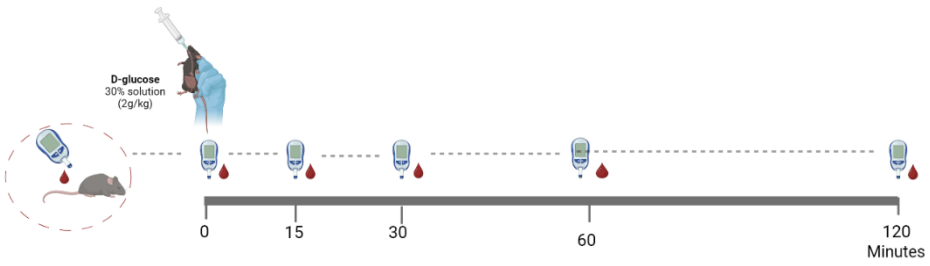

Figure 12. Schematic representation of oral glucose tolerance test (oGTT). The image was created with Biorender.

3.3. Biochemical analyses on plasma

3.3.1. Hepatic transaminases and lipid profile

To assess the potential effects of dietary intervention and of the treatments, plasmatic concentration of alanine aminotransferase (ALT) and aspartate aminotransaminase AST were evaluated. In details, plasmatic concentrations of ALT (#7018) and AST (#7036) were assessed with commercially available kits (FAR Diagnostic, Verona, Italy), according to the manufacturer's instructions.

For *article 2*, lipid plasmatic profile was monitored with colorimetric clinical assay kits (FAR Diagnostic, Verona, Italy) for the detection of total cholesterol (#7051) and triglycerides (#7136) levels.

3.3.2. Insulin plasmatic profile

Plasmatic levels of insulin were determined with enzyme-like immunosorbent assay kit ELISA (#EZRMI-13K, Millipore Corp., Billerica, MA, USA).

3.3.3. Pro-inflammatory cytokines panel

The evaluation of systemic inflammation markers was performed with different molecular techniques in the discussed articles. Specifically, in the article 1 the plasmatic levels of pro-inflammatory cytokines IL-6, IL-1β and INF-γ were assessed with ELISA commercially available kits. On the other hand, in the article 2 the same markers, along with the TNF-α and IL-17, were determined with Bio-Plex Pro™ Mouse Cytokine Th17 Panel A 6-Plex (#M6000007NY, Bio-Rad Laboratories, Inc., Hercules, CA 94547, USA), according to the manufacturer's instructions.

In details, **Bio-Plex technique** consists of a magnetic bead–based multiplex assay designed to measure multiple biomarkers in a minimal volume of plasma. Antibodies directed against the biomarkers are covalently coupled to magnetic beads, which are dyed with different fluorescent colours associated to the markers. Coupled beads, in the end, react with the plasmatic biomarkers of interest. After a series of washes, necessary for discarding the unbound protein, a sandwich complex is formed by adding a biotinylated detection antibody. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate, where the phycoerythrin is the as fluorescent

indicator. Data from the reactions are acquired using a Bio-Plex® System. which utilizes two lasers for simultaneous detection: a red laser (635 nm) to identify the beads, and a green laser (532 nm) to measure the fluorescent signal emitted by phycoerythrin. The intensity of the detected fluorescent signal is directly proportional to the concentration of the markers in the samples, which is expressed in pg/mL. Figure 13 outlines the steps of the BioPlex technique.

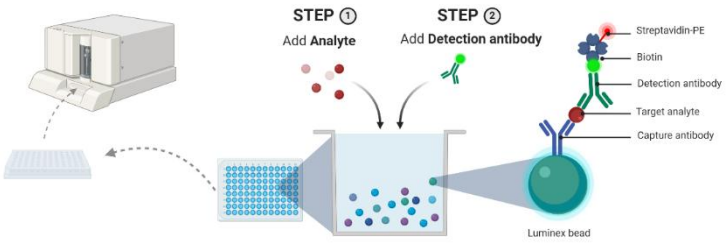

Figure 13. BioPlex principle and technique representation. The image was created with Biorender.

3.4. Western blot analyses

Semi-quantitative analyses of protein were performed in both article 1 and 2 on hepatic lysates, as previously reported [197–199].

3.4.1. Protein extraction

To obtain *total* protein extraction, about 0.05 g of hepatic tissue were homogenized with a Potter Evehjem homogenizer by adding a lysis buffer solution at 1:10 w/v ratio (NaCl 150 mM; Tris-HCl 50 mM pH7.5; EGTA 1 mM; Sodium Deoxycholate 0.25%; NP-40-IGEPAL 1%; Milli-q Water) containing protease and phosphatase inhibitors and centrifuged at 13,000× g for 25 min at 4 ◦C.

To obtain *cytosolic and nuclear* proteins, liver tissue was homogenized at 10% (w/v) in a specific lysis buffer (20 mM Hepes – KOH pH 7,9- 1 mM $MgCl_2$,0,5 mM EDTA,1 mM EGTA, 1% NP-40-IGEPAL) containing protease and phosphatase inhibitors and Dithiothreitol (DTT) with the aforementioned homogenizer, and centrifuged at 1000× g for 5 min at 4 ◦C. Supernatant were then collected and subjected to further centrifugation at 10,000× g for 40 minutes at 4°C, to isolate the cytosolic fraction. Meanwhile, the pellets, which contains the intact nuclei, were resuspended in an extraction buffer (20 mM Hepes – KOH

pH 7,9- 1,5 mM $MgCl_2$, 0,2 mM EDTA,1 mM EGTA, 20% glycerol 1% NP-40-IGEPAL) containing protease and phosphatase inhibitors and Dithiothreitol (DTT) and incubated on ice for 30 minutes to allow high salt extraction. In conclusion, a final centrifugation at 15,000× g for 20 minutes at 4°C allowed the isolation of the nuclear fraction (supernatants). Both the cytosolic and nuclear fractions are then harvested at −80°C until use.

Protein quantification was performed with the bicinchoninic acid assay (BCA assay), according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). This method relies on the colorimetric reaction that takes place between the Cu^{2+} ion and proteins. In details, Cu^{2+} ions are reduced in the presence of the proteins in alkaline conditions, that is visually seen as a blue complex. Next, the bicinchoninic acid reacts with the Cu^{2+} ions, forming purple complex. This BCA-copper complex is spectrophotometrically read at 562 nm: the absorbance is proportional to the protein concentration in the sample.

3.4.2. Sample preparation and electrophoresis

According to the results of the BCA assay, protein concentration was assessed and the corresponding volume to 50 µg was added in new tubes for each sample, along with Buffer 4x solution, dithiothreitol and bi-distilled water up to15 µL. Samples were boiled at 60 °C for 10 minutes and loaded into the into dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at the percentages of 8-10-12% for the running gel, while the stacking gel percentage was fixed at 4%. The electrophoresis was performed at constate voltage of (200 V) for 35 minutes.

3.4.3. Electro-transfer and immunodetection

Proteins were electro-transferred to a to polyvinylidene fluoride (PVDF) membrane (GE10600023 Amersham™ Hybond®® P Western blotting membranes, PVDF; Merck KGaA, Darmstadt, Germany), at constant voltage of 100 V for 70 min. Membranes were then incubated with Ponceau Red solution to confirm the correct transference of the proteins. After the removal of the Ponceau Red solution, membranes were washed under agitation 3 times for 5 minutes, with TBS-T solution (tris-buffered saline solution with 0,1% Tween®20 detergent) and incubated with a 10% w/v non-fat dried milk solution

prepared in TBS-T, under agitation, for 1 hour at room temperature. Excess of the blocking solution was then quickly removed, and membranes were incubated over-night with the primary antibody, prepared in TBS 1x solution (TBS 10 x diluted in Milli-Q water) containing NaN_3 as a preservative, under agitation at 4°C. Antibodies used are listed in the Table 3 (antibody dilution 1:500 or 1:1,000)

After a 3x5 minutes washing with TBS-T solution, membranes were incubated, under agitation, with horseradish peroxidase (HRP)-conjugated secondary antibodies (7074S, Anti-rabbit IgG, HRP-linked Antibody, 7076S Anti-rabbit IgG, HRP-linked Antibody, anti-goat 1721034S, Cell Signaling Technology, Inc., Denver, MA, USA) diluted in TBS 1x solution containing 5% non-fat dry milk (antibody dilution 1:5000 or 1:10,000) for 1 hour at room temperature .

Chemiluminescent detection of the proteins was assessed with Clarity and Clarity Max Detection Kit (Bio-Rad Laboratories, Inc., Hercules, CA 94547, USA) and quantified by using ImageLab Version 6.1.0 BioRad Laboratories Software and ImageJ software (ImageJ 1.54h; Java1.8.0_345[64 bit]); the results were normalized to standard diet fed mice values.

Code	**Antibody**	**Article**
PAB1293	anti-CML	1
KAL-KH025	anti-CEL	1
sc-365154	anti-RAGE	1
sc-13032	anti-Nrf2	1
Gtx15747	anti-Glo-1	1
8242S	anti-NFκB p65	1-2
AG-20B-0014-C100	anti-NLRP3	1-2
93709S	anti-Gasdermin	1
5174S	anti-GAPDH	1
4499S	anti-Histone H3	1
SAB4504299	anti- Tyr^{255}casein kinase II α	2
76040	anti casein kinase IIα	2
9246S	anti-$Ser^{32/36}$ IκBα	2

9242S	anti-total IκBα	2
24232S	anti-Caspase-1	2
sc-1511	anti-ICAM-1	2
4970S	anti-β actin	2
sc-73614	anti-vinculin	2
sc-25280	anti- PCNA	2

Table 3. List of primary antibodies used for Western blot technique. Primary antibodies were purchased from Abnova Corporation (Walnut, CA), Cosmo Bio (Carlsbad, CA), Santa Cruz Biotechnology, Inc. (Dallas,TX) , GeneTex, Inc. (Irvine, CA) Cell Signaling Technology, Inc. (Denver, MA), Adipogen Corporation(San Diego, CA).

3.5. Analyses on hepatic lysates

Hepatic lysates, obtained for the western blot technique, or freshly, according to the protocol, were used for the AGESs quantification and for the evaluation of antioxidant profile in article 1, and for the assessment of hepatic neutrophilic infiltration in article 2.

3.5.1. AGE quantification (Article 1)

AGEs quantification was performed in the laboratory of Prof. Dal Bello of the Department of Molecular Biotechnology and Health Sciences, University of Turin.

The quantification of free N"-carboxymethyl-lysine (CML) and N"-carboxyethyl-lysine (CEL in total liver extracts (n = 7/10 per group) was achieved by means of the liquid chromatography mass spectrometry (LC-MS). Specifically, after a hydrolysing step with 0.6 M trichloroacetic acid ($C_2HCl_3O_2$) and 6 M hydrochloric acid (HCl) for 12 h at 60° C, CML and CEL concentration were detected by LC-MS. The chromatographic separation was performed in an UltiMate™ 3000 HPLC system (Dionex, Milan, Italy) provided by a high-resolution LTQ Orbitrap mass spectrometer (Thermo Scientific, Rodano, Italy) with an atmospheric pressure interface and an electrospray ionization (ESI) source (Figure 14). The liver extracts were processed through a Phenomenex Synergi reverse-phase C18 column with dimensions of 150 × 2.1 mm and a particle size of 3 μm. The flow rate was set to 200 μL/min. The

composition of the gradient mobile phase was as follows: 95/5 to 40/60 in 25 min, 5 mM heptafluorobutanoic acid/acetonitrile. The monitored protonated molecular ions were 205.1188 m/z for CML and 379.2094 m/z for CEL. Calibration data obtained with CML and CEL standards were used for the quantitative determination of these analytes in the samples.

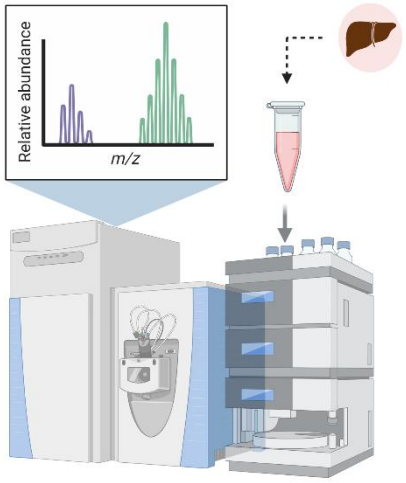

Figure 14. Liquid chromatography tandem mass spectrometry.

3.5.2. Glo-1 activity (Article 1)

Glo-1 enzymatic activity in total hepatic lysates was evaluated by spectrophotometric method, monitoring the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione for 2 min at 25 °C as previously performed [200, 201]. Specifically, an assay mixture containing 8 mM methylglyoxal (MGO) and 2 mM GSH in a sodium phosphate buffer (50 mM, pH 7.4) was incubated at 37 ◦C for 10 min to reach the equilibration of hemithioacetal formation before the addition of the protein extract (20–30 µg). One unit of activity is defined as the formation of 1 mmol of S-D lactoylglutathione/ min/mg cell protein.

3.5.3. Glutathione assay (Article 1)

Reduced glutathione (GSH) and total glutathione (GSSG) were spectrophotometrically detected (412 nm) in total hepatic homogenates according to Akerboom et al. [202].

Briefly, GSH and GSSG were determined throughout a kinetic assay: the catalytic amounts of GSH or GSSG and glutathione reductase generate a continuous reduction of 5,5′-dithiobis (2-nitrobenzoic acid, DTNB) by nicotinamide adenine dinucleotide phosphate (NADPH). The ratio of GSSG to GSH was used to estimate oxidative stress generated in our experimental procedure.

3.5.4. Myeloperoxidase assay (MPO) (Article 2)

Evaluation of *in vitro* myeloperoxidase activity was carried out as previously described [203]. About 0.1 g of hepatic tissue (n=8/10 group) were homogenized in 20 mM PBS (pH=7.4) and centrifuged (13000 x g, 10 min, 4°C) by means of Potter Evehjem homogenizer (1:5 w/v). Pellets were resuspended in 0.5 mL of 0.5% hexadecyltrimethylammonium bromide (HTAB) in PBS 50mM (pH=6) solution and underwent a final centrifugation step (13000 x g, 10 min, 4°C. Myeloperoxidase activity was assessed by measuring indirectly the 3.3 'dihydrochloride, 5.5'-tetramethylbenzidine (TMB) H_2O_2 dependant oxidation, that is visualised with the development of a blue colour. In details, 30 µL of the supernatants were plated on a 96-well plate. 180 µL of H_2O_2 (0.4 mM, prepared in 80 mM PBS, pH 5.4 to reach a concentration of 0.3 mM in the plate) were added to the each well and subsequently, 20 µl of TMB solution (18.4 mM, dissolved in water to reach a concentration of1.6 mM in the plate). After an incubation of 10 minutes at 37°C, TMB oxidation was measured at 650 nm every 2 minutes for 5 readings. Protein concentration was measured in the supernatants with the BCA assay. Data are expressed as optical densitometry (O.D.) at 650 nm for mg of protein.

3.6. Histological analyses and immunohistochemistry (Article 2)

3.6.1. Haematoxylin and eosin (H&E)

H&E staining was performed to estimate the inflammatory status of hepatic tissue.

Liver frozen tissue was cut in 10 µm cryostatic sections (Leica CM1950) and fixed in cold acetone for 2 minutes. Next, the sections were rinsed in distilled water, followed by an incubation step of 5 minutes in Mayer's haematoxylin solution. In order to stop the reaction, sections were rinsed in tap water for 5 minutes and counterstained with 0.05 % Eosin solution for 1 minutes followed

by a brief wash in distilled water. In conclusion, slices were dehydrated in ascending ethanol solutions (70-90-100%-1 minute) and fixed in xylene solution (1 minute). Slices were then mounted with DPX medium and coverslips. H&E staining was observed under a Zeiss Airyscan confocal microscope at the magnification of 10 and 20x (Zeiss, Göttingen, Germany).

3.6.2. Oil red O staining (ORO)

ORO staining was assessed for evaluating the local accumulation of lipids. For instance, the ORO solution is a lysochrome dye that is soluble in fat molecules, conveying its colouring to neutral fats and triglycerides. 10 µm thickness liver cryostatic sections were fixed for 10 minutes in cold 40 % formaldehyde solution. Slices were washed in 60% isopropyl alcohol and consequently stained in ORO for 15 minutes. Next, sections were washed in 60% isopropyl alcohol, followed by rinsing in distilled water. Slices were then counterstained with Harris' haematoxylin solution, mounted with 1:1 glycerol and water solution and coverslips. Stained tissues were viewed under an Olympus Bx4I microscope (10 and 40x magnification) with an AxioCamMR5 photographic attachment (Zeiss, Göttingen, Germany).

3.6.3. Immunohistochemical staining of MPO

Immunohistochemical detection of MPO was conducted on 10 µm liver cryostatic sections, fixed in cold acetone for 2 minutes. Endogenous peroxidase activity was blocked by adding H_2O_2 0.3% (diluted in PBS, 2 min). Next, a 30-minute incubation step with a 3% bovine serum albumin dissolved in PBS was performed to avoid non-specific binding. Slices were incubated with the primary antibody anti-MPO (1:30, #9535, Abcam, Boston, MA, USA), for 2 hours. Subsequently, 45 minutes of incubation with the anti-rabbit HRP-conjugated secondary antibody (1:400, #7074S) was performed. Diaminobenzidine (DAB) solution was used for chromogenically detecting MPO positive cells (2 minutes of exposure) in the tissue. Slices were counterstained with Mayer's haematoxylin solution for 5 minutes and viewed under a Zeiss Airyscan confocal microscope, at 10 and 20x magnification (Zeiss, Göttingen, Germany).

3.7. Real time q-PCR (qRT-PCR) (Article 2)

Total RNA was isolated from section of hepatic tissue (15 mg) by means of Animal Tissue RNA Purification Kit (#25700 Norgen Biotek Corporation

Thorold, ON, Canada), according to the manufacturer's instructions. RNA concentration was determined with a nanodrop Instrument (NanoDropOne, Thermo Fisher Scientific, Waltham,MA,USA). SensiFAST™ cDNA Synthesis Kit 50 reactions (#BIO-65053 Meridian Bioscience, USA) was used to obtained cDNA starting from RNA amount of 200 ng. Real-time qPCR was performed on 1 μL of cDNA with SensiFAST™ SYBR® No-ROX Kit (#BIO-98005, Meridian Bioscience, USA). CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA 94547, USA) was used for PCR reaction. Primers sequences (forward and reverse) sequences for the genes of interest (TNF-α, IL-1β, IL-6) are listed in Table 4 and were purchased from Biomers (Ulm, 89077, Germany). Relative gene expression was obtained after normalization to housekeeping gene GAPDH (#QT01658692, Mm_Gapdh_3_SG, QIAGEN Germantown, MD, EUA), using the formula $2^{-\Delta\Delta CT}$ as previously described [204]. Data are represented as fold change to standard diet group.

Forward TNF-α	5'-GCAGTTTCTGTCCCTTTCAC-3'
Reverse TNF-α	5'-CATTTGGGAACTTCTCATCCCT-3'
Forward IL-1β	5'-CTTTGAAGAGCCCATCCT-3'
Reverse IL-1β	5'-CCGTCTTTCATTACACAGGACAG-3'
Forward IL-6	5'-CTGATGCTGGTGACAACCAC-3'
Reverse IL-6	5'-TCCACGATTTCCCAGAGAAC-3'

Table 4. Primer sequences. 5' to 3' nucleotides sequences for forward and reverse primers of the investigated genes TNF-α, IL-1β, IL-6.

3.8. Zn-Spirulina composition and manufacturing (Article 1)

Arthrospira platensis (Spirulina) was cultivated in greenhouses in Unione Spirulina Biologica Italiana facility (USBI, Guidizzolo, Italy). In details, the alga

was obtained by filtration of its culture medium, which was enriched in zinc at the end of the Spirulina's growth period. The depotentiation method applied for ensuring the zinc enrichment was the antagonistic competition of the ions contained in the culture medium. Zinc assimilation was in keeping with the standard procedures that safeguard from any toxicity with a minimal increase in stress markers[205].

Following this step, 20 mL of the culture medium underwent a filtration through a pre-weighted 0.45 μM cellulose filter and dried at 48°C for 24 hours. Filter was reweighted and mineralized with nitric acid (1.5 mL) and sulfuric acid (1.5 mL) at 95° C for 30 minutes. A final filtration was performed, distilled water was added to reach a 50 mL volume and the solution was analysed with Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES) technique with the PerkinElmer Optima 8300 (Perkin Elmer Italia, Milan, Italy). In the table below (Table 5) the nutritional content of Zn-SP is listed.

Calories Kcal/100g Kj/100g	 348 1480
Total carbohydrates (g/100g) Dietary fiber Sugar	**24,2** < 1,0 <1,0
Total fat (g/100g) Saturated fat	**1,22** 0,56
Protein (g/100g)	60,05
Salt (g/100g)	2,09
Iron (mg/100g)	27
Potassium (mg/100g)	1640
Magnesium (mg/100g)	304
Phosphorus (mg/100g)	830
Zinc (Zn) (mg/100g)	200

Table 5. Zn-SP nutrients composition expressed in calories, macronutrients and micronutrients.

3.9. Statistical analyses

Data are expressed or as mean ± S.E.M for *n* observation, where *n* represents the number of animals studied. GraphPadPrism 7.0 software package was used for the data analyses (GraphPad Software 7.0, SanDiego, CA, USA). The normality of varibles' distributions was assessed with the Shapiro–Wilk test, while Bartlett's test was adopted for evaluating the homogeneity of the variances. To ensure the normality and the homogeneity of variances, a logarithmic transformation was employed to the data. One way ANOVA followed by Bonferroni's test were assessed. Non-parametric Kruskal-Wallis test, followed by Dunn's post-hoc analysis, was applied to data that were not normally distributed or exhibiting an heterogeneous in variance despite the logarithmic transformation. The standard level of significance was set up at at p value of < 0.05.

4. RESULTS

4.1. Evaluation of the potential effects of Zn-Spirulina administration in preventing metabolic inflammation

The AGE/RAGE cascade is a crucial event triggered by the consumption of unbalanced diets, eventually leading to harmful consequences associated with the accumulation of advanced glycation end products (AGEs). To mitigate these effects, novel antiglycation compounds are required, with natural products providing a valuable source of fascinating bioactive molecules. Cyanobacteria, particularly *Arthrospira platensis* (Spirulina), are a promising sources of high nutrient phytochemicals. Spirulina's antioxidant and ROS-scavenging properties may help to blunt AGEs accumulation, and its beneficial effects could be enhanced by zinc enrichment. However, the effects of Spirulina on glucose and inflammation in humans remain unclear, prompting further investigation on this green-blue alga's mechanism of action. This study aims to evaluate, for the first time, the antiglycation potential of a zinc-enriched Spirulina extract (Zn-SP) in a murine model of high-fat-high-sugar diet consumption.

4.1.1. Zn-SP ameliorates glucose handling capability impaired by HFHS chronic feeding

Chronic consumption of HFHS diet resulted in body weight gain, compared to SD fed mice (40.46 ± 0.76 *vs* 28.37 ± 0.18, $p<0.001$) that Zn-SP did not revert (40.86 ± 0.25). Dietary manipulation affected glucose handling capability, documented by higher fasting blood glucose levels (FBG-Figure15A) and glycaemia levels after 120 minutes the bolus of glucose, represented by the area under the curve (AUC-Figure15D); noteworthy, Zn-SP efficiently reduced both FBG levels and the AUC, hinting at a protective role in counteracting diet-induced alteration of glucose profile. Indeed, we documented a significant increase in the plasmatic levels of insulin in Zn-SP treated mice, compared to HFHS group (Figure 15B), which may explain the improved systemic glucose uptake.

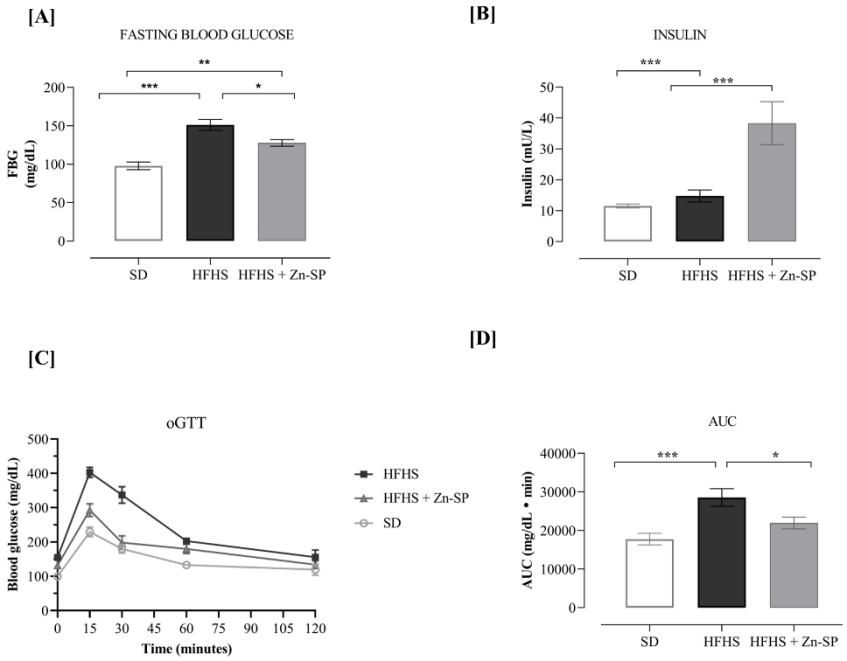

Figure 15. *Effects of dietary intervention and Zn-SP supplementation in mice*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 10–HFHS n = 20). 10 mice from the HFHS group received Zn-SP (350 mg/kg p.o.) three times a week for the last 7 week of the protocol. After an over-night fasting, fasting blood glucose and insulin were measured (A) and (B), and an oral glucose tolerance test was performed (C) and area under the curve (AUC) was calculated (D). Data are expressed as mean ± SEM. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.1.2. Zn-Spirulina alleviates hepatic injury preventing AGEs accumulation and enhances antioxidant and anti-glycation defences

Hyperglycaemic conditions evoked by HFHS consumption is recognized as one of the major triggers for the AGEs formation. We evaluated the accumulation of these molecules in both their free and bound-to-protein forms in the hepatic tissue. In details, we documented a higher accumulation of two of the most important AGEs, CML and CEL in protein bound forms, while for CEL the free form didn't reach a statistically significant level. (Figure 16A-D). Most notably, Zn-SP supplementation resulted in a reduction of both free and protein-bound CML and CEL back to values similar to those detected in SD group. In support of these results, a higher expression of RAGE receptor was assessed in HFHS group, meanwhile Zn-SP group presented a statistically significant reduction in the protein expression (Figure 16E).

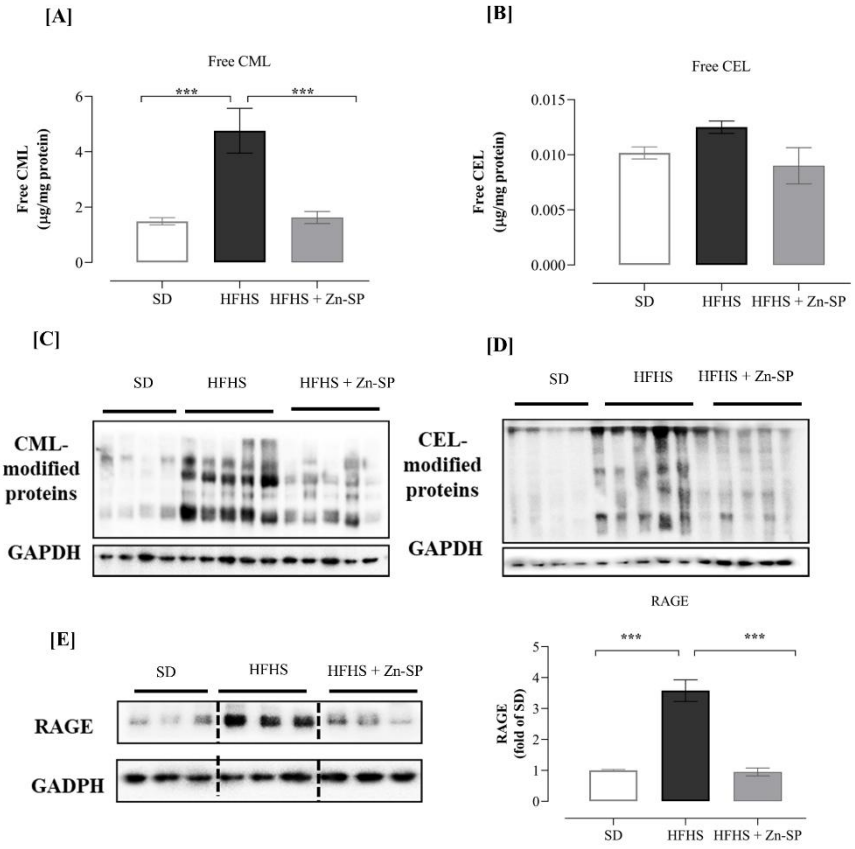

Figure 16. *Effects of dietary intervention and Zn-SP supplementation on AGEs hepatic accumulation*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 10–HFHS n = 20). 10 mice from the HFHS group received Zn-SP (350 mg/kg p.o.) three times a week for the last 7 week of the protocol. CML (A) and CEL (B) concentration were detected by HPLC/MS and the results were normalized to the amount of total protein. Western blotting analysis for CML-modified proteins (C), CEL-modified proteins (D), and RAGE (E). Densitometric analyses of the bands were normalized to corresponding GAPDH density. Data are expressed as mean ± SEM of 4–5 mice per group. Statistical significance: *** $p < 0.001$. All data are expressed as mean ± SEM. Statistical significance: *** $p < 0.001$.

Another key process to evaluate for unravelling the impact of AGEs on diet-induced metabolic abnormalities is to verify the antioxidant defences that could be compromised by dietary manipulation. We evaluated the protein expression of Nfr2, the most important transcription factor involved in the production of antioxidant enzymes such as glyoxalase 1 (Glo-1), documenting a reduction in the hepatic nuclear extracts of HFHS fed mice, compared to SD group, suggesting a decrease in the antioxidant defences (Figure 17A). Surprisingly, Zn-SP supplemented mice presented an increased in Nrf2 nuclear expression, compared to HFHS, hinting at a potentiation of its transcriptional activity. This

result is consistent with the evaluation of the expression of the downstream target gene, Glo-1, which was reduced in a statistically significant manner in HFHS, compared to SD, and restored back to levels recorded in SD group (Figure 17A).

Next, we evaluated the Glo-1 activity, to estimate the effective antioxidant efficiency as a result of the increased nuclear-to-cytosol ratio in Zn-SP group. We documented a statistically significant higher Glo-1 activity in Zn-SP group, compared to HFHS, which instead fails to be upregulated due to its higher cytosolic retention (Figure 17B). The antioxidant effect of Zn-SP was further strengthened by the reduced GSSG/GSH ratio, being known that GSH is a key co-enzyme in the Glo-1 mediated detoxifying reaction, compared to HFHS group, which presented a higher glutathione in its oxidized form (Figure 17C).

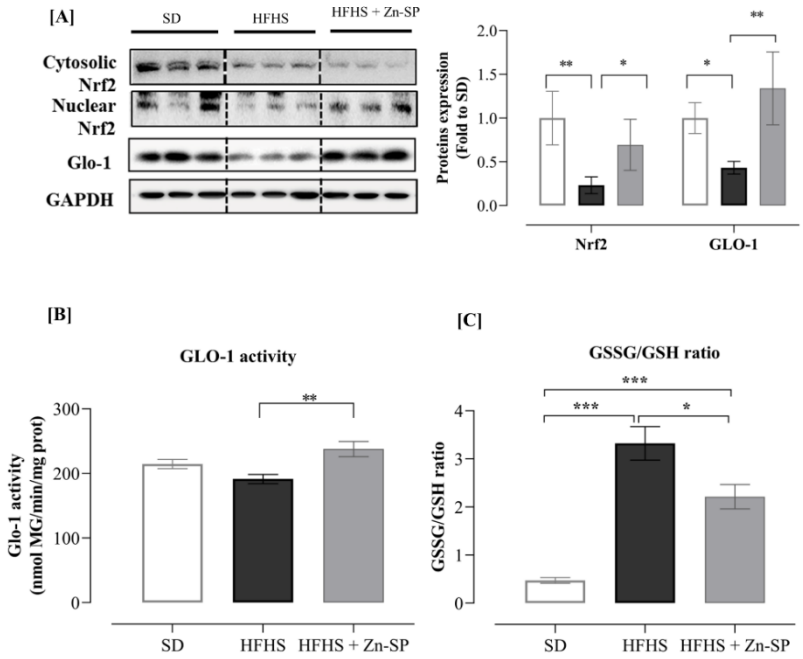

Figure 17. *Effects of dietary intervention and Zn-SP supplementation on hepatic antioxidant defences*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 10–HFHS n = 20). 10 mice from the HFHS group received Zn-SP (350 mg/kg *p.o.*) three times a week for the last 7 week of the protocol. Western blotting analysis for Nrf2 and Glo-1 (A). Densitometric analyses of the bands were normalized to corresponding GAPDH density. Data are expressed as mean ± SEM of 4–5 mice per group. Glo-1 enzymatic activity (B) GSSG/GSH ratio (C) were assayed spectrophotometrically. Data are expressed as mean ± SEM of 7-10 mice per group. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.1.3. Zn-Spirulina counteracts the overactivation of pro-inflammatory signaling in liver evoked by HFHS diet and the parallel increases of blood markers of inflammation

Diet-induced accumulation of AGEs resulted in the activation of pro-inflammatory signalling cascades at hepatic levels, as documented by an increased in the NFκB nuclear translocation and a higher expression of NLRP3 inflammasome and its downstream effector, gasdermin, compared to SD (Figure 18). Noteworthy, Zn-SP supplementation reduced the expression of these well-known proinflammatory mediators, confirming its key role as an anti-inflammatory compound.

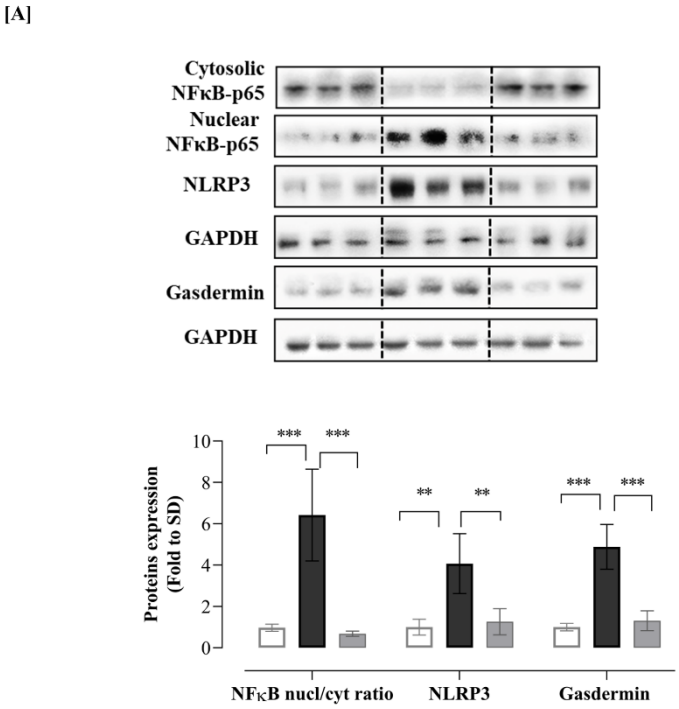

Figure 18. *Effects of dietary intervention and Zn-SP supplementation on pro-inflammatory signalling cascades.* Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 10–HFHS n = 20). 10 mice from the HFHS group received Zn-SP (350 mg/kg p.o.) three times a week for the last 7 week of the protocol. Western blotting analysis for NFκB-p65, NLRP3, Gasdermin. Densitometric analyses of the bands were normalized to corresponding housekeeping density. (GAPDH for total and cytosolic extracts, Histone H3 for nuclear proteins). Data are expressed as mean ± SEM of 4–5 mice per group. Statistical significance: **p < 0.01; *** p < 0.001.

The analyses at local levels were paralleled by the investigation of the effects of dietary manipulation and Zn-SP supplementation in plasma. For instance, as reported in Table 6, HFHS fed mice presented a robust increases of pro-

inflammatory cytokines IL-6, IL-1β and INFγ, supporting the evidence of the dramatic effects of chronic consumption of hypercaloric diet on the inflammatory profile. Most notably, Zn-SP group showed statistically significant reduced levels of these latter, hinting at an amelioration of systemic inflammation. To further strengthened the connection between peripherical (plasma) and local alterations (liver) we detected higher plasmatic levels of transaminases AST and ALT in HFHS fed mice, compared to SD, suggesting an organ injury and disfunction, that, on the other hand, was ameliorated by Zn-SP chronic administration (table6).

	SD	**HFHS**	**HFHS + Zn-SP**
IL-6 (pg/mL)	4,16±0,53	9,66±1,73**	3,86±0,68°°
IL-1β (pg/mL)	0,29±0,05	0,87±0,15***	0,20±0,03°°°
INF-γ (pg/mL)	0,66±0,14	4,03 ±0,75***	1,50±0,66°
AST (U/L)	16,15±1,64	89,29±12,22***	29,08 ±2,58°°°
ALT (U/L)	10,94±1,75	33,88±6,31***	14,97±2,06°°

Table 6. *Effects of dietary intervention and Zn-SP supplementation on systemic inflammation*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 10–HFHS n = 20). 10 mice from the HFHS group received Zn-SP (350 mg/kg p.o.) three times a week for the last 7 week of the protocol. Plasmatic levels of IL-6, IL-1β, INFγ were measured with ELISA kits and transaminases (AST-ALT) were detected spectrophotometrically with commercially available kits. Data are expressed as mean ± SEM of 8–10 mice per group. Statistical significance: **p < 0.01 vs SD; *** p < 0.001 vs SD; ° p< 0.05 vs HFHS, °°p < 0.01 vs HFHS; °°° p < 0.001 vs HFHS.

Overall, we evinced for the first time the antiglycation property of *Arthrospira Platensis,* suggesting a novel mechanism of action of the compound. Indeed, the abundant antioxidants and ROS scavengers phytocomplexes of this green-blue alga, corroborated by the enrichment in zinc, could combine their activity, resulting in the reduction of endogenous AGEs accumulation and in the disruption of AGE/RAGE cascade activation, that we observed in our study. Moreover, the beneficial effect of Zn-SP supplementation extends also to its ability to reinforce detoxifying mechanism of AGEs accumulation namely the Nrf2-Glo-1 system. Most notably, the downstream inactivation of two well-distinguished pro-inflammatory pathways, NFκB and NLRP3, consequent to the

unbalance of AGE/RAGE interaction driven by Zn-SP, highlighted the intriguing protective role of this latter in alleviating diet-induced hepatic and systemic inflammation, and in the consequent amelioration of glucose handling capability. In conclusion, with this study we identified a novel antiglycation agent, the zinc enriched Spirulina, and propose a new mechanism of action for this microalga as a non-medicinal intervention for halting metabolic inflammation.

4.2. Targeting casein kinase 2 (CK2) as a potential strategy for counteracting metaflammation

In the previous presented article, we identified a natural compound, Zn-SP, that counteracts diet-induced activation of NFκB and NLRP3. Nevertheless, these beneficial effects were a secondary consequence of the antiglycation property of Zn-SP, urging for further identify novel mediator that can directly affect the overactivation of these pathways, known for their deleterious effect in metabolic inflammation. For instance, on the second part of my doctoral project, I focused on the evaluation of a selective pharmacological target of casein kinase II (CK2), whose activity is crucial in promoting simultaneously the activation of different inflammatory cascades, such as NFκB. In support to this hypothesis, kinases are arising regulators governing a wide range of homeostatic cellular processes and their dysregulation has been proven in a plethora of human diseases. Specifically, CK2 dysfunctional regulation has already been proven in different inflammatory-driven pathologies and effective CK2 inhibitors with a good efficacy and safety profile are available. In the light of this, we unveil the CK2 role in the onset of metaflammation and the potential effect of CK2 inhibition with 4,5,6,7 tetrabromobenzotriazole (TBB) in a murine model of hypercaloric diet consumption.

4.2.1. Pharmacological inhibition of CK2 improves glucose and lipid profile.

HFHS fed mice presented a higher body weight compared to SD group (44,32 ± 0,82 *** vs 27,89 ± 0.46, $p<0.001$ vs SD), as a result of higher caloric intake, compared to SD (5,56 kcal/g vs 3.85 kcal/g). Despite a slight, but not statistic, decrease in the body weight in HFHS+TBB group, (42,44 ±0,97 vs 44,32 ± 0,82), pharmacological intervention with TBB did not impact on body weight gain.

HFHS chronic consumption resulted an altered glycaemic profile, evinced by higher fasting blood glucose levels and impaired response to oral glucose tolerance test, compared to SD fed mice (Figure 19A-C). Pharmacological intervention with TBB reduced, in a statistically significant manner, fasting blood glucose levels and overall, the glycaemia at the end of the oGTT,

compared to HFHS fed mice, hinting at its potential role in restoring diet-induced glucose intolerance.

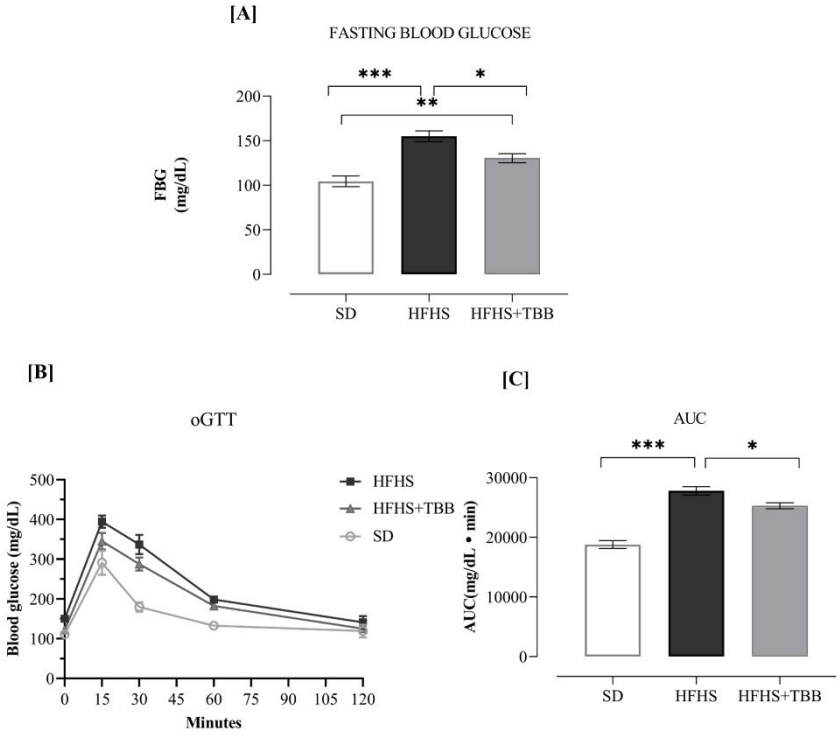

Figure 19. *Metabolic parameters at the end of the experimental protocol*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 15–HFHS n = 30). 15 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. After an over-night fasting, fasting blood glucose (A) and an oral glucose tolerance test was performed (B) and area under the curve (AUC) was calculated (C). Data are expressed as mean ± SEM. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Intriguingly, TBB administration effectively counteracted diet-induced alteration of systemic lipid profile. Indeed, the dramatic increase of total cholesterol and triglycerides (Figure 20 A-B), documented in the HFHS group in comparison to SD, was counteracted by the TBB compound, suggesting a potential protective role of the treatment in the alleviation of dyslipidaemia. In support of these systemic results, Oil red O staining of hepatic sections evinced the massive lipid local accumulation in HFHS fed mice, compared to SD fed ones, that was partially reverted by TBB administration (Figure 20C). Moreover, the quantification of ORO-stained lipid droplets confirmed the higher ORO positive area in HFHS group,

compared to SD fed mice, and the reduced accumulation of ORO dye in the liver of TBB treated mice (Figure 20D).

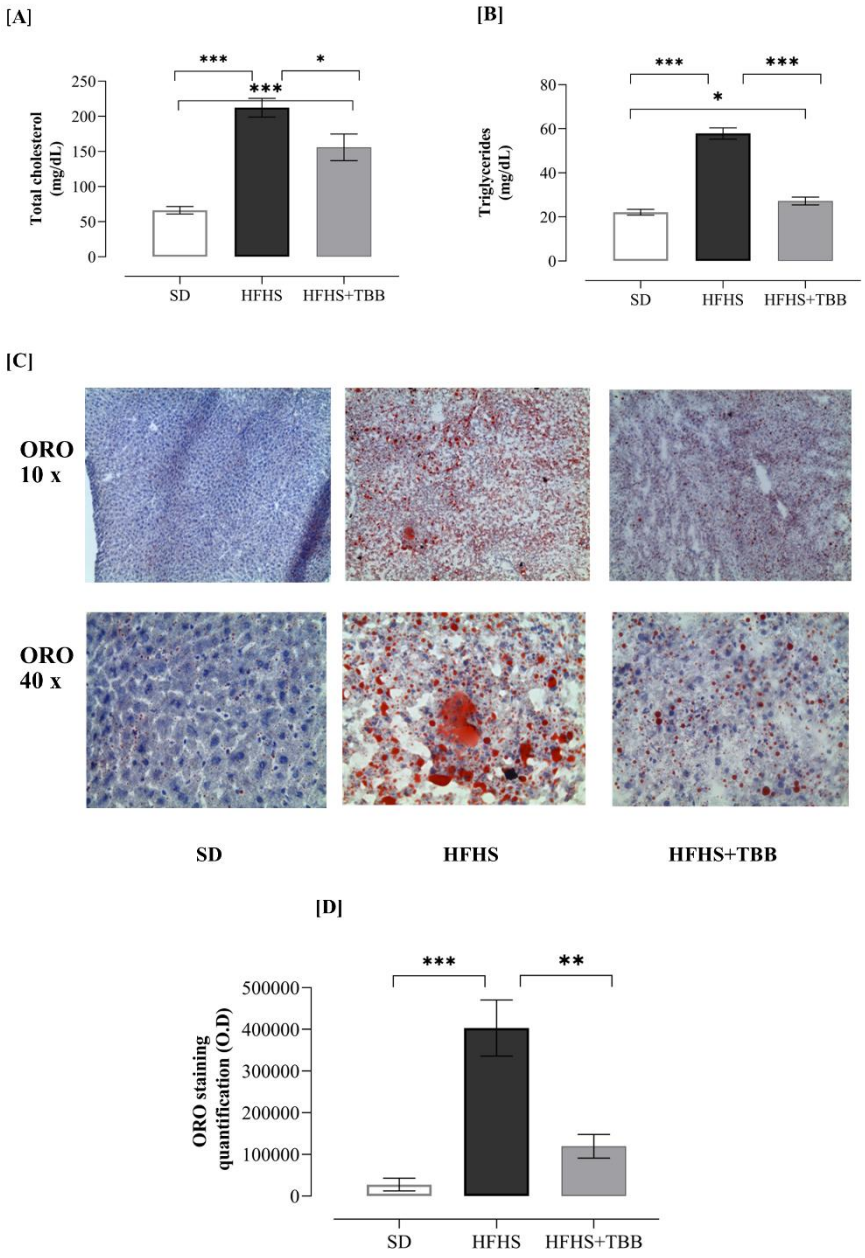

Figure 20. *Effects of HFHS chronic consumption and TBB treatment on lipid profile*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 12–HFHS n = 24). 12 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. Total cholesterol and triglycerides were measured (A) and (B) in plasma with commercially available kits; data are expressed as mean ± SEM of n=12/group; Representative photomicrographs of ORO hepatic sections. ORO staining was performed on 10 µm hepatic section *n*=4/5 mice/group (C) lipid accumulation was calculated (D) with ImageJ. on 40x

images, viewed under an Olympus Bx4I microscope with an AxioCamMR5 photographic attachment Data are expressed as mean ± SEM of n=4-5 mice/group. Statistical significance: **, $p<0.01$, *** $p < 0.001$.

4.2.2. TBB administration alleviates diet-induced hepatic injury.

Chronic exposure to HFHS diet resulted in a hepatic injury, as documented by statistically significant increase in plasmatic levels of transaminases AST and ALT, compared to SD fed mice (Figure 21 A-B). Interestingly, TBB intervention impacted on hepatic functionality, reducing in a statistically significant manner the levels of both AST and ALT. These systemic results on liver function were further supported with a qualitative analysis of local inflammation with immunohistochemistry techniques. For instance, we assessed a higher presence of inflammatory cells, in haematoxylin and eosin-stained tissues from HFHS group, compared to SD; noteworthy, TBB administration resulted in a reduction of the inflammatory status (Figure 21C). These qualitative results are in line with the detection, in HFHS fed mice, of a higher expression of ICAM-1 protein, a well-known adhesion molecule that recruits inflammatory cells in situ, compared to SD group, whereas the pharmacological intervention with TBB reduced its overexpression, suggesting a decrease in immune cells' enrolment (Figure 21A). In order to characterize the populations of immune cells involved, we evaluated the activity and expression of an indirect marker of leukocytes infiltration and lipid peroxidation, the myeloperoxidase (MPO). Indeed, both MPO activity and expression was increased by dietary manipulation, compared to SD, while HFHS+TBB group displayed a remarkable and statistically significant reduction of these levels (Figure 22B-C).

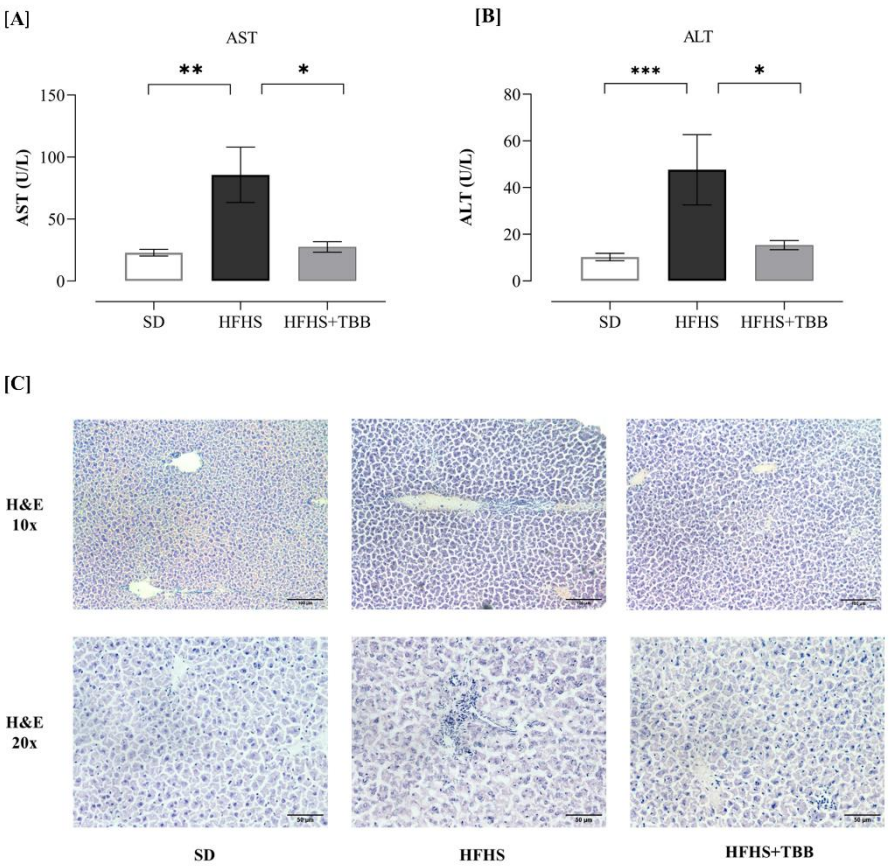

Figure 21. *Effects of HFHS chronic consumption and TBB treatment on hepatic inflammation.* Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 12–HFHS n = 24). 12 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. AST (A) and ALT (B) were detected in plasma with commercially available kits; data are expressed as mean ± SEM of n=8/10 group, statistical significance: * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$. H&E was performed on 10 μm hepatic section. Stained tissues were viewed under a Zeiss Airyscan confocal microscope at the magnification of 10 and 20x (Zeiss, Göttingen, Germany), n=4/5 mice/group.

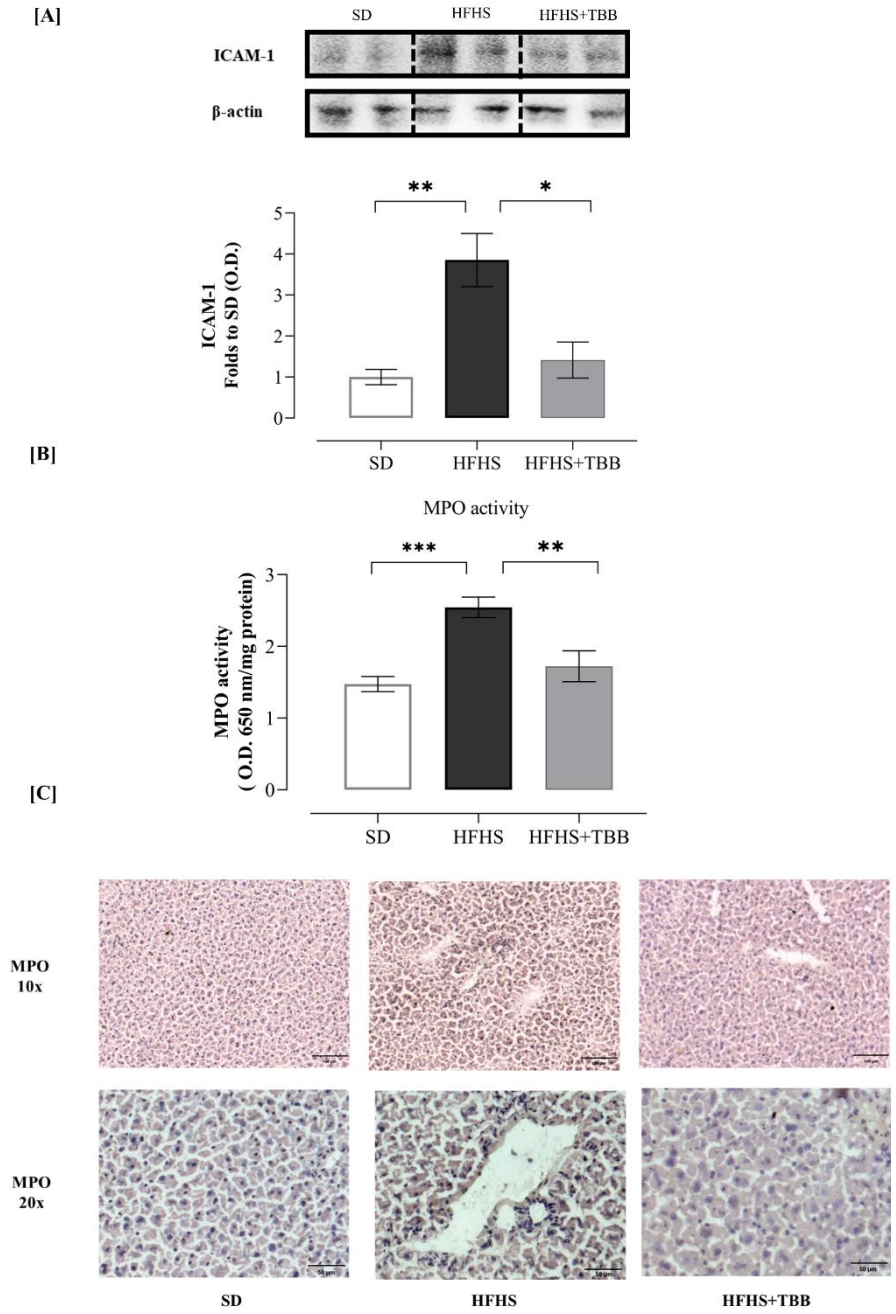

Figure 22. *Effects of HFHS chronic consumption and TBB treatment on ICAM-1 expression and leukocytes infiltration*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 12–HFHS n = 24). 12 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. 15 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. Western blot analysis of ICAM-1 normalized to β-actin (A) Densitometric analysis of the bands is expressed as relative optical density (O.D.). Data are expressed as mean ± SEM of 4-5 mice per group. MPO activity was assessed using an in vitro assay (B) and data are expressed as mean ± SEM of n=10 mice/group. Statistical analysis * $p<0.05$, ** $p<0.01$, ***$p<0.001$. (C) Representative photomicrographs of MPO immunostaining. Stained tissues (4/5 mice/group) were observed under a Zeiss Airyscan confocal microscope at the magnification of 10 and 20x (Zeiss, Göttingen, Germany).

4.2.3. CK2 inhibition ameliorates liver inflammation affecting the activation of pro-inflammatory signaling cascades.

Convincing evidences suggest CK2 as a crucial kinase that participates in the cross-talk of well-known cascades involved in the onset of metaflammation. Indeed, we assessed the contribution of CK2 in the activation of NFκB and NLRP3 cascades.

In HFHS group, we documented a statistically significant increase in the phosphorylation status on the serine residues 32/ 36 of IκBα (without changes in the total form), indicative of a higher degradation of the protein itself and a consequent reduced cytosolic retention of p65- NFκB subunit, compared to SD group (Figure 23A-B). In support of this, the nuclear-to-cytosol ratio was massively increased by HFHS feeding, providing evidence of a higher nuclear translocation of p65- NFκB subunit and of a boosted transcriptional activity of pro-inflammatory genes. Interestingly, TBB administration effectively counteracted the overactivation of IκBα, without affecting the expression of its total form, and reduced the nuclear expression of p65- NFκB, compared to HFHS group, hinting at a protective effect against the diet-induced activation of NFκB cascade.

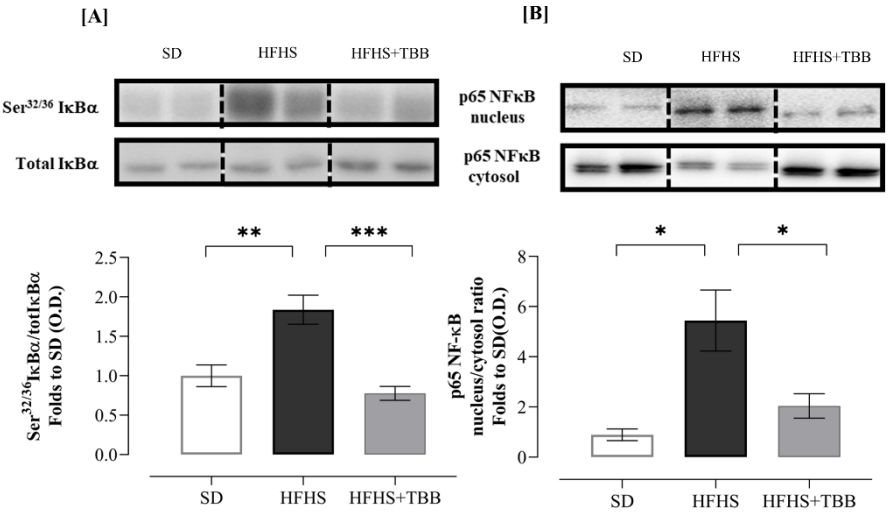

Figure 23. *Effects of HFHS chronic consumption and TBB treatment on NFκB cascade.* Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 12–HFHS n = 24). 12 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. Western blot analysis of $Ser^{32/36}$ IκBα (A) and p65- NFκB (B). Densitometric analysis of the bands is expressed as relative optical density (O.D.). Data are expressed as mean ± SEM of 4-5 mice per group. Statistical analysis: * $p< 0.05$, ** $p<0.01$, ***$p<0.001$.

Being *Nlrp3* a target gene of NFκB and the key pathogenic role of NLRP3 complex in metaflammation, we investigated the effect of the pharmacological inhibition of CK2 on NLRP3 and on its effector, caspase-1. We reported a statistically significant higher expression of NLRP3 following HFHS chronic exposure, compared to SD (Figure 24A). Moreover, HFHS group displayed an increased cleavage of caspase-1, compared to SD fed mice (Figure 24B). Most notably, pharmacological intervention with TBB impacted on the expression of both NLRP3 and cleaved caspase-1, suggesting an indirect protective role on inflammasome cascade.

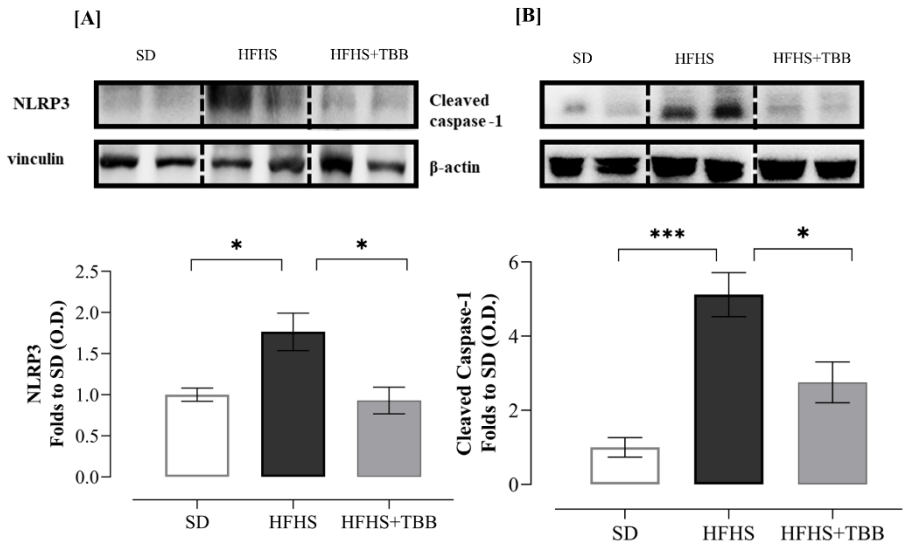

Figure 24. *Effects of HFHS chronic consumption and TBB treatment on NLRP3 inflammasome cascade*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 12–HFHS n = 24). 12 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. Western blot analysis of NLRP3(A), cleaved caspase-1(B) normalized to vinculin or β-actin. Densitometric analysis of the bands is expressed as relative optical density (O.D.). Data are expressed as mean ± SEM of 4-5 mice per group. Statistical analysis: * $p< 0.05$, ** $p<0.01$.

4.2.4. TBB administration counteracts the hepatic overactivation of CK2 α catalytic subunit

We documented the effect of HFHS chronic intervention on the activation of CK2 catalytic subunit α, as reported in Figure 25. Specifically, HFHS fed mice presented an increase in the phosphorylation of CK2α on Tyr255, when

compared to SD group. The dietary manipulation did not affect the expression of CK2 total form, evincing, in our model, an imbalanced kinase activity. This overactivation status was reverted in TBB treated mice, compared to HFHS, in a statistically significant manner. This relevant outcome suggests, for the first time, the impact of dietary manipulation on CK2 activation and confirms the effectiveness of TBB administration as a potent kinase inhibitor.

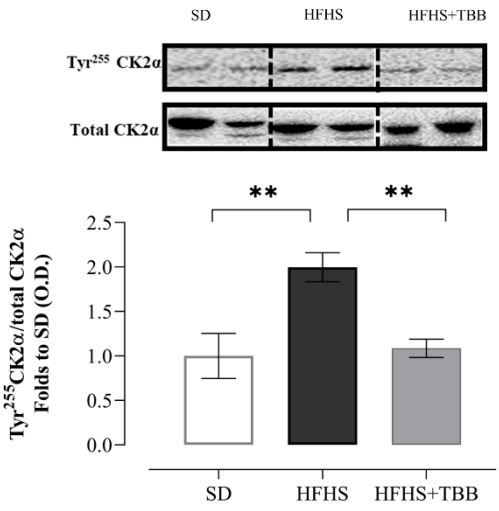

Figure 25. *Effects of HFHS chronic consumption and TBB treatment on CK2α activation.* Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 12–HFHS n = 24). 12 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. Western blot analysis of CK2α (phosphorylated and total forms). Densitometric analysis of the bands is expressed as relative optical density (O.D.). Data are expressed as mean ± SEM of 4-5 mice per group. Statistical analysis: ** $p<0.01$.

4.2.5. CK2 inhibition affects local and systemic cytokines levels impaired by HFHS chronic consumption

The detrimental effects of HFHS diet consumption were further confirmed by the qRT-pcr results on hepatic pro-inflammatory cytokines. For instance, when compared to SD group, HFHS fed mice displayed an increase in mRNA content of TNFα, IL-1β, and IL-6(Figure 26A-C). On the other hand, TBB intervention affected the mRNA cytokines levels, as documented by a reduction in a statistically significant manner.

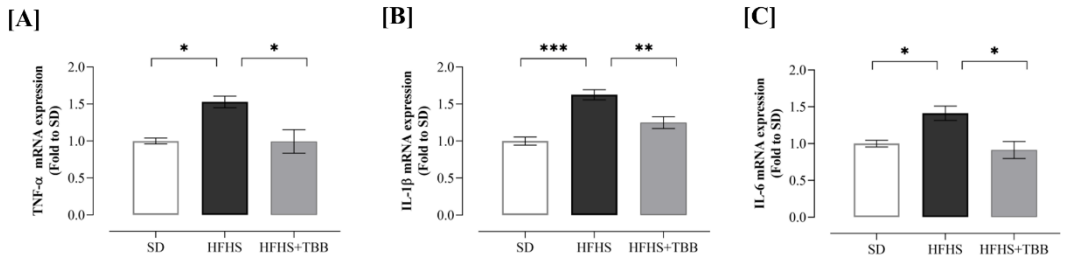

Figure 26. *Effects of HFHS chronic consumption and TBB treatment on mRNA expression of pro-inflammatory cytokines*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 12–HFHS n = 24). 12 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. RT-qPCR was performed for the: TNF-α(A), IL-1β(B), IL-6(C) genes. Relative gene expression was normalized to GAPDH gene, using the formula 2-ΔΔCT and folds change was determined by comparison to SD group. Data are expressed as mean ± SEM of 5-6 mice per group. Statistical analysis: * $p< 0.05$, ***$p<0.001$.

These fascinating outcomes of the effects of CK2 inhibition on local inflammation were paralleled by the systemic evaluation of inflammatory profile. As shown in Figure 27, HFHS presented a massive increase of plasmatic concentration of TNFα, IL-1β, IL-6, INF-γ and IL-17, compared to SD, suggesting the presence of a sterile inflammatory status evoked by hypercaloric diet consumption: noteworthy, TBB administration was effective in counteracting the abnormal increase of plasmatic cytokines, confirming the potential beneficial role of CK2 inhibition in ameliorating both local and systemic inflammation.

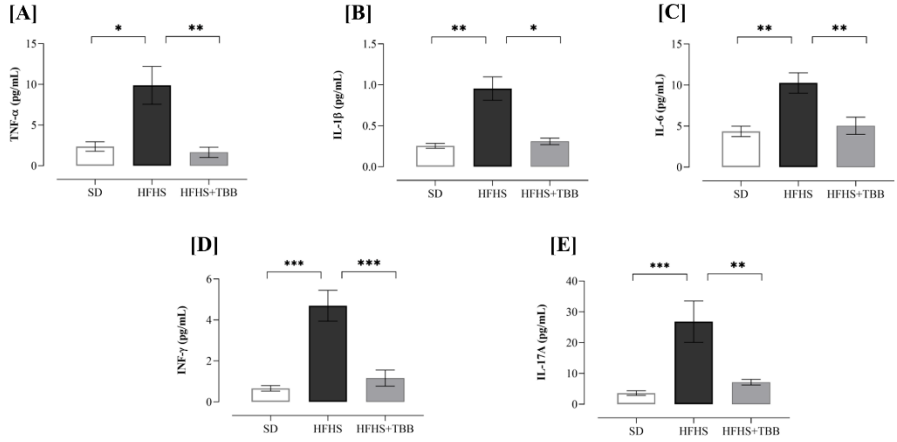

Figure 27. *Effects of HFHS chronic consumption and TBB treatment on systemic inflammatory profile*. Mice were fed with either SD or HFHS diet for 12 weeks (SD n = 12–HFHS n = 24). 12 mice from the HFHS group received TBB (2.5 mg/kg p.o.) for the last 8 week of the protocol. TNF-α (A), IL-1β (B), IL-6(C), INFγ(D), IL-17(E) were evaluated in plasma by using Luminex suspension bead-based multiplexed Bio-Plex Pro™ Mouse Cytokine Th17 Panel A 5plx XPL assay. Data are expressed as mean ± SEM from 5-10 mice per each group. Statistical analysis: * $p < 0.05$, *** $p<0.001$.

Altogether, with this work we demonstrated, the crucial involvement of casein kinase II (CK2) in the onset of metabolic inflammation, indeed confirming the central role of kinases in the pathogenesis of inflammatory disorders. The novelty of this study relies, most notably, in the beneficial effects consequent to the selective inhibition of this pleiotropic kinase, known for its almost unique effect on the simultaneous activation of different detrimental signalling cascades. For instance, the alleviation of hepatic inflammation, documented in TBB treated animals, was the direct effect of the disruption of CK2 downstream crosstalk with NFκB and NLRP3 and subsequent reduction of cytokines' local production and systemic release. One of the most fascinating outcome of this study was the identification of a CK2 inhibitor, TBB, responsible of the restoration of both glycaemic and lipid alterations, suggesting that the CK2-mediated attenuation of the latent chronic inflammation resulted in the amelioration of metabolic profile, convincingly strengthening the close parallelism between reduction in cytokines and improvement in metabolic functions. Overall, in this second set of experiments we recognized and

investigated a new potential therapeutic tool, casein kinase II (CK2) and assessed its involvement in the patho-mechanisms of metaflammation, and an effective pharmacological agent, TBB, as a valid therapeutical approach for blunting diet-induced metabolic abnormalities.

5. DISCUSSION

Nowadays, no one-size-fits-all medication has been found for the treatment of diet-induced related disorders, due to the complex etiopathogenesis of the diseases [206, 207]. The urgent need for novel therapeutical agents has prompt the scientific community to unravel the insight mechanisms that tightly regulate the onset of these pathologies, for identifying potential target to stop the global burden of these lifestyle associated disturbances. In the light of this, the crucial focus of my doctoral thesis was to strengthen and reinforce the central role of metaflammation as a key driver of metabolic associated disorders elicited by unbalanced nutrient overload, in order to define new potential regulators of this detrimental process. Thus, the results here reported clearly showed how it is possible to halt metabolic disturbances by targeting inflammatory-associated mediators adopting two distinct approaches. We demonstrated, on one side, that Zn-SP convincingly dampened the harmful effects associated with hypercaloric diet consumption, suggesting that the implementation of this supplement in individuals at lower risk for metabolic diseases' development, such as obesity and T2D, could be an easy-to-handle preventive approach for blunting severe metabolic disturbances. Despite a nutraceutical intervention could be important also for the introduction of high-quality nutrients in the dietary regimen, a pharmacological strategy has to be pursued for patients and people with high vulnerability to cardiovascular complications. Thus, on the other side, we deepened the investigation on one of the crucial hub of communication of the intracellular inflammatory system, the CK2 enzyme, which is a relevant breakthrough, thanks also to the availability of different drugs targeting its overactivation, among which TBB represents a valid candidate. The results presented in both sections of this doctoral thesis further confirm and extent the well-known pivotal roles carried out by the two detrimental regulators, the advanced glycation end products (AGEs) and the kinase CK2, suggesting novel potential tools for counteracting diet-induced harmful effects. For instance, we evinced, for the first time, the antiglycation property of zinc enriched Spirulina (Zn-SP), highlighting the protective effects of the green-blue alga and, most notably, pointing at a different and novel mechanism of action by which

Spirulina exerts its protective effect against metabolic dysfunctions. Indeed, we documented the ability of Zn-SP in blunting endogenous AGEs formation and consequent activation of its downstream interaction with RAGE. Our results further extend previous finding on the potential application of similar microalgae-derivatives, such as phycocyanin C [125], and offer a deeper insights on the molecular mechanisms underlying the protective effects of Spirulina that has been already documented in metabolic-driven alterations[135, 156]. In accordance with our study, a very recent work by Paramanya and colleagues, intriguingly highlighted the antiglycation activity of Spirulina by analysing *in vitro* the effects of an aqueous extract on the glycation of bovine serum albumin mediated by hyperglycaemic conditions [208]. These data confirmed that the microalga's beneficial outcome of counteracting AGEs accumulation is not attributable to only one component, but instead to a multifaceted action orchestrated by his plethora of components, in contrast to other approaches whose antiglycation activity is ascribable to a single component. Moreover, our alternative strategy for blunting AGE/RAGE cascade further supports the concept of the natural realm as a resourceful field for the identification of novel antiglycation compounds and potentially enlarge the current non-pharmacological options for counteracting their endogenous accumulation, such as pyridoxamine, benfotiamine and carnosine [209, 210]. The identification of an alternative tool could represent a valuable scenario, particularly given the absence of drugs approved targeting RAGE. Moreover, the zinc enrichment is a further element that boosts Spirulina's effectiveness as an antiglycative compound, owing its effect to its role as essential co-factor in enzymatic reactions [149, 150]. Another fascinating and innovative outcome of the present PhD project is that Zn-SP boosted the antioxidative defences altered by hypercaloric chronic consumption, documenting the protective effect of Zn-SP on Nrf2-Glo1 axis at hepatic level. This effect suggests a complementary mechanism of action of the tested extract in promoting AGEs-detoxifying defences: the Zn-SP driven reduction of hepatic AGEs accumulation dampened AGE/RAGE interaction, affecting ROS production, GSH availability and restoring Glo-1 detoxification cycle. In support to this, the expression of Glo-1 gene is regulated by Nrf2, which in turns we documented to be in a higher into the nuclei of Zn-SP treated mice, compared to HFHS fed ones, hinting at an

increased Nrf2-mediated transcriptional activity for Glo-1. Moreover, our findings proved how it is possible to halt the activation of NFκB/NLRP3 cascade by reducing the oxidative status and consequent activation of both signalling cascades, with a non-selective targeting that prevents the pro-oxidative status that promote both NFκB and NLRP3 activation [211, 212]. Nevertheless, these results clearly strengthened the mutual correlation between low-grade inflammation and metabolic disturbances, documented here by the beneficial effects of Zn-SP in the parallel amelioration of systemic inflammatory and glycaemic profiles, and propose the supplementation with *Arthrospira Platensis* enriched in zinc as the driver of the restoration of metabolic inflammation in a murine model of hypercaloric diet consumption.

In the second set of data presented in my doctoral thesis, we investigated the beneficial outcome of the pharmacological targeting of CK2 in the context of chronic hypercaloric dietary challenge in mice. From an in-depth focus of effects of TBB administration, it emerges that the beneficial role of CK2 inhibition could be assumed at two different levels of analysis, namely the impact on hepatic and systemic inflammation and the effects on metabolic profile. Indeed, we documented, for the first time, the diet-induced overactivation of CK2α catalytic subunit at hepatic level, convincingly supporting the scientific evidence of aberrant activity CK2 in the pathogenesis of inflammatory-associated diseases [175, 213, 214] and the inhibitory effect of TBB administration in dampening CK2 abnormal activation. The beneficial outcomes consequent to this event were the further inactivation of the NFκB/NLRP3 cascades and the reduction of transcription pro-inflammatory cytokines at hepatic level. These findings confirmed our hypothesis to halt simultaneously the activation of detrimental mediators by targeting an upstream regulator, CK2, which governs the cross-talk of several intracellular signalling pathways. Furthermore, it has to be underlined that these data are consistent with our recent findings on the investigation of CK2 in the septic context, that evinced a beneficial effect of CK2 inhibition on CK2/ NFκB/NLRP3 axis [175]. Overall, the amelioration of local inflammation, mediated by TBB administration, documented also by the downregulation of ICAM-1 and MPO activity and expression, demonstrates the relevant increase of neutrophils driven by dietary manipulation, as we and other as already proven

in this experimental conditions[215, 216], reinforcing the concept of metaflammation introduced almost twenty years ago by Hotamisligil, as a condition characterized by immune cell's infiltration at a subacute level [5]. The ultimate effect, and one of the most relevant recorded in TBB treated mice was the restored inflammatory profile at systemic level, that resulted in the amelioration metabolic profile, dramatically affected by chronic overnutrition. For instance, these positive effects could be potentially ascribable by the indirect protective mechanisms evoked by CK2 inhibition, that blunting hepatic inflammation by reduction CK2 overaction and consequent effects on its downstream regulators, resulted in the reduction of pro-inflammatory status in both in metabolic organs, such as the liver, and overall, in the consequential higher proficiency of metabolic processes, such as glucose handling capability and lipid profile, documented in our experimental protocol. However, recent concerns suggest a potential involvement of CK2 in metabolic processes, confirming once more the pleiotropic effects of this kinase on cell fate. Indeed, Pack and colleagues unravelled the potential implications of CK2 in the gluconeogenesis process, assuming a direct effect of the kinase on the enzyme fructose-1,6-biphosphatase (FBP1), and proving how the acute pharmacological inhibition of CK2 reduced FBP1 activity and ameliorated glycaemia levels in mice [217]. The amelioration of both cholesterol and triglycerides plasmatic levels concentrations paralleled by the qualitative and quantitative analyses of hepatic ORO staining, could be also explained, most intriguingly, as the consequences of CK2 indirect effects on the sterol regulatory element-binding proteins SREBP1 and SREBP2, pivotal mediators guiding the transcriptional machinery of fatty acids and cholesterol [218], specifically throughout CK2 mediated effects on mammalian target of rapamycin complex 1 (mTORC1). In addition, CK2 is recognised as an inhibitor of LPIN1 (phosphatidic acid phosphatase lipin 1), negative regulator of SREBP1 [219, 220]. This dual and complementary effects of CK2 on the promotion of the nuclear retention of SREBP1 could explain the beneficial effects of TBB administration on systemic lipid profile. Furthermore, another master regulator of fatty acid production, FASN (fatty acid nuclear synthase) has been recognised to be a CK2 target, following insulin stimulation in obese mice [221]. Overall, the protective effects of targeting CK2 in the framework of diet-induced inflammation highlights a

novel pharmacological tool for the alleviation of metaflammation thanks to its interplay with pro-inflammatory cascades, and on the other side, urge to better elucidate the tight mechanisms in that led to the CK2-mediated amelioration of glucose and lipid handling processes.

Overall, the experimental results achieved during my doctoral project and here discussed, are in agreement and further broaden most recent literature data that highlight the distinct and unique position of metaflammation in the pathogenesis of metabolic disorders [1, 222]. For instance, the proposed interventions tested in our preclinical models, Zn-SP and TBB, fit into the innovations of two wide fields of the pharmacological research specifically the development of innovative nutraceutical approaches and new small molecules kinase inhibitors (SMKIs). It has to be noticed that despite these two realms are very distinct and far from each other, however, represent crucial sources of novel compound for a broad variety of clinical disorders. Specifically, what could be underlined as "novel" for the area of microalgae is the fact that they depict a potential source of treatments that, thanks to their multifactorial composition, could affect with a synergic mechanism of action, different aspects of complex conditions such as the harmful consequences of hypercaloric diet consumption, here tested. On the other hand, the emerging reality of kinase inhibitors, alongside with the concept of drug repurposing, represent nowadays one of the most promising categories in drug discovery and consequent bench-to-bedside process. In particular, there are 80 FDA approved SMKIs, and among them, more than twenty are used in the treatment of multiple diseases, hinting at the central role of kinase in governing different pathological contexts [78]; moreover, the trend in kinase approval has been considerably increased over the past twenty years, when at the beginning of this century, only one SMKI was approved, while in 2020, eight candidates received the approvals [223].

Nevertheless, some limitations of the present PhD project must be acknowledged. The protective supplementation with Zn-SP should be analysed in the context of a prophylactic strategy for individuals at low-risk of developing metabolic disturbances, for which well-adjusted lifestyle management and high-quality nutrients balance should be enough to achieve protection against diet-induced alterations. Zn-SP must not be considered as a medicine, and for this

reason, not useful for patients who already developed relevant metabolic derangements. In this clinical setting, therapeutical interventions are needed, and thus, the pharmacological inhibition of CK2 could represent an interesting therapeutic strategy to be pursued. With concern to the protective role of CK2 inhibition, we have consistently proven the ability of CK2 in governing the interplay among NFκB/NLRP3 mediators, ascribing the protective effects of CK2 inhibition mainly to its action in contracting their simultaneous activation. On the other hand, it must be noted that other inflammatory cascades could be involved in CK2 cross-talk, for this reason it is unlikely entirely limiting the beneficial effect of CK2 on NFκB cascade, but it should be questionable the potential consequences of CK2 on other mediator, such as the JAK/STAT signalling pathway. For instance, an in-depth analysis and elucidation of other potential mediators could, in turn, increase and widen the prospect of CK2 as a pivotal mediator in the orchestration of cell homeostasis and further accelerate the ongoing drug discovery focused on this promising kinase, and moreover, justify its application in the metabolic context.

In conclusion, the PhD project confirm and further extend findings on the role of metaflammation as valuable and resourceful area of investigation that provides fascinating mediator to unveil and to target for halting the metabolic consequences of a chronic nutrient overload. Besides, it offers preliminary findings identifying new valid approach for its therapeutical targeting. Novel compounds could be part of the auspicial extension of the therapeutic options needed for managing the epidemic proportion assumed by life-style associate disorders, such as CK2 kinase inhibitors, or from the perspective of a preventive intervention, supplementation with zinc enriched *Arthrospira Platensis*. A graphical resume focusing on the diet-induced molecular pathways investigated in this PhD thesis, the AGE/RAGE axis and the CK2-mediated crosstalk with inflammatory cascades, is represented in Figure 28. This panel highlights also the effects on these mediators evoked by the two tools tested in our preclinical dietary models, the Zn-SP and the CK2 inhibitor, TBB.

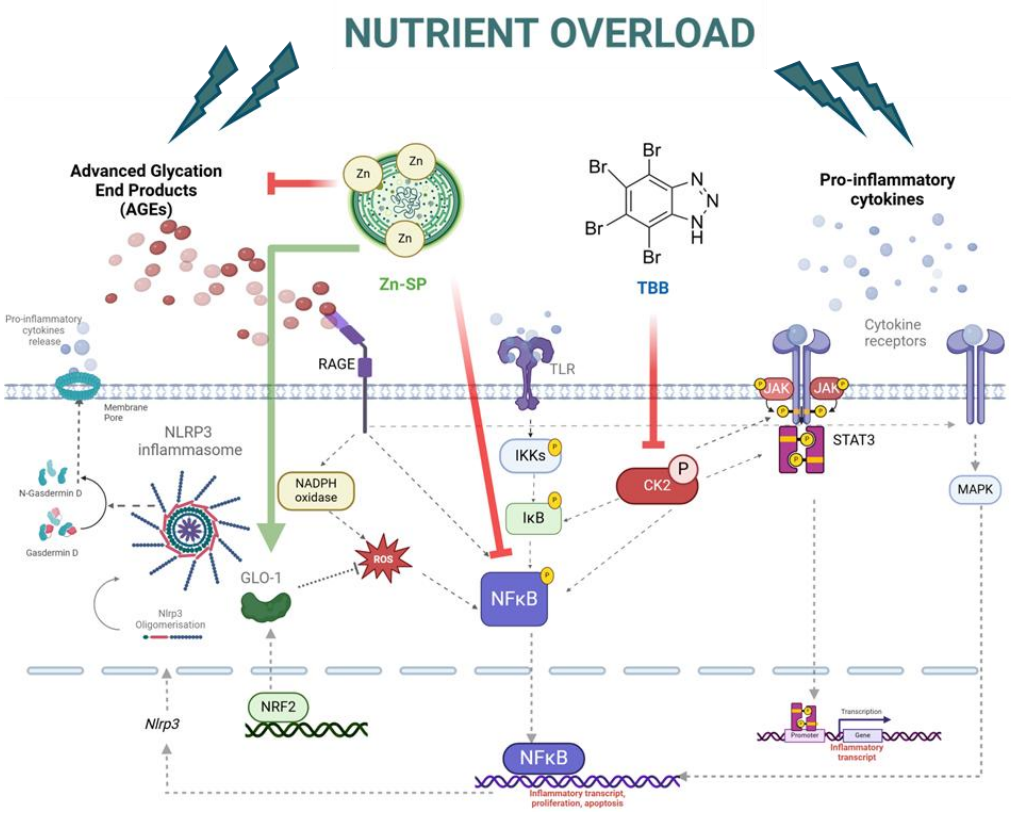

Figure 28. Representative scheme of the molecular pathways triggered by dietary manipulation investigated in the PhD thesis, the Advanced Glycation End Products (AGEs) and casein kinase II (CK2), alongside with the protective effects of Zn-Spirulina (Zn-SP) and the CK2 inhibitor here tested, TBB.

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