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**Unravelling the pathogenic role of Advanced Glycation End Products in chronic
inflammation and related diseases: molecular insights and strategic interventions**

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To my parents Laura and Gianluca,

SUMMARY

Advanced glycation End Products (AGEs) are a class of compounds which are formed through a non-enzymatic reaction between reducing sugars and proteins, lipids, or nucleic acids, playing a critical role in promoting inflammation, oxidative stress, and tissue damage. This is particularly relevant in metabolic disorders like obesity and insulin resistance, where AGE accumulation contributes to the exacerbation of systemic inflammation, a condition termed metabolic inflammation or “metaflammation”. This thesis explores the pathogenic role of Advanced Glycation End Products (AGEs) in chronic inflammation and associated diseases, with a focus on both molecular mechanisms and potential therapeutic interventions. The first part of the thesis investigates how different types of hypercaloric diets and simple sugars contribute to AGE accumulation in metabolically active organs. Using preclinical models, the thesis evaluates the impact of these diets on mitochondrial dysfunction, metabolic derangements, and the activation of inflammatory pathways in organs such as the liver and skeletal muscle. Furthermore, it explores the anti-glycation properties of natural compounds, including dietary fibres (FOS) and zinc-enriched *Spirulina platensis*, which show promise in preventing AGE-induced metabolic dysfunctions. Additionally, the thesis examines the role of Spleen Tyrosine Kinase (SYK) in metabolic inflammation. When activated, the receptor for AGEs (RAGE) triggers several pro-inflammatory pathways through a cascade of phosphorylation events, which present potential targets for pharmacological intervention. The repurposing of the SYK inhibitor fostamatinib, originally used for autoimmune disorders, is explored as a therapeutic option for diet-induced metabolic diseases. The research demonstrates fostamatinib's ability to reduce inflammation and improve insulin sensitivity by inhibiting key signalling pathways involved in AGEs-induced damage.

The second part of the thesis focuses on the role of AGEs in childhood cancer survivors (CCS), who are at increased risk of long-term complications, including cardiovascular diseases and secondary malignancies, due to prior cancer treatments. These survivors often exhibit systemic inflammation and premature aging, conditions in which AGEs are hypothesized to play a critical role. Observational studies in CCS reveal elevated plasma levels of AGEs, suggesting that they may be involved in driving the chronic inflammatory state observed in these individuals. The research also identifies potential biomarkers of premature aging, providing insight into the molecular pathways underlying the late effects of cancer therapy.

Overall, this thesis offers new insights into the pathophysiological role of AGEs in both metabolic diseases and cancer survivorship, highlighting the potential of dietary interventions, natural compounds, and pharmacological agents to mitigate AGE-induced damage. Moreover, it proposes novel research directions, including the exploration of AGE reduction strategies through diet modification and the use of nutraceutical or pharmacological approaches to improve long-term health outcomes.

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1. BACKGROUND

1.1 Advanced Glycation End Products (AGEs)

Advanced glycation end-products (AGEs) are a diverse and complex group of compounds formed through various mechanisms and from different precursors. Typically, AGEs are produced through a non-enzymatic reaction between the carbonyl groups of reducing sugars and the free amino groups found in nucleic acids, proteins, or lipids.

The following sections will provide a brief overview of the processes involved in AGE formation and their mechanism of action, to help contextualize the results discussed in this thesis.

1.1.1 AGEs formation and classification

AGEs can be formed exogenously and ingested with the food (dietary AGEs) or formed endogenously. The predominant process of endogenous AGEs is through the complex, multistage glycation process Maillard reaction, from the name of the first scientist, Louis Camille Maillard, who firstly described the formation of AGEs in 1912 when he studied the reaction between glucose and glycine in processes like baking or broiling. Subsequent discoveries in food chemistry identified the Maillard reaction end-products as key contributors to the rich taste, aroma, and savour of thermally processed foods high in sugar and protein¹. As reported, the Maillard reaction is one of the most extensively studied mechanisms leading to AGE formation. However, the structural diversity of AGEs highlights the wide range of pathways and mechanisms involved in their production *in vivo* such as the already mentioned non-enzymatic Maillard reaction as well as, among others, the polyol-pathway and lipid peroxidation, as briefly described in **Figure 1**.

To form AGEs, the end-products generated during the Maillard and polyol pathways must undergo further rearrangements, resulting a diverse class of reactive carbonyl compounds. Some well-known examples of these carbonyls include glyoxal, methylglyoxal, glyceraldehyde, glycolaldehyde, diacetyl, and 1- and 3-deoxyglucosone. These reactive carbonyls can then react with available amine groups on lysine and arginine residues producing a wide variety of AGEs. The most studied endogenous AGE is carboxymethyl-lysine (CML)².

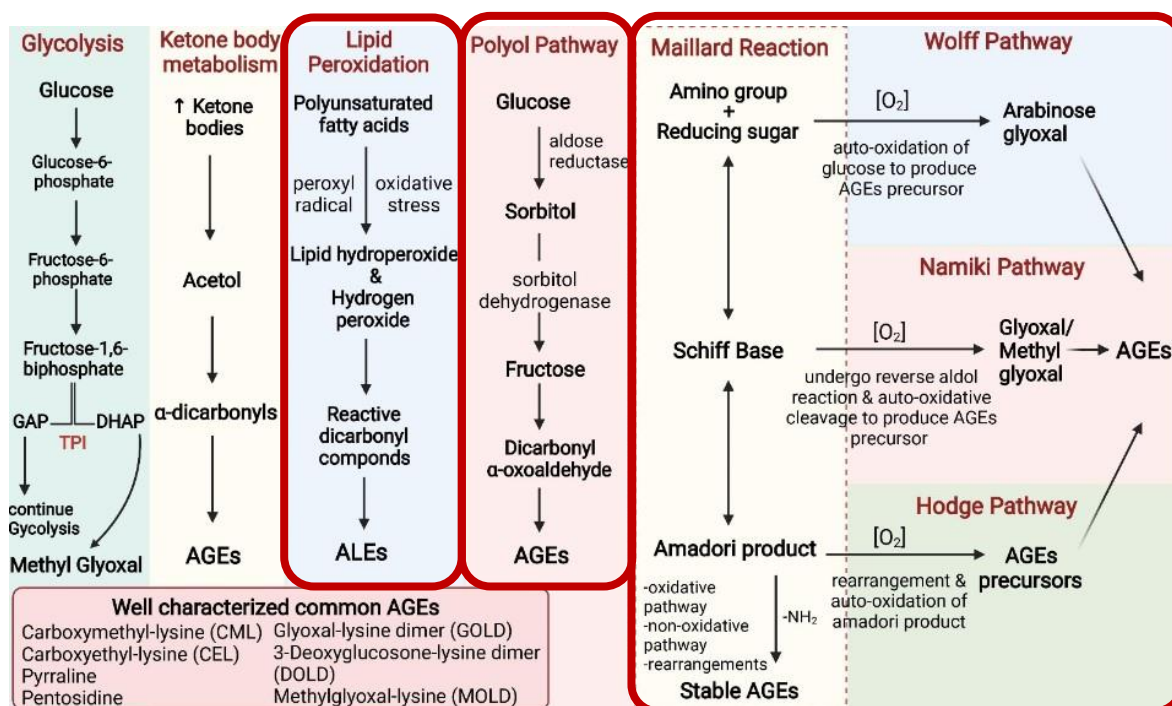


Figure 1 Pathways of AGEs format formation. The **Maillard reaction** is the primary pathway for the formation of advanced glycation end-products (AGEs) that involves several mechanisms: the metal-catalyzed autooxidation of glucose (Wolff pathway), the reverse aldol reaction and autooxidation of Schiff bases (Namiki pathway), and the non-oxidative cleavage of Amadori products (Hodge pathway), all of which generate reactive dicarbonyls and AGEs precursors that contribute to AGEs formation. In the **polyol pathway**, an excessive amount of glucose is reduced to sorbitol by the enzyme aldol reductase, which is then converted to fructose by sorbitol dehydrogenase. Fructose and its metabolites, being potent glycating agents, lead to the production of AGEs. The **lipid peroxidation** of polyunsaturated fatty acids leads to the formation of lipid peroxides, which first converts to reactive dicarbonyls and ultimately results in the formation of advanced lipid peroxidation end products (ALEs). Adapted from: Khalid M, Petroianu G, Adem A. Advanced Glycation End Products and Diabetes Mellitus: Mechanisms and Perspectives. *Biomolecules*. 2022. GAP: glyceraldehyde-3-phosphate; TPI: triose phosphate isomerase; DHAP: dihydroxyacetone phosphate

To date, there is no single way to classify and categorize AGEs, but various classifications have been proposed based on different criteria.

- **Endogenous and Exogenous AGEs**

As previously underlined, the first criterion for defining an AGE is its origin, categorizing AGEs into compounds that are either absorbed from food (exogenous and dietary AGEs, or dAGEs) or generated within the organism. Endogenous AGEs are typically formed in various tissues and body fluids under normal physiological conditions through glycation reactions. However, pathological conditions can lead to excessive accumulation of AGEs. In healthy individuals, the levels of glycated plasma protein products are typically below 3%. In contrast, in pathological conditions such as diabetes, this level can increase by up to three times, resulting in the development of a pathological phenotype. Dietary AGEs naturally occur in uncooked animal-derived foods, but their concentration increases with certain processing methods, particularly thermal treatments. Furthermore, processed foods in modern diets are often rich in sugars and proteins that undergo the Maillard reaction during thermal processing, leading to rapid AGE formation. Initially, it was believed that dAGEs were poorly absorbed by cells, which led to a significant underestimation of their impact on human health. However, *in vivo* studies revealed

a substantial accumulation of these compounds when the animals were fed with AGE-supplemented foods. Depending on their chemical characteristics, approximately 10-30% of ingested dietary AGEs are absorbed into the systemic circulation. The unabsorbed AGEs are transported to the colon, where they can disrupt gut microbiota homeostasis and induce inflammatory responses that affect gut integrity. This localized inflammatory response is linked to an increase in systemic inflammatory cytokines^{3,4}.

- *Fluorescence and Cross linking of AGEs*

Considering the diversity of chemical structures and the ability to emit fluorescence, AGEs can be divided in:

- non-fluorescent and cross-linked compounds (GOLD, MOLD,);
- fluorescent and cross-linked AGEs (pentosidine and crossline);
- fluorescent and non-cross-linked molecules (argpyrimidine);
- non-fluorescent and non-cross-linked AGEs (CEL, CML and pyrraline)

- *Low molecular weight AGEs (LMW) and high molecular weight (HMW)AGEs*

AGEs can be further classified based on their molecular weight into LMW-AGEs (<12KDa), which are protein-bound proteins, and HMW-AGEs (>12KDa), which are free proteins or peptide-bound molecules. Compounds such as CML can be absorbed via simple diffusion, while peptide-bound AGEs use peptide transporters to cross the epithelium. This means that LMW-AGEs are readily distributed throughout the body. Their accumulation can be observed mainly in the liver, kidneys, lungs, heart, and spleen.

1.1.2 Mechanisms of action

Two main mechanisms contribute to AGEs-induced pathophysiology in diabetes mellitus. AGEs exert harmful effects either directly by forming cross-links with proteins or indirectly by binding to cell surface receptors. AGEs interact with various receptors, including Toll-like receptors (TLR), scavenger receptors, G-protein-coupled receptors and pattern recognition receptors (PRR)⁴. The most significant receptor is the receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily, originally identified for its ability to bind AGEs. As a PRR, RAGE can recognize specific ligand structures. RAGE encodes a 404-amino acid protein weighing 45–55kDa, with three domains: an extracellular region (N-terminal V-type and two C-type immunoglobulin domains), a hydrophobic transmembrane domain, and a cytoplasmic domain (full length RAGE, fl-RAGE). The V-type domain binds extracellular ligands, while the cytoplasmic tail is essential for intracellular signalling⁵.

In addition to full-length RAGE, several naturally occurring isoforms of RAGE have been identified (**Figure 2**). These arise from alternative splicing and proteolytic cleavage of fl-RAGE. Truncated forms, such as the N-terminal truncation, lack the ligand-binding domain and cannot interact with AGEs. The C-terminal truncation forms soluble RAGE (sRAGE), including endogenous secretory RAGE (esRAGE) from alternative splicing and cleaved RAGE (cRAGE) from proteolysis by metalloproteases. sRAGE lacks a transmembrane domain and acts as a decoy receptor by binding RAGE ligands in the extracellular space, preventing fl-RAGE from activating intracellular signalling.

The membrane-bound full-length RAGE is fundamental in mediating RAGE signalling in response to AGEs, leading to proinflammatory and pro-oxidative activation: details on these mechanisms will be explained in the next chapter.

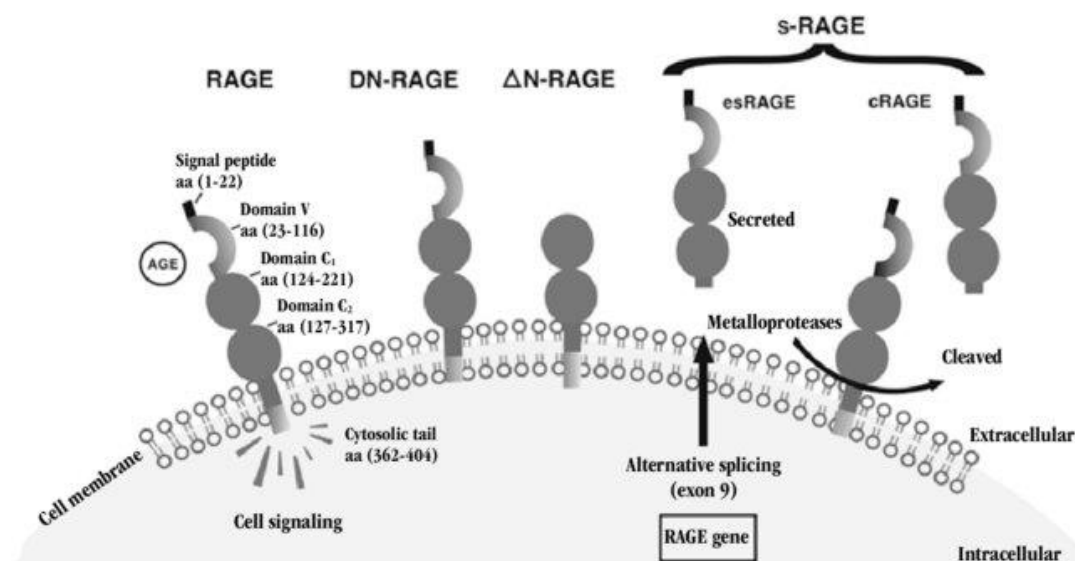


Figure 2 RAGE isoforms resulting from alternative splicing and proteolytic cleavage RAGE: receptor of advanced glycation end-products; DN-RAGE: dominant negative RAGE; Δ N-RAGE: delta-RAGE isoform without domain V; sRAGE: soluble RAGE isoforms; esRAGE: endogenous secretory RAGE; cRAGE: cleaved RAGE; AGE: advanced glycation end-product. Adapted from: Mauren Isfer Anghebem Oliveira et al. RAGE receptor and its soluble isoforms in diabetes mellitus complications. *J. Bras. Patol. Med. Lab.* (2013).

1.1.3 AGEs' detoxification systems

Accumulation of endogenous AGEs is limited by detoxification pathways that include AGE-R1, the glyoxalase system (Glo) and the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf-2).

AGE-R1

AGE-R1 is a 48 kDa membrane protein involved in the oligo-saccharyl-transferase (OST) system⁶. It regulates oxidative stress by inhibiting NADPH oxidases and protein kinase C- δ phosphorylation. Reduced AGE-R1 expression disrupts AGE-RAGE signalling, leading to nuclear translocation of NF κ B p65. AGE-R1 also prevents AGE-induced phosphorylation of p66shc, a protein linked to oxidative stress and apoptosis, which is elevated in diseases like diabetes and atherosclerosis⁷. Additionally, AGE-R1 influences sirtuin-1 (SIRT1), a key regulator of cellular stress responses⁸.

Glyoxalase system

The glyoxalase system consists of two enzymes, glyoxalase 1 (Glo-1) and glyoxalase 2 (Glo-2), with glutathione (GSH) as a cofactor. Glo-1, a zinc-dependent enzyme made of two identical subunits, is the rate-limiting step in the system, converting methylglyoxal to S-D-lactoyl-glutathione. Glo-2 hydrolyzes this compound to D-lactate and GSH. Glo-1 activity is compromised in oxidative stress due to reduced GSH levels, and its gene expression is regulated by antioxidant response elements (ARE) and hypoxia inducible factor 1 α (HIF1 α).

Hypoxia, by enhancing anaerobic glycolysis, increases methylglyoxal (MG) formation and reduces Glo-1 expression, worsening dicarbonyl stress⁹.

Nrf2

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key defence mechanism against oxidative, inflammatory, and carbonyl stress. Nrf2 boosts the production of antioxidant enzymes that protect against glycation and help recycle glutathione. Under normal conditions, Nrf2 is bound to Keap1. During oxidative stress, Nrf2 dissociates from Keap1, migrates to the nucleus, and binds to AREs to promote the transcription of protective genes, including enzymes like GSH reductase and peroxidase¹⁰.

1.2 AGEs and inflammatory disorders: a focus on obesity and other related metabolic derangements

1.2.1 AGEs and inflammation

AGEs play a significant role in the development and progression of inflammatory disorders. When binding to their receptor RAGE, AGEs trigger a cascade of pro-inflammatory signalling pathways and the activation of NADPH oxidase¹¹, which in turn increases the reactive oxygen species (ROS) production, generating a pro-oxidative status that further exacerbates AGEs endogenous production (**Figure 3**). The positive feed-forward loop elicited by ROS production triggers the activation of several downstream effectors, overall leading to the activation of NF- κ B signaling pathway. NF- κ B is a protein transcription factor that regulates innate immunity and cytokine gene expression, exacerbating inflammatory responses. Upon stimulation, NF- κ B translocate to the nucleus acting as a master regulator of inflammation^{12,13}. RAGE-induced inflammation also triggers the NLRP3 inflammasome, a member of the Nod-like receptor (NLR) family. Once induced, the NLRP3 inflammasome leads to caspase-1 activation, resulting in the secretion of pro-inflammatory cytokines, interleukin-1 β (IL-1 β) and IL-18. It is important to underline that the NLRP3 activation signal could also be triggered by ROS: for this reason, the oxidative environment generated by AGEs accumulation is a trigger stimulus that enhances NLRP3¹⁴. In addition to promoting inflammation, AGEs impair the function of proteins and cells within the immune system, further perpetuating inflammatory responses¹⁵. Persistent activation of these pathways can damage tissues and exacerbate conditions like rheumatoid arthritis, inflammatory bowel disease, atherosclerosis and metabolic derangement, in particular obesity.

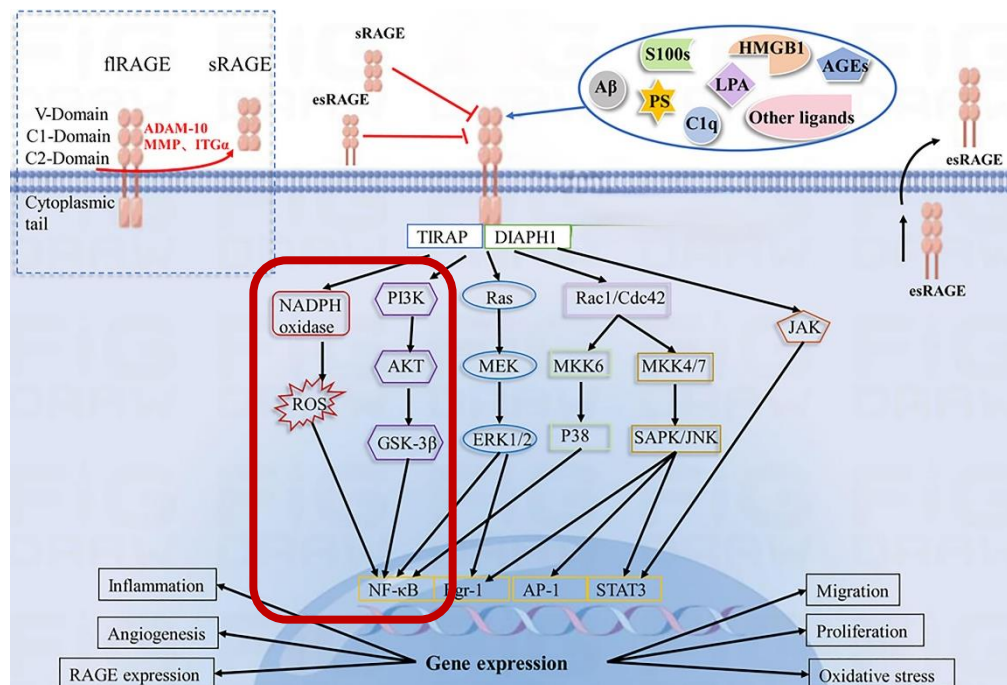


Figure 3 Schematic illustration of the RAGE-ligand pathways. RAGE interacts with several ligands, including AGEs, triggering the activation of several signalling pathway. This leads to changes in gene expression towards inflammation, oxidative stress, angiogenesis, proliferation, migration and upregulation of RAGE expression. The main pathways evaluated in this thesis are evidenced in the bold rectangle. NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase; PI3K: phosphoinositide 3-kinase; AKT: protein kinase B; ROS: reactive oxygen species; GSK-3 β : glycogen synthase kinase-3 beta. NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells. Adapted from: Dong H et al. Pathophysiology of RAGE in inflammatory diseases. *Front Immunol.* (2022)

Obesity is recognized not merely as a condition of excessive fat accumulation but as a complex, systemic disorder that impacts various metabolic pathways and organs. The intricate interplay between adipose tissue, muscle metabolism, and other key organs such as the brain and liver, together with environmental and psychosocial factors, underscore the systemic and complex nature of obesity¹⁶. Obesity fundamentally develops as an imbalance between caloric intake and energy expenditure¹⁷. When energy intake surpasses the energy utilised by the organism, it is stored as fat in the adipose tissue (AT). As previously mentioned, obesity is characterized by a state of low-grade chronic inflammation, often termed "metabolic inflammation" or "metaflammation"¹⁸. White adipose tissue (WAT) functions as a complex secretory organ, where immune cells, particularly macrophages, within adipocytes produce and release various cytokines, known as adipokines (such as TNF- α , leptin, resistin, visfatin, and IL-6)¹⁹. In the last 20 to 30 years, research has revealed that obesity-related inflammation, with increased accumulation and inflammatory polarization of immune cells, involves not only the adipose tissue but multiple organs, such as skeletal muscle, liver, gut, pancreatic islets, and brain. This inflammatory process is believed to contribute to metabolic dysfunctions associated with obesity, ultimately resulting in insulin resistance and type 2 diabetes mellitus²⁰.

1.2.2 AGEs, obesity and metabolic disorders

A growing body of evidence demonstrated the direct interference of either exogenous or endogenous AGEs on different metabolic pathways contributing to alterations of lipid metabolism and to the onset of a low-grade chronic inflammation and insulin resistance, comprehensively known as "metabolic inflammation" or "metaflammation"²¹. Exogenous AGEs are derived from environmental factors: particularly, highly processed foods characteristic of "Western" diets, air pollutants, and tobacco smoke are major contributors to pathological accumulation, all of which are intricately associated with the development of obesity and other metabolic disorders. Indeed, the increased use of Western-style diets, which in addition to the well-established macronutrient differences (eg, higher fat content), also contain high levels of exogenous AGEs boosting their accumulation. Remarkably, increased dietary AGE intake results in a significant elevation of circulating AGEs, including CEL, methylglyoxal (MGO), and CML, providing evidence that orally ingested AGEs are absorbed and circulated throughout the body²². Endogenous AGEs are normally formed when the body processes sugars²³, but they can accumulate pathologically due to impaired metabolism, unbalanced protein turnover rate, hyperglycemia, enhanced oxidative stress status and aging. In particular, the *in vivo* formation and deposition of AGEs in tissues can be facilitated by the consumption of dysregulated and hypercaloric diets²⁴. Indeed, increased carbohydrate and/or lipid substrate availability promotes the addition of a carbonyl group from a reducing sugar to a free amino group of a protein rearranging, as already described, to the irreversible AGEs.

Moreover, several studies have suggested a potential link between AGEs and insulin resistance. One proposed mechanism is the glycation of insulin itself, which may impair its effectiveness. Insulin, being a short-lived protein, is susceptible to glycation during its formation and storage in the pancreas²⁵. Supporting this, mice infused with mono-glycated insulin showed a 20% reduction in glucose tolerance compared to those given non-glycated

insulin²⁶. Additionally, insulin modified by methylglyoxal (MG) has been shown to reduce glucose uptake in adipocytes and skeletal muscle cells, impairing insulin's normal function. MG also disrupts insulin signalling by inhibiting tyrosine phosphorylation of IRS and PI3 kinase activation in pancreatic β -cells²⁷. In a study with KK-A(y) mice, a model of obesity and T2D, a positive correlation was found between serum AGEs and insulin levels. Treatment with the anti-glycative compound reduced fasting insulin levels in a dose-dependent manner, improving insulin sensitivity and further supporting AGEs' role in insulin resistance²⁸.

1.2.3 Epidemiology of obesity and current treatments

Overweight is defined by the World Health Organization (WHO) as a Body Mass Index (BMI, calculated as $[\text{weight (kg)}/\text{height}^2 (\text{m}^2)]$) of 25 or higher, and obesity as a BMI of 30 or higher²⁹. Although BMI does not give a precise indication about body composition and fat distribution, which are fundamental markers for obesity-associated comorbidities, it is still the internationally accepted standard method to define obesity both in epidemiological research, as well as clinical settings³⁰. In 2022, 1 in 8 people worldwide were living with obesity, with adult obesity having more than doubled and adolescent obesity having quadrupled since 1990³¹.

Obesity is a significant risk factor for other metabolic abnormalities³², a cluster of conditions associated with an increased risk of cardiovascular diseases (CVD) and type 2 diabetes (T2D).

The primary strategy for managing obesity involves preventing weight gain and promoting significant lifestyle changes. This includes the consumption of diets rich in fibres and lean proteins, with a low percentage of saturated fats, while discouraging the consumption of sugar-sweetened beverages, refined grains, red and processed meats, and excessive alcohol^{33,34}. Additionally, guidelines recommend obese patients engage in regular physical activity minimizing sedentary behaviours³⁵. Although lifestyle modifications are essential for weight management, there is a high risk of weight regain due to metabolic adaptations and the potential for low patient compliance. Consequently, pharmacological and surgical interventions should also be considered. Anti-obesity medications (AOMs) are recommended in patients with a BMI equal or above 30 kg/m² or with a BMI of 27 kg/m² with a concomitant weight-related condition (e.g., type 2 diabetes mellitus, hypertension, hyperlipidemia, or sleep apnea)³⁶. There are 5 drugs currently approved both by the Food and Drug Administration (FDA) and by the European Medicine Agency (EMA) for long-term use (**Table 1**).

Table 1 *Approved drugs currently used for the pharmacological treatment of obesity (indicated for patients with a BMI ≥ 30 kg/m² or with a BMI ≥ 27 kg/m² with a concomitant weight-related condition). EMA: European Medicine Agency; FDA: Food and Drug Administration*

DRUG	TRADE NAME	YEAR of APPROVAL	MECHANISM of ACTION	DOSE (for adults)
Orlistat	Xenical/ Alli	1999 (FDA) 1998 (EMA)	Gastric and pancreatic lipase inhibitor	120mg (Xenical) 60mg (Alli, over the counter medication)
Naltrexone/ Bupropion	Mysimba/ Contrave	2014 (FDA) 2015 (EMA)	Bupropion is DA and NE reuptake inhibitor and releasing agent / Naltrexone is an opioid antagonist	7.2mg naltrexone and 78mg bupropion
Liraglutide	Saxenda	2014 (FDA) 2015 (EMA)	GLP-1 analogue	0.6mg – 3mg (increasing dosage)
Semaglutide	Wegovy	2021 (FDA) 2021 (EMA)	GLP-1 analogue	0.25mg – 2.4mg (increasing dosage)
Tirzepatide	Zepbound/ Mounjaro	2023 (FDA) 2023 (EMA)	GIP and GLP-1 analogue	2.5 – 15mg (increasing dosage)

The approved AOMs reported in the Table target peripheral and central pathways to decrease energy intake by reducing appetite and increasing satiety. Additionally, they also act in the gastrointestinal tract, where they slow gastric emptying³⁷. Orlistat has mainly peripheral effects, inhibiting gastric and pancreatic lipases, thus decreasing dietary fat absorption³⁸. The mechanism of action of the combination of Naltrexone (opioid receptor antagonist that inhibits the POMC -proopiomelanocortin- pathway inhibitor) and Bupropion (antidepressant, norepinephrine and dopamine reuptake inhibitor that directly stimulate POMC cells) is not completely elucidated but the two drugs work synergistically to increase POMC peptide production, and therefore decrease food intake³⁹. In addition, the combination Naltrexone/Bupropion (NB) acts on the reward pathways, increasing activity in various cortical areas in response to food cues. Finally, glucagon like peptide receptor (GLP1-R) agonists, Liraglutide and Semaglutide act centrally by decreasing appetite, and peripherally, on the pancreas by increasing insulin secretion and on the gastro-intestinal tract leading to decreased intestinal motility and delayed gastric emptying⁴⁰. Tirzepatide, sometime also referred as “twincretins” analogue, is a dual agonist for the glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) receptors^{41,42}. The dual agonism ability decreases hyperglycemia significantly more than GLP-1 agonist agents and reduces the patient's appetite⁴³. All anti-obesity medications have demonstrated effectiveness in improving both weight and various metabolic parameters. However, the extent of their efficacy and the specific effects they elicit can vary significantly depending on the particular pharmacological agent employed. In clinical trials⁴⁴, the average reduction in body weight achieved with these medications is typically around 10%. Notably, some

agents can produce even more substantial weight loss. For instance, Tirzepatide at a dosage of 15 mg has been associated with a maximum weight reduction of up to 20%⁴⁴. The most frequently observed adverse effects of the discussed AOMs, occurring in more than 10% of the population, are primarily related to the gastrointestinal tract. These include symptoms such as abdominal distress, abdominal pain, constipation, diarrhoea, nausea, and vomiting⁴⁵.

1.3 The interplay between AGEs and kinases

The diet-induced inflammatory environment is worsened by the accumulation of both exogenous and endogenous advanced glycation end products (AGEs). When AGEs bind to their receptor, they activate signalling pathways that elevate oxidative stress and inflammation. In response to this inflammatory state, kinases are activated, which further contribute to metabolic dysfunction. These kinases also interfere with insulin signalling by phosphorylating critical components of the insulin receptor pathway, which eventually exacerbates insulin resistance⁴⁶ (**Figure 4**). As an example, the AGE/RAGE axis can directly interfere with the JAK/STAT pathway: after treatment of macrophages with different AGE precursors for 72h they observed an increase in JAK2 and STAT1 phosphorylation⁴⁷. Moreover, the silencing of the RAGE gene prevented the phosphorylation of ERK1/2 and p38 MAPK in keratinocytes⁴⁸.

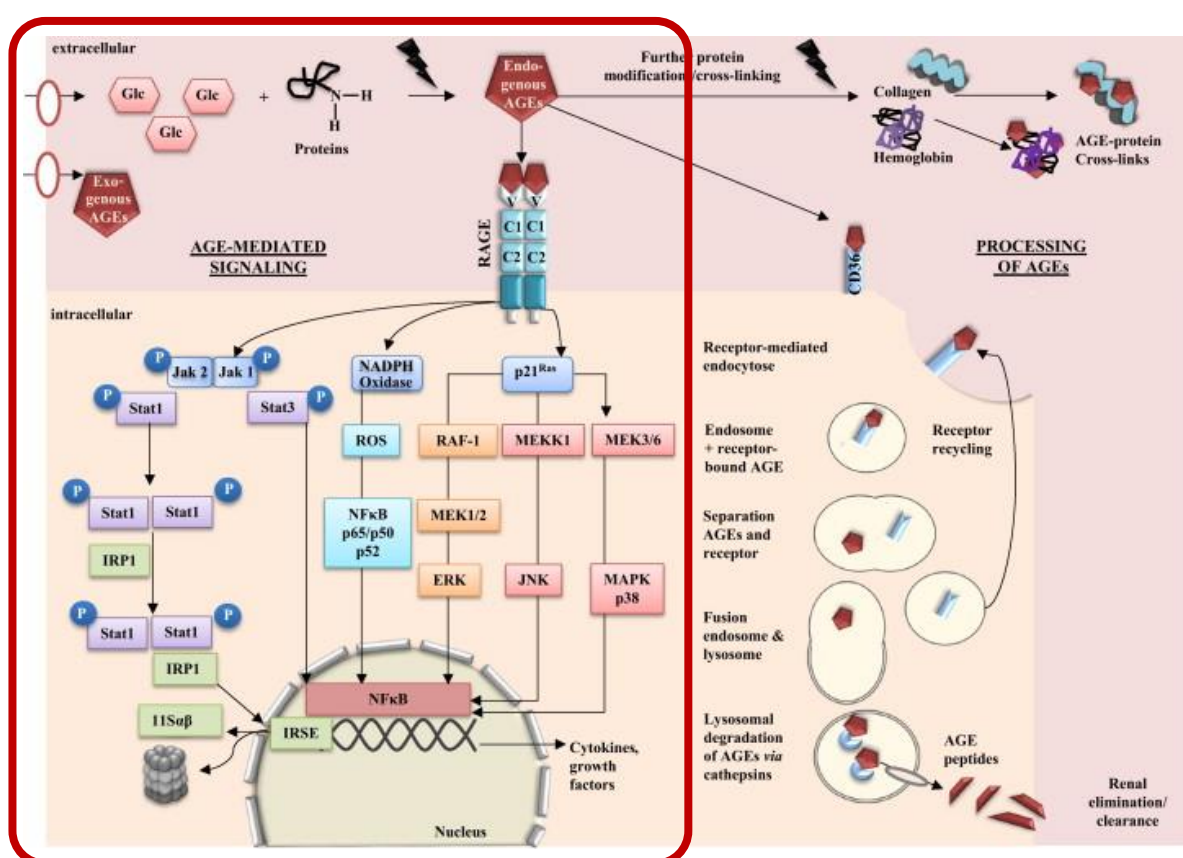


Figure 4. AGE–RAGE interaction stimulates a various number of kinases-mediated signaling cascades, including Jak/Stat, mitogen activated protein kinases (MAPK), such as p38, extracellular regulated (ERK)-1/2 and c-Jun N-terminal kinase (JNK). Modified from: Ott C, Jacobs K, Haucke E, Navarrete Santos A, Grune T, Simm A. Role of advanced glycation end products in cellular signaling. *Redox Biol.* (2014)

The human *kinome* (the complete set of protein kinases encoded in the genome) comprises 518 distinctive serine/threonine and tyrosine kinases, which are key components of virtually every mammalian signal transduction pathway. Therefore, the action of protein kinases and protein phosphatases is essential for multiple physiological responses as well as pathological processes. Consequently, kinases provide a compelling target family for the development of small molecule inhibitors, which could be used as tools to delineate the mechanism of action for biological processes and potentially be used as therapeutics to treat human diseases. The

US FDA has approved 72 small molecule therapeutic protein kinase inhibitors, nearly all of which are orally effective⁴⁹. 62 of these drugs are approved for the management of neoplasms (50 against solid tumors, eight against nonsolid tumours such as leukaemia, and four against both types of tumors). Eleven of the FDA-approved protein kinase inhibitors are prescribed for the treatment of nonneoplastic diseases. For example, (i) netarsudil is employed for the treatment of glaucoma, (ii) ruxolitinib, ibrutinib, and belumosudil are prescribed for the management of graft vs. host disease, (iii) fostamatinib is prescribed for the management of chronic immune thrombocytopenia, (v) baricitinib, tofacitinib, and upadacitinib are employed for the treatment of rheumatoid arthritis. Moreover, sirolimus and ibrutinib are approved therapeutics for both neoplastic and nonneoplastic diseases.

As recently documented^{50,51}, one of the main activators of several key immune-related signalling pathways is the spleen tyrosine kinase (SYK), which has emerged as a potential target for the treatment of autoimmune diseases. SYK protein is a 72 kDa kinase, which mainly acts via interaction of its tandem SH2 domains with immunoreceptor tyrosine-based activation motifs (ITAMs), changing its conformation once activated. The SYK inhibitor Fostamatinib, as just reported, was recently approved for the treatment of immune thrombocytopenia (ITP), autoimmune haemolytic anaemia and IgA nephropathy⁵². Fostamatinib is a prodrug that is converted to the active metabolite R406, which inhibits SYK in an ATP-competitive manner with high selectivity and potency (IC₅₀ 41 nM). SYK is highly expressed not only in hematopoietic cells but also in adipocytes as well as skeletal muscle and hepatic cells^{53,54}. Moreover, activated SYK can promote the activation of several inflammatory cascades, including the NLRP3 inflammasome. As we and other have convincingly demonstrated both genetic and pharmacological inhibition of NLRP3 inflammasome significantly attenuates the metabolic abnormalities and the related organ injury/dysfunction caused by chronic exposure to an hypercaloric diet^{55,56}.

1.4 AGEs as potential inducers of inflammation in childhood cancer survivors

1.4.1 Late effects of cancer therapy and childhood cancer survivors

During the last 40 years, the chance of recovery of children affected by cancer (childhood cancer survivors, CCS) has progressively increased and today, considering globally all types of neoplasm, it is around 80%. Thanks to advances in the treatments, Hodgkin Lymphoma (HL) is nowadays one of the most curable cancers, in particular when diagnosed in paediatric patients, with the number of children and adolescents who recover exceeds 90%⁵⁷. However, the concept of healing refers only to the primary tumours, as HL survivors are subject to late effects of cancer therapy that can compromise their quality and expectancy of life. Survivors of HL show a 5.1-fold increased risk of death from causes other than HL⁵⁸. After 50 years of age, survivors of HL reach a certain cumulative mortality 15-20 years earlier than the general population (**Figure 5**).

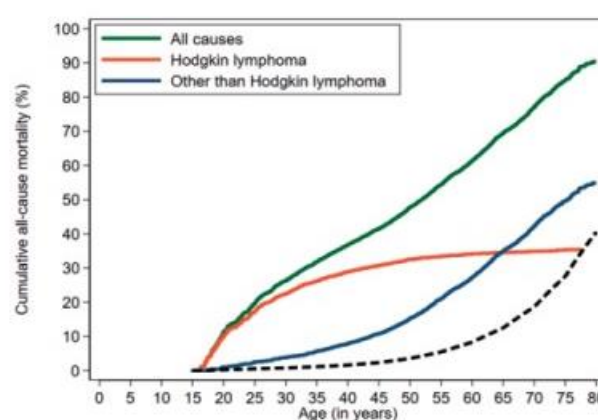


Figure 5 Cumulative mortality from all causes, Hodgkin lymphoma (HL), and other than HL by age. Solid lines represent the observed cumulative mortality, and dashed line represents the expected other-cause mortality based on general population rates. Source: de Vries S, Schaapveld M, Janus CPM, et al. Long-Term Cause-Specific Mortality in Hodgkin Lymphoma Patients. *J Natl Cancer Inst.* (2021)

The main pathologies, accounting for 65.7% of this excess mortality, are cardiovascular disease (CVD) and second malignancies (SM). The risk for cardiac disease among HL survivors is significantly increased 5 years or more after treatment and includes coronary artery disease and ventricular dysfunction. Moreover, survivors of paediatric HL demonstrate a higher prevalence of modifiable cardiovascular risk factors, including metabolic syndrome, hyperlipoproteinemia, insulin resistance, hypertension and endothelial dysfunction, factors involved in the accelerated onset of atherosclerosis⁵⁹. For what concerns SM, breast and lung cancers are the most frequent among HL survivors, typically occurring after 5 to 10 years after patients are off therapy.

1.4.2 Systemic inflammation in CCS: a possible role for AGEs?

Exposure to chemo- and radiotherapy leads to inflammation, to accumulation of senescent cells and to the increase of reactive oxygen species (ROS) and DNA mutations. After irradiation or chemotherapy, long-term alterations could persist. Radiation therapy (RT) direct effects include disruption of atomic structures, leading to chemical and biological modifications; moreover, it induces water radiolysis, the generation of ROS and NOS and mitochondrial dysfunction⁶⁰. Radiation exposure also activates an immune response, consisting in the activation of M1 pro-inflammatory macrophages, the recruitment of

neutrophils and lymphocytes and the production of pro-inflammatory cytokines, such as interleukin 1 (IL1), IL-6 and TNF α . These immune system alterations could persist also after the suspension of radiation therapy⁶¹. On the other hand, anthracyclines, and in particular doxorubicin (DOX), induce massive accumulation of ROS and RNS⁶².

As a direct consequence, all the aforementioned conditions (CVD and SM), which may affect the health of CCS, share inflammation as a well-recognized pathogenetic mechanism. Indeed, it was recently demonstrated childhood cancer survivors have a pro-inflammatory status characterized by increase in inflammatory markers: C-reactive protein and fibrinogen, as well as a shift toward memory and activated T lymphocytes and a higher expression of activation marker CD38. These alterations suggest a chronic immune activation in cancer survivors patients compared with age-matched individuals⁶³.

Even though, in the last years, many studies have focused on the epidemiology and the risk factors involved in the development of CVD and second cancers in CCS, still little is known about the biological and molecular mechanisms driving the onset of these pathologies in this class of subject. In our recent publication⁶⁴ we showed a marked increase of AGEs plasma levels in adult survivors of pediatric acute lymphoblastic leukemia who had received chemotherapy and allogeneic hematopoietic stem cell transplantation conditioned with total body irradiation, compared with the normal population. Given the AGEs-induced pro-oxidant state and low-grade, chronic inflammation through the binding and activation of their receptor RAGE, they may represent the missing link between past anti-cancer therapies and the inflammatory status currently observed in CCS.

1.4.3 Premature aging in childhood cancer survivors and the role of AGEs

As reported, after irradiation or chemotherapy, long-term alterations persist. In addition, CCS are at risk of developing frailty prematurely. Frailty is a condition characterized by premature aging processes and vital organ failure, which predispose to the early development of chronic age-related diseases, frequent hospitalization and early mortality. From a clinical point of view, frailty is diagnosed when three or more of the following signs are manifested:

1. low lean muscle mass
2. fatigue
3. low energy expenditure
4. slow walking speed
5. weakness.

Frailty is a condition seen in both the elderly and childhood cancer survivors (CCS). In older adults, it tends to result from a combination of genetic factors and lifestyle choices, while in CCS, it is more likely linked to organ damage caused by cancer treatments. The St. Jude Lifetime Cohort study monitored frailty trends among CCS over five years and discovered that its prevalence doubled during this period. By the end of the study, frailty was more common in CCS, with an average age of 30, than in adults over 65, significantly raising their risk for chronic health issues and mortality⁶⁵.

Also in this context, AGEs may play a role. Protein glycation occurs *in vivo* in physiological conditions at a slow and continuous pace, which accelerates with age, driving AGE

accumulation in tissues; for this reason, AGEs have been associated with the development of age-related diseases. Aging is a progressive structural and functional decline across multiple organ systems, associated with increased susceptibility to disease and mortality⁶⁶. The free radical theory of aging, postulated in 1959 by Denham Harman, identifies oxidative stress as the main cause of the time-dependent impairment of cellular functions. Insufficient activity of the endogenous antioxidant system, which occurs in aging organisms, leads to ROS accumulation, a condition that favours glycoxidation reactions; in addition, defects in the detoxification mechanisms due to reduction in the proteolytic capacities of cells and loss of glyoxalase activity, contribute to AGE accumulation. For what concerns age-related diseases, AGEs play a role in the pathogenesis of many chronic conditions, like type 2 diabetes mellitus, neurodegenerative diseases, chronic renal failure, sarcopenia, osteoporosis, cardiovascular disease and cancer⁶⁷.

2. AIMS OF THE THESIS

Metabolic disorders and inflammation are intricately linked through a network of biochemical pathways, where AGEs and kinases play crucial roles^{1,50}. The diet-induced inflammatory environment is aggravated by exogenous and endogenous accumulation of AGEs. AGEs, binding to their receptors (RAGE), trigger signalling pathways that increase oxidative stress and inflammation, and kinases are activated in response to this inflammatory state. These kinases interfere as well with insulin signalling by phosphorylating key components of the insulin receptor pathway, exacerbating insulin resistance and promoting the metabolic disturbances seen in metabolic syndrome⁴⁶.

Data in the literature also reported a fundamental role of AGEs and chronic inflammation in human pathological conditions, specifically in paediatric cancer survivors⁶⁸. In childhood cancer survivors, normal cells exposed to chemotherapy and radiotherapy exhibit permanent molecular alterations in DNA structure and function, as well as disruptions in normal cellular repair mechanisms. These changes ultimately contribute to increased systemic inflammation^{69,70} and to an increased risk of chronic health conditions, such as cardiovascular diseases and secondary malignant tumour.

In this context, my research was divided into two areas of investigation, both closely linked by the pro-inflammatory role of Advanced Glycation End Products.

PART 1 (Preclinical in vivo and ex vivo studies)

The first part of this PhD thesis aims to:

- Elucidate how different types of hypercaloric diets and simple sugars (fructose and galactose) contribute to the accumulation of AGEs and investigate the pathophysiological responses of various metabolically active organs.
- Investigate whether the oral administration of naturally derived compounds, such as dietary fibres and the green-blue algae *Spirulina platensis*, can prevent AGE accumulation and the onset of diet-related dysfunctions.
- Evaluate the role of Spleen Tyrosine Kinase (SYK) in a dysmetabolic context and assess the efficacy and mechanism of action of its inhibitor Fostamatinib, to suggest its potential repurposing for the treatment of metabolic diseases.

PART 2 (Human observational studies)

The second part of the research focuses on the role of chronic inflammation, closely linked to AGEs accumulation, in the pathogenesis of late complications following anticancer treatments. Specifically, this part aims to:

- Explore the pro-inflammatory and pro-oxidative status in survivors of childhood Hodgkin lymphoma.
- Evaluate potential biomarkers of premature aging in this cohort.

To test our hypotheses, we based this thesis on the work published in the following articles from my PhD studies (*studies I, II, III, and V*), as well as on datasets that are still unpublished (*studies IV and VI*).

- I. **Eleonora Aimaretti***, Guglielmina Chimienti*, Chiara Rubeo, Rosa Di Lorenzo, Lucia Trisolini, Federica Dal Bello, Atefeh Moradi, Massimo Collino, Angela Maria Serena Lezza, Manuela Aragno, Vito Pesce. **Different Effects of High-Fat/High-Sucrose and High-Fructose Diets on Advanced Glycation End-Product Accumulation and on Mitochondrial Involvement in Heart and Skeletal Muscle in Mice**. *Nutrients* (2023)

* equally contributed to this work.

- II. Fidèle Almasri*, Debora Collotta*, **Eleonora Aimaretti**, Nadine Sus, Manuela Aragno, Federica Dal Bello, Carola Eva, Raffaella Mastrocola, Rikard Landberg, Jan Frank, Massimo Collino. **Dietary Intake of Fructooligosaccharides Protects against Metabolic Derangements Evoked by Chronic Exposure to Fructose or Galactose in Rats**. *Mol Nutr Food Res* (2023).

* equally contributed to this work.

- III. **Eleonora Aimaretti***, Elisa Porchietto*, Giacomo Mantegazza, Giorgio Gargari, Debora Collotta, Giacomo Einaudi, Gustavo Ferreira Alves, Enrica Marzani, Alessandro Algeri, Federica Dal Bello, Manuela Aragno, Carlo Cifani, Simone Guglielmetti, Raffaella Mastrocola, Massimo Collino. **Anti-Glycation Properties of Zinc-Enriched *Arthrospira platensis* (Spirulina) Contribute to Prevention of Metaflammation in a Diet-Induced Obese Mouse Model**. *Nutrients* (2024)

* equally contributed to this work.

- IV. The first part of this thesis includes also a partial set of unpublished data. The full manuscript will be submitted shortly.

Eleonora Aimaretti*, Elisa Porchietto*, Gustavo Ferreira Alves, Giacomo Einaudi, Chiara Rubeo, Enrica Marzani, Debora Collotta, Raffaella Mastrocola, Natasha Irrera, Manuela Aragno, Carlo Cifani, Massimo Collino. **Potential repurposing of fostamatinib in diet-induced metabolic disease: an *in vivo* study**.

* equally contributed to this work.

- V. **Eleonora Aimaretti***, Francesco Felicetti*, Federica Dal Bello, Filippo Gatti, Alessandro Godono, Francesca Saba, Giacomo Einaudi, Massimo Collino, Franca Fagioli, Manuela Aragno*, Enrico Brignardello*. **Advanced Glycation End Products and their related signaling cascades in adult survivors of childhood Hodgkin Lymphoma: a possible role in the onset of late complications**. *Free Radic Biol Med*. (2021)

* equally contributed to this work.

- VI. The second part of the thesis contains preliminary data on a second cohort of childhood Hodgkin Lymphoma survivors with the aim of evaluating potential biomarkers of premature aging in this cohort.

3. STUDY DESIGNS

The methodological framework of this thesis involved two approaches:

- **PART 1 Preclinical in vivo and ex vivo studies** This section aimed to evaluate the impact of hypercaloric diets on AGEs selective accumulation in organs and tissues and the corresponding pathophysiological responses, including inflammation. Furthermore, the study aimed to assess the efficacy of natural compounds or pharmacological interventions in mitigating these effects.
- **PART 2 Human observational studies** This study aimed to investigate the clinical role of AGEs and chronic inflammation in the pathogenesis of late complications of anticancer treatments in two distinct populations of childhood cancer survivors. The study also aimed to identify potential new biomarkers of premature aging in these patients.

3.1 Preclinical studies (**PART 1**)

The studies presented in this thesis were conducted either in three weeks old C57BL/6J OlaHsd male mice (**studies I, III and IV**; Envigo RMS Srl, Udine, Italy) or seven weeks old male Sprague-Dawley rats (**study II**; Janvier Labs, Le Genest-Saint-Isle, France). The animals were housed under conventional laboratory conditions at $25 \pm 2^{\circ}\text{C}$ and light/dark cycle (12/12 h) automatically controlled. Animals were provided with food and water ad libitum for the duration of the entire protocol. Body weight and food intake were recorded weekly with a precision scale. The *in vivo* procedures described were carried out in compliance with the ARRIVE guidelines and approved by the local Animal Use and Care Committees and the National Authorities (more details are provided in each specific article).

3.1.1 Studies I, III and IV

3.1.1.1 Dietary manipulation

After 1 week of acclimatisation, seventy mice were randomly assigned to the following dietary regimens for 12 weeks:

- **standard diet (SD, $n=15$)**. The standard diet (3,85 kcal/g) provided 10% kcal from fat, 70% kcal from carbohydrates (corn starch, maltodextrin) and 20% kcal from protein.
- **high-fat high-sugar diet (HFHS, $n=40$)**. The HFHS diet (5,56kcal/g) supplied 58% kcal from fat (Coconut Oil, hydrogenated), 25,5% kcal from carbohydrates (sucrose, maltodextrin) and 16,5% kcal from protein.
- **high fructose diet (HFr, $n=15$)**. This diet was isocaloric with the SD diet and it provided 10% kcal from fat, 70% kcal from carbohydrates (of which 60% were from fructose) and 10% from corn starch, and 20% kcal from protein.

Food pellets were obtained from Research Diet (New Brunswick, NJ 08901, USA) with the following codes: D12450K, D12331, and D02022704. After 4 or 5 weeks of dietary manipulation (5 weeks for study III and 4 weeks for study IV), two sub-groups on the HFHS

diet regimen began receiving either a natural compound, *Arthrospira platensis* (Spirulina) enriched in zinc (**Zn-SP**, $n=10$; Unione Spirulina Biologica Italiana, USBI, Curtatone, Mantova, Italy), or a drug treatment, **Fostamatinib disodium hexahydrate** ($n=15$; CliniSciences, Nanterre, France), for the remaining weeks. Spirulina was administered at a dosage of 350 mg/kg three times a week, a dose selected based on previous in vivo protocols^{71,72}. The zinc enrichment in our Zn-SP formulation corresponded to a daily intake of approximately 0,3 mg/kg body weight, which is below the levels reported to cause toxicity in mice. Further details of the zinc enrichment and composition of the natural extract can be found in **Table 2**.

Table 2. Zinc-enriched *Arthrospira platensis* (spirulina) nutritional content administered to a subset of HFHS-fed animals in study III

<i>Components</i>	<i>Content and composition</i>
Calories (Kcal/100 g)	348
Total carbohydrate (g/100 g)	24.2
Dietary fibres	<1.0
Sugar	<1.0
Total fat (g/100 g)	1.22
Saturated fat	0.56
Protein (g/100 g)	60.05
Salt (g/100 g)	2.09
Iron (Fe) (mg/100 g)	27
Potassium (K) (mg/100 g)	1640
Magnesium (Mg) (mg/100 g)	304
Phosphorus (P) (mg/100 g)	830
Zinc (Zn) (mg/100 g)	200

Fostamatinib was administered orally at a dose of 30 mg/kg/day, following previous in vivo studies that demonstrated this dosage does not cause severe adverse effects and can improve functional and biochemical disturbances associated with experimental chronic inflammation^{73,74}.

A schematic representation of the in vivo protocols with mice is reported in **Figure 6**.

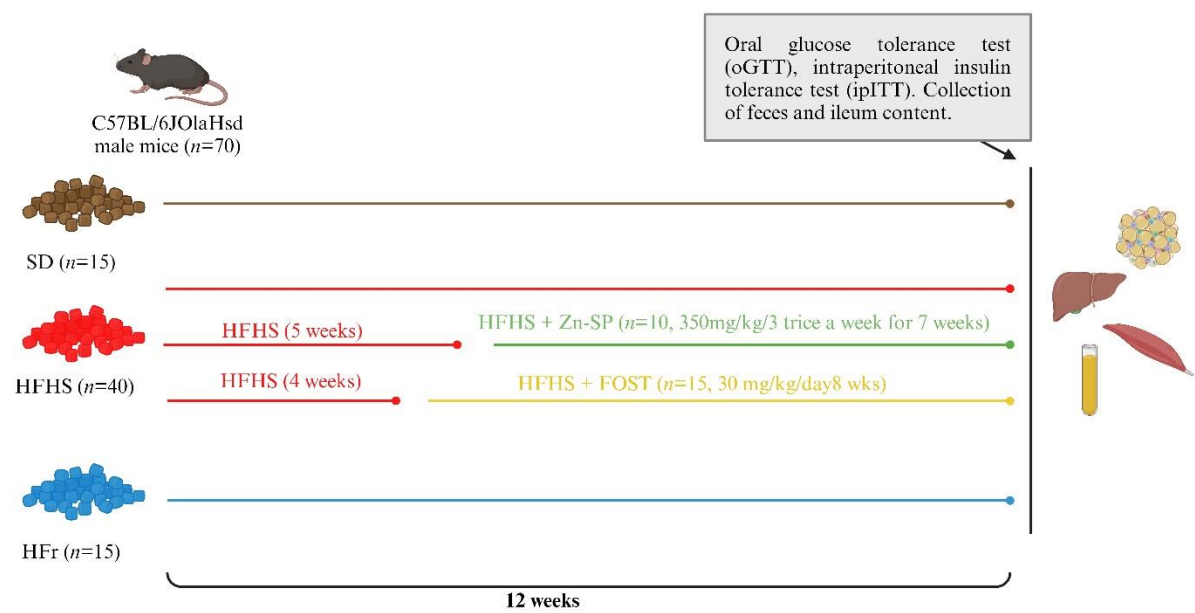


Figure 6. Timeline of the experimental design in mice studies (I, III and IV). C57BL/6Olasd male mice were randomly selected to receive either a standard diet (SD, n=15), a high-fat high sugar diet (HFHS, n=40) or a high-fructose diet (HFr, n=15). After 4 or 5 weeks of dietary, two sub-groups on the HFHS diet regimen were treated orally either with a natural compound, *Arthrospira platensis* (Spirulina) enriched in zinc (Zn-SP, n=10; 350mg/kg/3days a week) or a drug treatment, Fostamatinib disodium hexahydrate (n=15; 30mg/kg/day), for the remaining weeks. (Created with biorender.com).

A summary of the different dietary manipulations and of the interventions of each study are reported in **Table 3**.

Table 3. Schematic representation of the different dietary and interventional approaches of the mice studies. The entire duration of the in vivo protocol was of 12 weeks with, in total, 5 different groups of animals.

	SD (n=15)	HFHS (n=15)	HFr (n=15)	interventions
<i>Study I</i>	•	•	•	---
<i>Study III</i>	•	•	---	Zn-SP (350mg/kg/3d a week, <i>per os</i>) for 7 wks; n=10
<i>Study IV</i>	•	•	---	Fostamatinib (30mg/kg/day, <i>per os</i>) for 8 wks; n=15

3.1.1.2 *In vivo* procedures

Oral glucose tolerance test (oGTT)

After 12 weeks of dietary manipulation, the oGTT was performed following a 12h fasting period overnight. Briefly, after measuring the basal fasting blood glucose, we administered 2g/kg of glucose (30% aqueous solution, Sigma Aldrich, St. Louis, MO, USA) by oral gavage and we measured the serum glucose by tail puncture after 15, 30, 60 and 120 minutes with a conventional glucometer (Accu-check Aviva, Roche Diabetes Care Italy S.p.A, Italy).

Intraperitoneal insulin tolerance test (ipITT) - performed only in studies I and IV -

At the end of the 12-weeks protocol, following a 6h fasting period, the insulin tolerance test was performed⁷⁵. Shortly, glucose at the basal level was measured and, right after, insulin was administered intraperitoneally at a dose of 0,75 IU of insulin/kg of body mass. Glycemia was then evaluated after 15, 30, 60 and 90 minutes with the same glucometer employed in the oGTT.

3.1.1.3 Plasma and organ collection

After overnight fasting, mice were anesthetized with isoflurane (IsoFlo, Abbott Laboratories, North Chicago, IL 60064, USA) and euthanized by cardiac puncture and exsanguinated. Blood samples were collected in microtubes containing 5% ethylene-diamine-tetra acetic acid (EDTA) and then centrifuged at 5000 g at room temperature to separate plasma which was directly snap-frozen. Organs were collected in tubes and frozen in liquid nitrogen or harvested in optimal cutting temperature compound (OCT; Merck Millipore, Burlington, Massachusetts, USA). Samples were then stored at -80°C for further analyses.

3.1.2 Study II

3.1.2.1 Dietary manipulation

Forty-two male Sprague-Dawley rats (250–274 g) were purchased from Janvier Labs (Le Genest-Saint-Isle, France) at 7 weeks of age. After a 12 days familiarization period, the rats were randomly divided into the following dietary regimens for 8 weeks:

- **control group (CNT, n=6)**. The control diet (2,95 kcal/g) provided 5% kcal from fat (sunflower oil) and 40% from carbohydrate (starch, cellulose).
- **fat-enriched diet (FAT, n=6)**. The fat enriched diet (4,30 kcal/g) provided 20% kcal from fat (sunflower oil) and 40% carbohydrate (starch, cellulose).

All the other diets were isocaloric with the fat-enriched one (4,30 kcal/g), with the addition of two monosaccharides, galactose (GAL) or fructose (FRU) and the enrichment of 10% of fructooligosaccharides (FOS), a fermentable dietary fiber. The remaining groups are:

- **FAT + 10% fructooligosaccharides (FAT+FOS, n=6);**
- **FAT +25% galactose (FAT+GAL, n=6);**
- **FAT +25% galactose + 10% fructooligosaccharides (FAT+GAL+FOS, n=6);**
- **FAT +25% fructose (FAT+FRU, n=6);**
- **FAT +25% fructose + 10% fructooligosaccharides (FAT+FRU+FOS, n=6).**

All the diets provided 25% kcal from proteins. Further details on the diet compositions can be found in article n° III.

A schematic representation of the *in vivo* protocols with mice is reported in **Figure 7**.

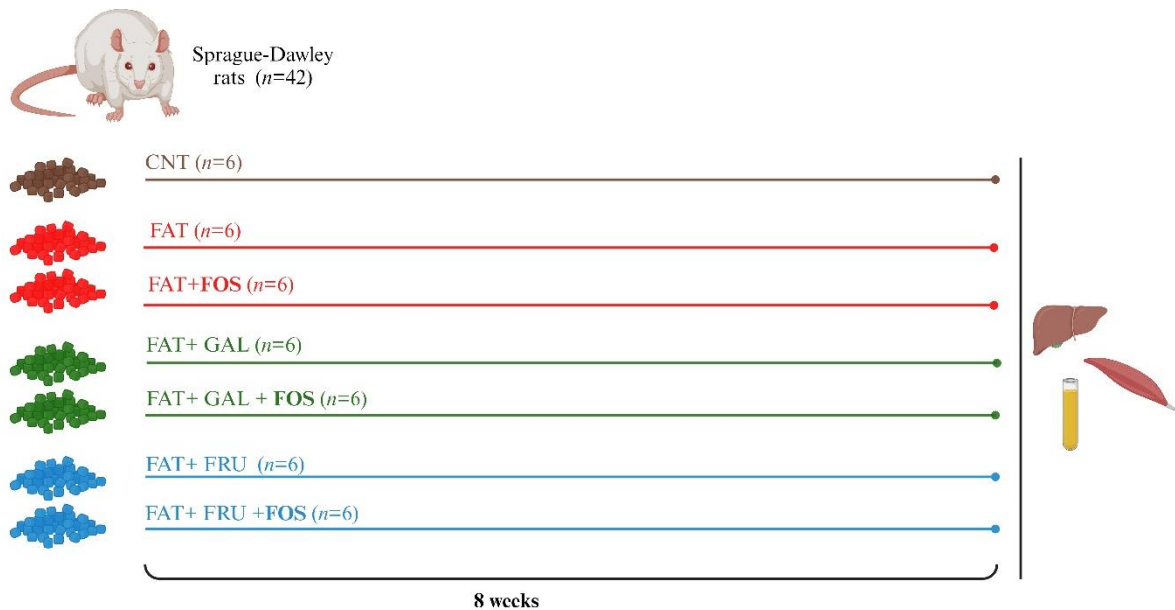


Figure 7. Timeline of the experimental design in the rat study (//). Sprague-Dawley rats were randomly selected to receive for 8 weeks either a standard diet (SD, $n=6$), fat enriched diet (FAT, $n=6$), a fat-enriched diet with 25% galactose (FAT+GAL) or a fat-enriched diet with 25% fructose (FAT+GAL). Additionally, the potential efficacy of 10% fructooligosaccharides (FOS), a fermentable dietary fiber, was assessed in the three different fat-enriched diets (FAT+FOS, FAT+GAL+FOS, FAT+FRU+FOS). (Created with biorender.com).

3.1.2.2 Plasma and organ collection

Following an 8-week intervention period, the rats underwent an overnight fast and were subsequently anesthetized using CO₂ before being euthanized via decapitation. Blood samples were collected into heparin-coated Monovettes (Sarstedt, Germany). Tissues were excised, placed in cryotubes, and rapidly frozen in liquid nitrogen. All samples were immediately stored at -80°C for subsequent analysis. Additionally, skeletal muscle and liver tissues were embedded and preserved in optimal cutting temperature (OCT) compound.

3.2 Human observational studies (PART 2)

The two observational presented in this thesis are part of a collaboration with the Transition Unit for Childhood Cancer Survivors at “Città della Salute e della Scienza” Hospital in Turin (Italy).

3.2.1 Study V

In a cohort of patients regularly monitored at the Transition Unit for Childhood Cancer Survivors, 20 individuals who had survived Hodgkin Lymphoma (HL) were recruited for this study. The inclusion criteria were as follows:

- a previous diagnosis of Hodgkin Lymphoma before the age of 25 years;
- age at enrolment between 18 and 40 years;
- completion of therapy at least 5 years prior to enrolment.

Exclusion criteria included a history of allogeneic hematopoietic stem cell transplantation (HSCT), glucose metabolism disorders (such as diabetes mellitus or impaired fasting glucose), a body mass index (BMI) greater than 25 kg/m^2 , active smoking or cessation less

than one-year prior, ongoing illness, and current treatment with anti-inflammatory drugs (both steroidal and non-steroidal) or other medications that could potentially interfere with the study outcomes. For comparison, 40 healthy volunteers were recruited during routine occupational health evaluations, following the same exclusion criteria. The study received approval from the hospital's Ethics Committee (approval no. CS2/1174), and informed consent was obtained from all participants.

3.2.2 Study VI

Similarly to the previous study, after signing the informed consent, subjects routinely followed at the Transition Unit for Childhood Cancer Survivors with the listed characteristics were enrolled ($n=35$):

- a. history of Hodgkin Lymphoma, diagnosed before 30 years of age
- b. oncological therapies terminated between 2010 and 2017
- c. age at the time of the study between 18 and 40 years

Two control populations consisting of healthy subjects, matched by sex, were enrolled:

- a. individuals aged between 18 and 40 years ($n=51$)
- b. individuals aged between 50 and 65 years ($n=49$)

The same exclusion criteria defined for the study n° V were also applied in the present study. The study received approval from the hospital's Ethics Committee (approval no. 0069523)

In both studies, following a blood sampling of 15mL of whole blood, we collected for each individual plasma and peripheral blood mononuclear cells.

3.2.3 Plasma Separation

Blood samples were collected into EDTA containing tubes. Plasma was isolated by centrifugation at 1500 RPM for 15 minutes and subsequently aliquoted and stored at -80°C .

3.2.4 Peripheral Blood Mononuclear Cell (PBMC) Extraction

PBMCs were extracted from whole blood using Ficoll-Paque Plus (GE Healthcare Europe GmbH, Germany) and gradient centrifugation at 1400 RPM for 35 minutes at room temperature. This process separates the blood into three layers: the top plasma layer, an intermediate layer containing PBMCs, and a bottom layer of polymorphonuclear cells and erythrocytes. Following isolation, PBMCs were washed twice with 0.9% saline solution and stored at -80°C for further analyses.

3.3 Experimental *ex vivo* procedures

The specific techniques employed in each study are thoroughly described in the corresponding publications. A concise description of the main experimental methodologies used is here provided. Some techniques are common to both preclinical *in vivo* studies and observational human studies; however, the key differences, if present, will be emphasized.

3.3.1 Measurement of biochemical markers

Plasma samples from mice were used to determine the levels of the transaminase-aspartate aminotransferase (AST) and alanine aminotransferase (ALT), cholesterol, triglycerides and

lactate dehydrogenase (LDH). Markers were measured using commercially available clinical assay kits (FAR Diagnostics, Verona, Italy) according to the manufacturer's instructions.

3.3.2 Systemic inflammation determination

In *studies I, II and IV*, systemic cytokine levels (IL6, IL1 β , IL10, IL17, IFN γ , TNF α) were measured in plasma samples using either ELISA (enzyme-linked immunosorbent assay) kits, following the provided manufacturers' indications (details on the products used can be found in the articles) or the Luminex-based Bio-Plex Pro™ Mouse Cytokine assay (6-Plex Th17 panel, #M6000007NY). This method allows for the simultaneous detection of multiple cytokines by employing fluorescently dyed microspheres, also known as beads, with each colour corresponding to a specific cytokine. The principle is similar to a traditional ELISA, but instead of a solid surface, it relies on fluorescent beads coated with antibodies specific to the target biomarker. When mixed with the sample, these beads bind to the relevant cytokines. After removing unbound substances through a series of washes, biotinylated detection antibodies are added to form a so-called sandwich complex. This complex is then tagged with streptavidin-phycoerythrin (SA-PE), a fluorescent marker. The intensity of the fluorescent signal, detected by the Bio-Plex 200 reader (Bio-Rad, Hercules, California, USA), correlates directly with the concentration of the cytokines in the sample, which is reported in pg/mL.

3.3.3 Enzymatic assays

Myeloperoxidase (MPO) activity assay

In the *study II*, approximately 100 mg of samples from the liver and gastrocnemius muscles were homogenized in a 1:5 ratio (w/v) with ice-cold 20 mM sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 13,000 g for 10 minutes at 4 °C. The resulting supernatants were discarded, and the pellets were retained. Next, 500 μ L of hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM sodium phosphate buffer, pH 6.0) was added, and the pellets were resuspended. Subsequently, a second centrifugation (13,000 g for 10 minutes at 4 °C) was carried out. The resulting supernatants (30 μ L) were used to assess myeloperoxidase (MPO) activity by measuring the hydrogen peroxide (H₂O₂)-dependent oxidation of 3,3',5,5'-tetramethylbenzidine (TMB). This reaction involved adding 180 μ L of H₂O₂ (final concentration 0.3 mM) in 0.08 M sodium phosphate buffer (pH 5.4) and 20 μ L of TMB (final concentration 1.6 mM) dissolved in Milli-Q water. In its oxidized form, TMB produces a blue color, which was detected using a spectrophotometer. The 96-well plate was incubated for 10 minutes at 37 °C, and the optical density (O.D.) was measured every two minutes at 650 nm (EnSight, PerkinElmer, Waltham, MA, USA). Protein content in the final supernatant was also measured using the BCA protein assay kit (refers to chapter 4.3.4 for further details). MPO activity was expressed as optical density at 650 nm normalized to the protein concentration (mg of protein).

Glyoxalase-1 (Glo-1) Activity Assay

The activity of Glo-1 was determined in protein extracts (as described in chapter 4.3.4) from mouse liver using a spectrophotometric method based on McLellan *et al*⁷⁶. The assay consisted of a 200 μ L reaction mixture containing 8 mM methylglyoxal (MG) and 2 mM

glutathione (GSH) in 50 mM sodium phosphate buffer (pH 7.4). This mixture was incubated at 37°C for 10 minutes to allow hemithioacetal formation to equilibrate before adding 20–30 µg of protein extract. The increase in absorbance at 240 nm, indicating the production of S-D-lactoylglutathione, was monitored using a microplate spectrophotometer (EnSight, PerkinElmer, Waltham, MA, USA) as an indicator of Glo-1 activity.

Glutathione Assay

Reduced and total glutathione were assayed spectrophotometrically (412 nm, read with EnSight spectrophotometer) in liver total homogenates according to the method of Akerboom *et al*⁷⁷. In brief, the levels of both reduced (GSH) and oxidized (GSSG) glutathione were measured using a kinetic assay. This method involves small amounts of GSH or GSSG, along with the enzyme glutathione reductase, which reduces 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). The GSSG-to-GSH ratio was calculated as a direct indicator of oxidative stress.

The last two assays described were performed in *study III*.

3.3.4 Western Blot

Western blot analyses were performed on frozen tissues (*studies I, II, III, IV*) or on pelleted peripheral blood mononuclear cells (*study V*).

Sample preparation

Briefly, around 50 mg of tissue or the pellet of cells were homogenized with a lysis buffer, containing protease and phosphatase inhibitors, and centrifuged at 13000g for 25 minutes at 4°C. In *study III*, to isolate cytosolic and nuclear proteins, liver tissue was homogenized at 10% (w/v) in a buffer and subjected to centrifugation at 1000g for 5 minutes at 4°C. The supernatant was then centrifuged again at 10000g for 40 minutes at 4°C to obtain the cytosolic fraction. The pellet, containing intact nuclei, was resuspended in an extraction buffer and incubated on ice for 30 minutes to facilitate high-salt extraction, followed by centrifugation at 15000g for 20 minutes at 4°C to isolate the nuclear proteins. Supernatants were then collected and total protein content quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA) and following the manufacturer's instructions. Once total protein extractions were prepared and quantified, 50 or 60µg of proteins were prepared to be separated by electrophoresis. Samples were prepared in microtubes by adding 50/60µg of proteins, 4µL of sample buffer solution (sample buffer 4x + dithiothreitol) and ultrapure distilled water (*quantum sufficit* to reach 16 µL). Samples were heated at 95°C for 5 minutes and then loaded into dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking gel) and a (8%, 10% or 12% running gel).

Electrophoresis, blotting and immunodetection

Electrophoresis was performed using the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad Laboratories Inc, Hercules, CA, United States), containing running buffer and constant voltage at 200 V, for approximately 35 min (R.T.). Proteins were then transferred to a polyvinylidene difluoride membrane or to a nitrocellulose membrane (Amersham™ Hybond®; Merck KGaA, Darmstadt, Germany) through an electro-transfer at constant

voltage of 100 V for 70 min. The blots were then blocked with 5% non-fat dried milk (A0830, PanReac Applichem, Darmstadt, Germany) and incubated with primary antibodies overnight at 4°C. All the primary antibodies are reported in **Table 4**.

Table 4 List of primary antibodies used for Western blot technique in studies I, II, III, IV and V

Antibody	Code	Dilution	Distributor
rabbit anti-NFκB p65	8242	1:1000	Cell Signalling (Danvers, Massachusetts, USA)
rabbit anti-ASC	67824	1:1000	Cell Signalling
mouse anti-NRLP3	AG-20B-0014-C100	1:1000	AdipoGene Life Sciences, San Diego, USA
rabbit anti-Gasdermin D	93709	1:1000	Cell Signalling
mouse anti-cleaved-IL1β	52718	1:1000	Cell Signalling
mouse anti-CML	PAB1293	1:1000	Abnova Corporation (Walnut, California, USA)
mouse anti-CEL	KAL-KH025	1:1000	Cosmo Bio (Carlsbad, California, USA)
mouse anti-RAGE	sc-365154	1:1000	Santa Cruz Biotechnology (Dallas, Texas, USA)
mouse anti-Nrf2	sc-13032	1:1000	Santa Cruz Biotechnology
mouse anti-Glo1	Gtx15747	1:1000	GeneTex, Inc. (Irvine, California, USA)
p47phox (NADPHox)	4301	1:1000	Cell Signalling
rabbit anti-Ser307 IRS-1	2381	1:1000	Cell Signalling
mouse anti-total IRS-1	3194	1:1000	Cell Signalling
rabbit anti-Ser473 AKT	4060	1:2000	Cell Signalling
rabbit anti-total AKT	9272	1:1000	Cell Signalling
rabbit anti-Ser9 GSK-3β	9322	1:1000	Cell Signalling
rabbit anti-total GSK-3β	9315	1:1000	Cell Signalling
rabbit anti-Tyr525/526 Syk	2710	1:1000	Cell Signalling
rabbit anti-total Syk	13198	1:1000	Cell Signalling
DRP1	H00010059-M01	1:1000	Abnova
MFN2	9482	1:1000	Cell Signalling
PGC-1	NBP1-04676PCP	1:1000	Novus Biologicals, (Minneapolis, USA)
TFAM	7495S	1:1000	Cell Signalling
SOD2 (MnSOD)	13194S	1:1000	Cell Signalling
Peroxisredoxin 3, PRXIII	LFPA0030	1:1000	Ab Frontier (Seoul, Republic of Korea)
rabbit β-actin	A2066	1:1000	Sigma-Aldrich (St. Louis, MO, USA)
rabbit anti-GAPDH	2118	1:1000	Cell Signalling

Blots were then incubated with a secondary antibody (Cell Signalling; anti-mouse #7076; anti-rabbit #7074) conjugated with HRP (dilution 1:10000) and developed using the ECL detection system (Bio-Rad Laboratories S.r.l., California, USA). The immunoreactive bands were analyzed by the Bio-Rad Image Lab Software™ 6.0.1 and results were normalized to standard diet.

3.3.5 Quantitative real time - polymerase chain reaction (qRT-PCR)

qRT-PCR was performed both in preclinical *study II* and in the first observational study (*study V*).

Total RNA was extracted from liver (10 mg) using Total Tissue RNA Purification Kit (#3700, Norgen Biotek Corporation, Thorold, ON, Canada) according to manufacturer's instructions. This kit is designed for isolating total RNA from a variety of sources, such as cells, bacteria, yeast, viruses, and fluids like plasma/serum, blood, saliva, cerebrospinal fluid (CSF), and others. They enable the extraction of high-quality, pure RNA with optimal RIN values and A260/A280 ratios, making them ideal for downstream applications like qRT-PCR, RT-PCR, microarrays, NGS, and more. The homogenization was conducted using gentleMACS™ Octo Dissociator (Miltenyi Biotec) with a specific program (duration:00m:53s, rpr:2753). Instead, approximately 30mg of gastrocnemius muscle or pelleted PBMC were lysed and processed to isolate total RNA using the standard phenolchloroform method (TRIzol Reagent, #15596026, Thermo Fischer, Waltham, MA, USA). The RNA concentration and purity were measured using a nanodrop (Titertek-Berthold) machine.

The obtained total RNA was retro-transcribed using the SensiFAST cDNA SynthesisKit (#BIO-65054 Meridian Bioscience, USA) according to manufacturer's instructions. Transcript levels were determined by Real time qPCR using the SensiFAST SYBR No-ROX Kit (#BIO-98005 Meridian Bioscience, USA) with 10 ng of cDNA per well. PCR reaction was carried out on the CFX Connect Real-Time PCR Detection System (Bio-Rad) in the following conditions:

- A. 95 °C for 2 min (polymerase activation)
- B. 95 °C for 5 s (denaturation)
- C. 60-65 °C for 10 s (annealing)
- D. 72 °C for 5-20 s (extension)

Steps B., C. and D. were repeated for 40 cycles and fluorescence was recorded at the end of each cycle. Relative gene expression was obtained after normalization to housekeeping genes using the formula $2^{-\Delta\Delta CT}$ as previously described⁷⁸ and folds changes were determined by comparison to the relative control group. The GAPDH, CCL2, and CXCL10 primers were purchased from Eurofins Scientific (Nantes, France). β -actin (QT01680476), beta-2-microglobulin (QT00088935), interleukin 6 (QT00083720), advanced glycosylation end product-specific receptor (AGE-R) (QT00000119), NADPH oxidase 1 (NOX-1) (QT00025585), NLR family, pyrin domain containing 3 (NLRP3) (QT01666343), p65/RELA (QT01007370), interleukin 1 β (QT00021385), AGER1/DDOST (OST-48) (QT00081466), gasdermin-D (GSDMD) (QT01155448) were purchased from Qiagen (Hilden, Germany). Negative controls for reverse transcription (no reverse transcriptase enzyme) and for qPCR (no complementary deoxyribonucleic acid) were included in the experiments.

3.3.6 Quantification of AGEs by Ultra-High-Performance Liquid Chromatography (UHPLC)-Tandem Mass Spectrometry

Tissue homogenates of *studies I, II and III* or plasma samples from both observational studies (*studies V and VI*) (50 μ L) were hydrolysed with 500 μ L of 0.6 M trichloroacetic acid and

50 μ L of 6 M hydrochloric acid for 2 hours at 60°C. The samples were analysed using an ultra-high-performance liquid chromatography (UHPLC) system coupled to a triple quadrupole mass spectrometer (AB-Sciex Triple Quad 5500, Milan, Italy), equipped with an electrospray ionization (ESI) Turbo Ion source. Separation of analytes was achieved on a reversed-phase C18 column (Kinetex 100 $\times$ 2.1 mm, 1.7 μ m particle size, Phenomenex Torrance, California, USA) with a flow rate of 0.35 μ L/min. A gradient mobile phase ranging from 95/5 to 40/60 in 25 minutes, composed of 5 nM heptafluorobutanoic acid/acetonitrile, was used. The eluent from the column was directed to the Turbo Ion source with nitrogen as the sheath (GS1) and curtain (CUR) gases, and air as the auxiliary gas (GS2). The source voltage was maintained at 4.5 kV in positive ion mode, with CUR set to 26 arbitrary units (arb), GS1 at 45 arb, and GS2 at 50 arb. The heated capillary was kept at 275°C. MRM transitions for N ϵ -(carboxymethyl)-lysine (CML) included m/z 205 $\rightarrow$ 84, with a declustering potential (DP) of 100 V, entrance potential (EP) of 7 V, and collision energy (CE) of 32 V. Quantification of the analytes was achieved using calibration curves for CML and methylglyoxal-derived hydroimidazolone-1 (MGH1) at concentrations of 10, 50, 100, 250, 300, and 500 μ g/L. The concentration of analytes in the samples consistently fell within the linear calibration range. The coefficient of variation for the measurements ranged from 8% to 14%.

3.3.7. Imaging methodologies for lipid visualization and cellular marker detection

Oil Red O staining

Oil Red O staining is a histological technique that was used in *study II* to visualize hepatic lipid droplets on 14 μ m liver cryostatic sections. The dye used is lipophilic, meaning it selectively binds to lipid-rich areas, staining them in red. Stained tissues were viewed under an Olympus Bx4I microscope (400 $\times$ magnification) with an AxioCamMR5 photographic attachment (Zeiss, Gottingen, Germany).

Immunofluorescence

Double immunofluorescence was performed in *study IV* for GLUT-4 in a dilution 1:20 (rabbit anti-insulin regulatable glucose transporter 4, cod. CBL243, EMD Millipore Corporation, Temecula, CA 92590, USA) and dystrophin in a dilution 1:600 (Monoclonal Anti-Dystrophin antibody produced in mouse, D8168, Merck KGaA, Darmstadt, Germany) on 10 μ m cryostatic sections of gastrocnemius muscles. After blocking, sections were incubated with a mix of primary antibodies overnight at 4°C degree. After washing, sections were incubated with a mix of labelled secondary antibodies with the following concentrations: 1:200 Goat F(ab')₂ Anti-Rabbit IgG (Abcam, Boston, MA, USA), 1:400 Anti-Mouse IgG FITC antibody produced in goat (Sigma-Aldrich Company Ltd., St. Louis, Missouri, USA). The images were colour-combined and assembled into photomontages using ImageJ (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

3.4 Statistical analysis and data interpretation

Statistical analysis is briefly described in this section and detailed in corresponding section of each paper. Data were expressed as dot-plots or as bars for each study and as mean $\pm$ SD/SEM or median with range of n observations, where n represents the number of animals/experiments/subjects studied. The standard distribution of data was verified by

Shapiro-Wilk normality test and the homogeneity of variances by Bartlett test. The statistical analysis was performed by one/two-way ANOVA, followed by Bonferroni's post-hoc test or by the t-test. Data that were not normally distributed, non-parametric statistical analysis was applied through Kruskal-Wallis followed by Dunn's post hoc-test or through Mann-Whitney test. A P value <0.05 was considered significant. Statistical analysis was performed using GraphPad Prism® software version 7.05 (San Diego, California, USA).

4. RESULTS

In this section, a comprehensive summary of each study will be provided to emphasize the key findings and to underscore their significance within the broader context of the thesis project. The aim is mainly to highlight how each study contributes to the overall objectives and framework of the entire PhD project. For deeper exploration, full text of each study is included toward the end of the thesis.

PART 1 (Preclinical studies)

4.1 STUDY I

Different Effects of High-Fat/High-Sucrose and High-Fructose Diets on Advanced Glycation End-Product Accumulation and on Mitochondrial Involvement in Heart and Skeletal Muscle in Mice

Eleonora Aimaretti*, Guglielmina Chimienti*, Chiara Rubeo, Rosa Di Lorenzo, Lucia Trisolini, Federica Dal Bello, Atefeh Moradi, Massimo Collino, Angela Maria Serena Lezza, Manuela Aragno and Vito Pesce

* equally contributed to this work

Nutrients 2023; 15, 4874.

The consumption of energy-dense, fat- and sugar-rich diets, coupled with a sedentary lifestyle, can lead to metabolic syndrome, type 2 diabetes (T2D), non-alcoholic fatty liver disease and cardiovascular diseases⁷⁹. These conditions are associated with chronic low-grade inflammation and increased production of reactive oxygen species (ROS). Recent research has shed light on how particular nutrients impact metabolic disturbances. Among these, fructose stands out, as its consumption has surged in recent decades due to shifts in dietary habits, particularly the increased intake of sugar-sweetened drinks and processed foods high in sugar⁸⁰.

This study aims to compare the effects of a diet enriched on both fats and sugars and of a diet with high concentration of fructose on:

- formation of advanced glycation end-products (AGEs)
- mitochondrial function
- tissue-specific damage, particularly in the heart and skeletal muscles, to explore the broader implications of modern dietary habits.

The most relevant **results** include:

- ***The two diets induced comparable metabolic impairments and inflammation*** The HFHS and HFfr diets determined an impaired response to both the oGTT and ITT, indicating a condition of glucose and insulin intolerance induced by the two diets (Fig 2). The HFHS led to elevated inflammation and oxidative stress, evidenced by over-activation of oxidative (NADPH oxidase) and inflammatory (NLRP3 inflammasome) pathways.

- ***Differential accumulation of advanced glycation end-products (AGEs) in plasma and in the heart induced by the two diets*** Both diets increased the accumulation of AGEs, specifically N ϵ -(carboxymethyl)lysine (CML) in plasma. CML levels were raised significantly in the hearts of HFHS-fed mice, but not in the hearts of HFr-fed mice.
- ***Differential tissue adaptation to oxidative stress: mitochondrial responses in heart and skeletal muscle under dietary stress*** Results demonstrated the heart's remarkable ability to adapt to the heavy mitochondrial oxidative stress induced by an HFHS diet, as mitochondrial biogenesis was significantly impacted but effectively compensated by changes in mitochondrial dynamics. In contrast, the skeletal muscle did not show such adaptation, with a substantial reduction in mitochondrial biogenesis that was not effectively offset by any mitochondrial adjustments. On the other hand, the milder oxidative stress caused by the HFr diet produced tissue-specific effects in both the heart and muscle. In the heart, a noticeable decrease in mitochondrial biogenesis was partially balanced by increased fission, while in the TA, a slight reduction in biogenesis was offset by a tendency toward increased fusion, highlighting the distinct adaptability of each tissue to the diet.

The first study presented in this thesis emphasize for the first time the distinct responses of the heart and skeletal muscle to different types of dietary-induced impairments. Both diets resulted in an accumulation of advanced glycation end-products (AGEs) with a more pronounced increase observed in the hearts of HFHS-fed mice. It is well reported that the interaction of AGEs with their receptor enhances oxidative stress through ROS production by NADPH oxidases inside the mitochondria, affecting the functionality of the mitochondrial respiratory chain⁸¹. Despite the enhanced AGEs-induced NADPH oxidase expression, the heart demonstrates a notable adaptability to HFHS-induced oxidative stress, through a new balance of mitochondrial dynamics and an increased expression of PRXIII (peroxiredoxin III). This finding is consistent with the hypothesis according to which PRXIII cooperates with MQC (mitochondria quality control) processes in the heart through the regulation of mitochondrial ROS removal and oxidative damage prevention⁸². On the other hand, the skeletal muscle did not accumulate AGEs to the same extent of the heart exhibiting limited capacity for such adaptation. In contrast, the HFr diet induces milder oxidative stress, which triggers tissue-specific mitochondrial responses, with the heart showing increased fission and skeletal muscle displaying enhanced fusion, reflecting different adaptive mechanisms. In conclusion, the study highlights for the first time distinct tissue-specific responses to dietary-induced impairments, with the heart showing greater adaptability to oxidative stress through enhanced mitochondrial dynamics and PRXIII expression, while skeletal muscle demonstrates limited adaptation. Furthermore, these findings underscore the differential impact of modern diet on mitochondrial function in different organs.

STUDY II

Dietary Intake of Fructooligosaccharides (FOS) protects against Metabolic Derangements Evoked by Chronic Exposure to Fructose or Galactose in Rats.

Fidèle Almasri*, Debora Collotta*, Eleonora Aimaretti, Nadine Sus, Manuela Aragno, Federica Dal Bello, Carola Eva, Raffaella Mastrocola, Rikard Landberg, Jan Frank*, Massimo Collino*

* equally contributed to this work

Molecular nutrition & food research 2024; 68(4), e2300476.

This study was conducted in the frame of the European Project “CarbQ4Health project under the umbrella of the Joint Programming Initiative “A Healthy Diet for a Healthy Life” (JPI HDHL) (2019–02201) and of the ERA-NET Cofund HDHL INTIMIC (GA N° 727565 of the EU Horizon 2020 Research and Innovation Programme).

As also demonstrated in **study I**, excessive consumption of simple sugars can lead to the accumulation of advanced glycation end products (AGEs). This accumulation is a key factor in the development of low-grade chronic inflammation, which negatively affects lipid metabolism and contributes to various metabolic disorders⁸³. Among the most consumed sugars, there are fructose and galactose. While the detrimental effects of fructose on AGEs accumulation and metabolic health have been well-documented, the specific impact of galactose on AGE formation remains largely unexplored, leaving a significant gap in our understanding of how different sugars influence chronic inflammation⁸⁴.

Recent interest has grown in the role of fructooligosaccharides (FOS), a type of prebiotic fiber, which offer protective effects against the negative consequences of excessive sugar consumption by promoting a healthier gut microbiome and potentially reducing systemic inflammation⁸⁵. However, the interaction between fructooligosaccharides and sugars concerning AGEs accumulation and metaflammation, has not yet been fully investigated.

The second study here presented aims to:

- investigate the ability of the two sugars to exacerbate the effects of a fat diet and to identify differences in the accumulation of AGEs and activation of inflammatory pathways.
- investigate the potential efficacy of the addition to the diet of FOS in counteracting these effects.

The main highlights of this project were:

- ***FOS ameliorated metabolic parameters and liver and muscular functionality*** The different diets did not result in significant differences in body weight gain or fasting blood glucose. However, there were notable changes in lipid profiles, with the fat and sugar -enriched diets increasing plasma triglycerides and cholesterol. FOS

consumption significantly reduced these levels. The same trend was also observed for the hepatic transaminases ALT and AST.

FOS also demonstrated protective effects in the liver and in the skeletal muscle, reducing the lipid accumulation induced by the diets.

- ***AGEs accumulation was prevented by FOS supplementation in the liver and in the skeletal muscle*** The intake of both fructose and galactose led to a substantial increase in tissue accumulation of AGEs, which was significantly mitigated by FOS. As a result, there was a marked increase in the expression of RAGE in the liver and gastrocnemius muscles of rats consuming a Westernized diet containing fructose or galactose. However, when FOS was simultaneously included in the diet, this RAGE upregulation was significantly reduced.
- ***Local inflammation induced by the dysregulated diets was dampened by FOS*** As expected the diets evoked a massive infiltration in the two organs examined of immune cells, which contribute to the development of metabolic inflammation. FOS supplementation resulted in a reduction of local expression of the chemoattractant chemokines measured.

This study underscores the detrimental local effects of excessive simple sugar consumption in metabolically active organs (specifically liver and gastrocnemius) by promoting the accumulation of AGEs and the activation of inflammatory pathways, adding substantial new evidence for galactose. Indeed, we found that the intake of either fructose or galactose in a fat-enriched diet evoke the accumulation of AGEs, which may be due not only to the well-known non-enzymatic Maillard reaction, but also to an accelerated glycolysis⁸⁶. In fact, both galactose and fructose lead to the production of the glycolysis reactive intermediates, such as glyoxal (GO) and methylglyoxal (MGO), which are AGEs precursors.

We and others have previously described the crucial role of the AGE-RAGE pathway as a key cascade at the interface between inflammation and dysregulated lipid handling^{87,88}. For instance, AGEs induce a deregulated activation of triacylglycerol and cholesterol synthesis, resulting in intracellular lipid accumulation with reduced muscle efficiency and hepatic fibrosis. Thus, the lipid derangements that we observed mainly in the liver, might be explained at least in part by the related accumulation of AGEs.

FOS supplementation has already been demonstrated to lower serum and liver lipids, as well as hepatic markers of metabolic inflammation⁸⁹. Here, for the first time, we also demonstrated that the supplementation with the prebiotic FOS effectively prevented AGEs accumulation and RAGE overexpression, thus mitigating local inflammation. These findings suggest that improving a dysregulated diet with dietary fibres could be an effective strategy for protecting against sugar-induced metabolic inflammation and improving overall metabolic health in the context of a Westernized diet.

4.2 STUDY III

Anti-glycation properties of zinc-enriched *Arthrospira platensis* (Spirulina) contribute to prevention of metaflammation in a diet-induced obese mouse model

Eleonora Aimaretti*, Elisa Porchietto*, Giacomo Mantegazza, Giorgio Gargari, Debora Collotta, Giacomo Einaudi, Gustavo Ferreira Alves, Enrica Marzani, Alessandro Algeri, Federica Dal Bello, Manuela Aragno, Carlo Cifani, Simone Guglielmetti, Raffaella Mastrocola* and Massimo Collino*

* equally contributed to this work

Nutrients, 2024; 16(4):552

This study was conducted in collaboration with the Unione Spirulina Biologica Italiana (USBI). USBI represents a distinct collaboration among Italian Spirulina producers. The Italian Organic Spirulina Union is dedicated to advancing the quality and innovation of independent Spirulina algae cultivation in Italy. By implementing rigorous cross-verification of both the production processes and the final product, USBI ensures the superior quality of Italian Spirulina⁹⁰.

As demonstrated in **study 2**, the consumption of natural fibres can mitigate the adverse effects of advanced glycation end products accumulation in various organs. To further explore this, we aimed to confirm the hypothesis *in vivo* by administering *Arthrospira platensis* (Spirulina), a blue-green algae known for its well-established anti-inflammatory and antioxidative properties^{91,92}, to investigate whether its bioactive components also possess anti-glycative effects. Additionally, in this study, we fed the animals a hypercaloric diet with elevated levels of both fat (58%) and simple sugars (25,5%) to enhance endogenous AGE formation, thereby allowing us to evaluate the efficacy of the supplementation under more challenging conditions.

The study investigates the anti-glycation properties of zinc-enriched Spirulina and its effects on preventing metabolic inflammation (metaflammation) in a diet-induced obese mouse model. The most promising results are here reported:

- ***Improvement in Glucose Homeostasis and hepatic function after 7 weeks of supplementation with Zn-SP*** Mice on a high-fat, high-sugar diet supplemented with Zn-SP showed significant improvements in glucose metabolism. Zn-SP helped restore fasting blood glucose levels and improved overall glucose tolerance, suggesting a protective effect against diet-induced hyperglycemia and insulin resistance. Moreover, Zn-SP treatment led to a reduction in markers of hepatic dysfunction, reducing the circulating levels of ALT (alanine transaminase) and AST (aspartate transaminase).
- ***Zn-SP reduced AGE Accumulation by interfering with their formation and promoting their degradation*** Zn-SP supplementation significantly prevented the

accumulation of advanced glycation end products in the liver and reduced the expression of RAGE. Moreover, when Zn-SP was chronically administered to HFHS mice, the hepatic nuclear translocation of Nrf2 was restored, leading to a markedly upregulated expression of Glo-1 protein, the main AGE-detoxifying enzyme, paralleled by enhanced enzymatic activity and reduction in the GSSG/GSH ratio.

- ***Zn-SP reduced local and systemic Inflammation*** The reduction in AGE accumulation contributed to lower levels of local and systemic inflammation. Zn-SP supplementation resulted in decreased plasma levels of pro-inflammatory cytokines such as TNF- α , IL-6 and IL-1 β , which are typically elevated in obesity and contribute to metaflammation.
- ***Normalization of Gut Microbiota Composition induced by the oral administration of Zn-SP*** The high-fat, high-sugar diet led to dysbiosis, a disruption in the normal gut microbiota balance, while Zn-SP treatment helped restore a correct composition, potentially contributing to the overall metabolic improvements observed in the study.

This study confirms that natural supplements, thanks to their composition, can effectively mitigate the endogenous formation of AGEs and their associated negative effects in *in vivo* models. Spirulina phytocomplex includes several antioxidant molecules such as phycocyanins, carotenoids, tocopherols, and phenols, and its efficacy in counteracting oxidative stress has been largely demonstrated in animal models and in human trials⁹³. Here, we reported, for the first time, that the antioxidant effect of Zn-SP may be mediated by the enhancement of the transcriptional activity of Nrf2 in the liver, thus confirming the antioxidant properties of the spirulina phytocomplex, which contribute, along with its anti-inflammatory properties, to protection against diet induced metabolic derangements. Most notably, we documented for the first time that the beneficial antioxidants and anti-inflammatory effects of Zn-SP supplementation are due, at least in part, to its antiglycation properties, reducing the levels of two main AGE derivatives, CML and CEL but also through the downregulation of their receptor RAGE.

In conclusion, administering Zn-SP on a hypercaloric diet resulted in significant improvements in glucose and lipid metabolisms, liver function, and reduction in systemic inflammation. The supplementation also prevented AGE accumulation by enhancing glyoxalase-1 activity and restored gut microbiota balance. These findings suggest that Zn-SP is a promising intervention for combating metabolic disorders exacerbated by high-fat, high-sugar diets.

4.3 STUDY IV

Potential repurposing of fostamatinib in diet-induced metabolic disease: an in vivo study.

Eleonora Aimaretti*, Elisa Porchietto*, Gustavo Ferreira Alves, Giacomo Einaudi, Chiara Rubeo, Enrica Marzani, Debora Collotta, Raffaella Mastrocola, Natasha Irrera, Manuela Aragno, Carlo Cifani, Massimo Collino

* equally contributed to this work

The data here reported are unpublished.

As noted in the introduction, activation of the AGE-RAGE pathway, whether through the ingestion of exogenous AGEs or consumption of a dysregulated diet that enhances endogenous AGE formation, can trigger various pro-inflammatory and pro-oxidative cascades, involving multiple kinases⁹⁴, including those in the SYK (spleen tyrosine kinase) signalling pathway⁹⁵. To explore the potential for pharmacologically inhibiting the over-activation of kinase-mediated signalling pathways induced by AGE/RAGE interaction, we conducted an in vivo study under the same dietary conditions as **studies 1 and 3**.

SYK is a crucial enzyme involved in immune-related signalling pathways and is highly expressed in various cells, including adipocytes, skeletal muscle, and hepatic cells. It plays a significant role in promoting inflammation, particularly through the activation of the NLRP3 inflammasome, which contributes to metaflammation and insulin resistance⁹⁶. Fostamatinib, a SYK inhibitor, has been recently approved for the treatment of immune thrombocytopenia (ITP), autoimmune haemolytic anaemia and IgA nephropathy⁹⁷. Fostamatinib is a prodrug that is converted to the active metabolite R406, which inhibits SYK in an ATP-competitive manner with high selectivity and potency (IC₅₀ 41 nM). So far, no experimental data have been reported on the potential role of the SYK signalling pathway in the pathogenesis of diet-induced metabolic derangements and the therapeutic relevance of its pharmacological inhibition. Thus, here we investigated:

- the role of SYK activation in a context of diet induced metabolic alterations, focusing in particular on the skeletal muscle.
- the potential beneficial effects of fostamatinib in a murine model of diet-induced obesity and insulin resistance.

The data obtained are here summarised and the main figures reported:

- ***Fostamatinib reduces body weight gain induced by the high-fat high-sugar diet***
C57BL6 mice fed with the experimental high-fat high-sugar (HFHS) diet for 12 weeks had greater body weight gain than standard diet-fed littermates (SD). The body weight gain, recorded weekly, was slightly, but not significantly, reduced by Fostamatinib administration in the first 5 weeks of the drug treatment. The reduction became significant in the last 3 weeks.

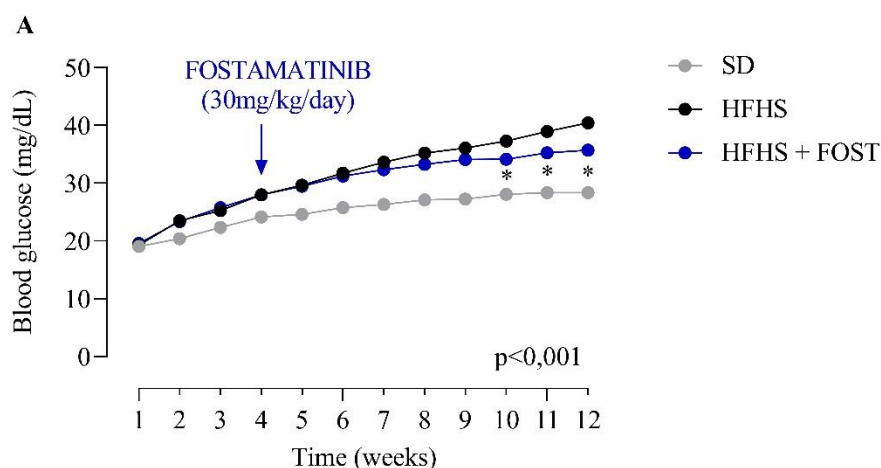


Figure 8 Body weight Weekly body weight (A) was evaluated after the dietary manipulation and the administration of Fostamatinib (30mg/kg) (n=15 per each group). All the data are expressed as mean $\pm$ SEM. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test. * $p < 0,05$ vs HFHS.

- **Amelioration of systemic insulin resistance following 8 weeks of fostamatinib administration** The oral glucose tolerance test (oGTT) and the insulin tolerance test (ITT) confirmed the ability of fostamatinib to counteract the development of the HFHS-induced systemic glucose and insulin resistance.

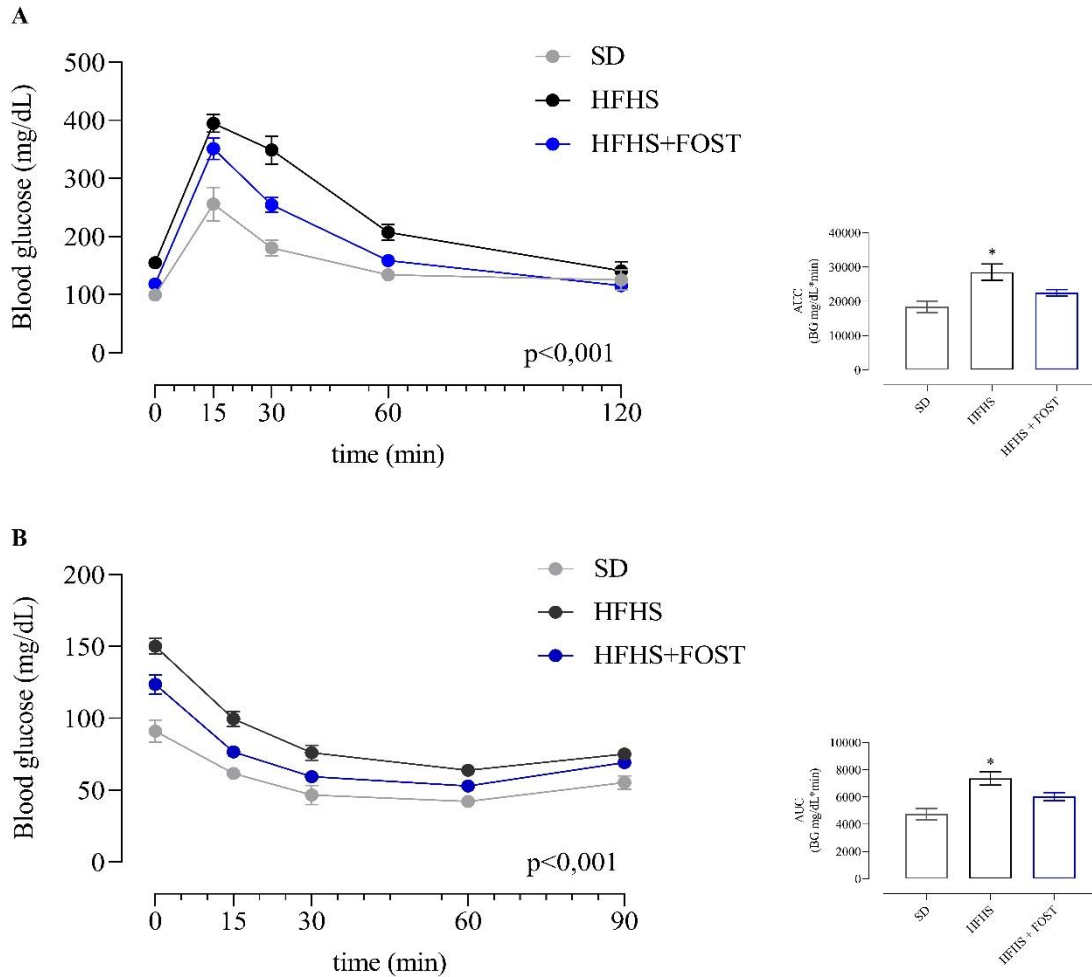


Figure 9 . Oral glucose tolerance test (oGTT; A) and intraperitoneal insulin tolerance test (iITT, B). All parameters were measured after 12 weeks of dietary manipulation and the administration of Fostamatinib (30mg/kg) following 12- or 6-hours fasting period respectively. All the data are expressed as mean $\pm$ SEM for n=8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test. * $p < 0,05$ vs SD HFHS+FOST.

- ***Fostamatinib induced an amelioration of insulin sensitivity by restoring a correct GLUT4 translocation*** GLUT4 (Glucose Transporter Type 4) is a transmembrane protein that facilitates the transport of glucose into cells, predominantly in skeletal muscle and adipose tissue. Upon insulin stimulation, GLUT4 translocates from intracellular storage vesicles to the plasma membrane, where it mediates the uptake of glucose from the extracellular environment into the cell. GLUT4 immunofluorescence analysis confirmed a limited GLUT-4 membrane translocation in the skeletal muscle of mice exposed to HFHS. On the contrary, fostamatinib administration resulted in a significant improvement of GLUT-4 membrane translocation, as shown by a continuous and homogeneous co-localization of GLUT-4 with the sarcolemma marker dystrophin, like that recorded in the SD group.

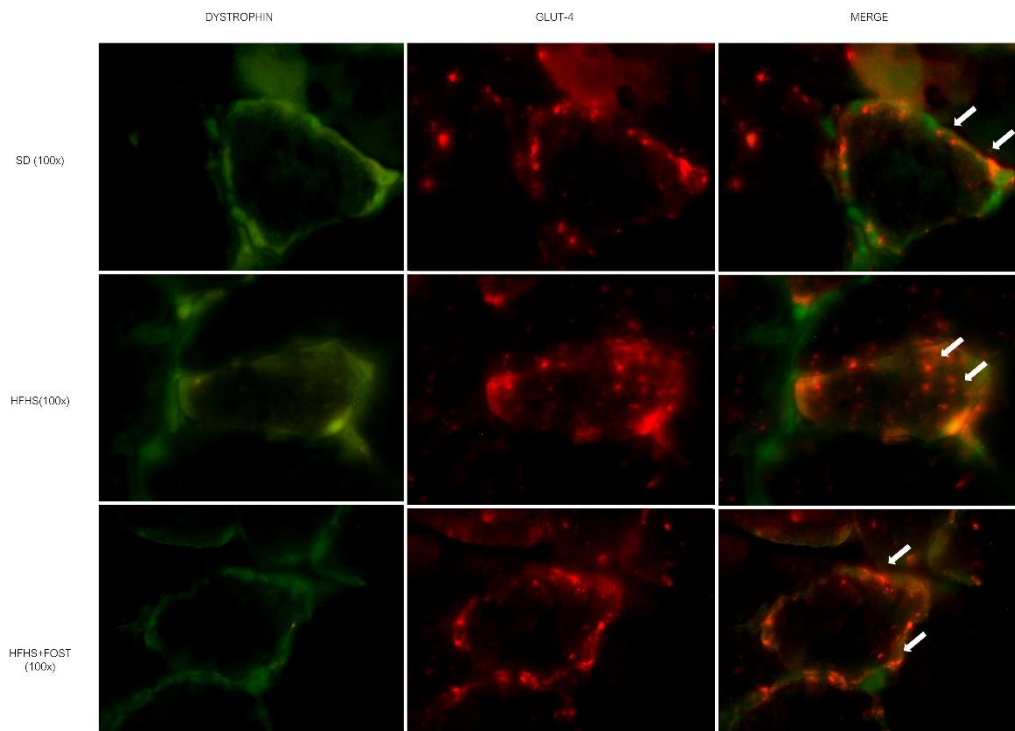


Figure 10 Representative images of GLUT4 translocation on sarcolemma of gastrocnemius muscle. After 12 weeks of dietary manipulation and 8 weeks of Fostamatinib administration in the treatment group, gastrocnemius muscles were collected in optimal cutting temperature (OCT) compound. 100x magnification photomicrographs of immunofluorescence analyses for GLUT4 and dystrophin on gastrocnemius sections from SD, HFHS, and HFHS+FOST mice performed on 4–5 mice per group.

- ***Fostamatinib induced a clear downregulation of local and systemic inflammation***
HFHS-fed mice showed elevated systemic inflammation, with increased levels of IL-6, IL-1 β , IL-17, IFN γ , and TNF α compared to those on a standard diet. Remarkably, Fostamatinib treatment completely reversed the diet-induced cytokine surge in the bloodstream.

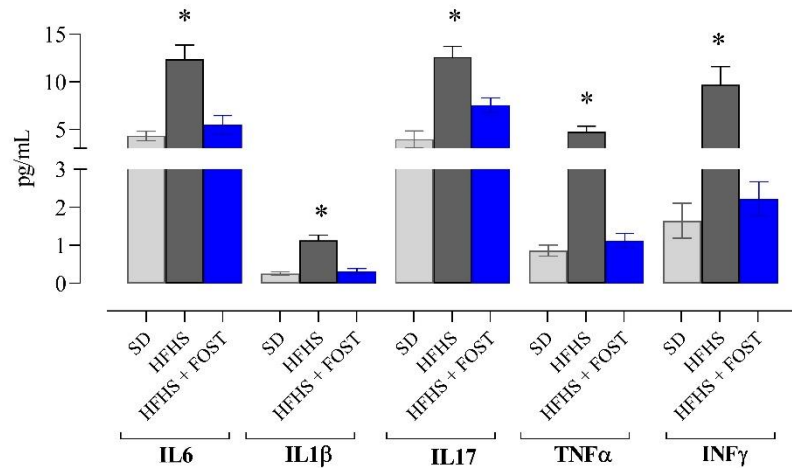


Figure 11 Effect of fostamatinib on plasmatic cytokines during the 12weeks dietary manipulation. Plasma levels of IL6, IL-1 β , IL-17, INF γ and TNF α were determined. Data are expressed as mean $\pm$ SEM of $n = 10/15$ mice per group. Cytokines were statistically analyzed by one-way ANOVA followed by Bonferroni's post hoc test, * $p < 0,05$ vs SD/ HFHS+FOST.

In this study, we demonstrated a key role for the tyrosine kinase SYK in regulating metabolic inflammation and insulin resistance. We presented preliminary findings suggesting, for the first time, that fostamatinib could offer a promising opportunity for drug repositioning in the clinical management of diet-induced metabolic disorders. SYK has previously been shown to regulate pro-inflammatory pathways, including the NLRP3 inflammasome, which has been clearly implicated in initiating and promoting sterile inflammation in dysmetabolic conditions over the past decade^{1498,99}. Recently, it was demonstrated that NLRP3-derived IL1 β decreases GLUT4 expression and impairs its insulin-induced translocation to the sarcolemma, playing a key role in the development of insulin resistance¹⁰⁰. Most notably, our findings showed that SYK inhibition was associated with improved systemic insulin sensitivity. This improvement may be partly due to the indirect effect of SYK inhibition in down regulating the formation and activation of the NLRP3 inflammasome complex, which in turn helps restore GLUT4 translocation and reduce insulin resistance.

Overall, these findings suggest that fostamatinib could be a novel pharmacological approach for repositioning in the treatment of metabolic diseases, offering potential advantages in terms of cost and safety.

PART 2 (Human observational studies)

The *studies V and VI* reported in this section were conducted in collaboration with the Transition Unit for Childhood Cancer Survivors at “Città della Salute e della Scienza” Hospital in Turin (Italy).

4.4 STUDY V

Advanced glycation end products and their related signalling cascades in adult survivors of childhood Hodgkin lymphoma: A possible role in the onset of late complications

Francesco Felicetti*, Eleonora Aimaretti*, Federica Dal Bello, Filippo Gatti, Alessandro Godono, Francesca Saba, Giacomo Einaudi, Massimo Collino, Franca Fagioli, Manuela Aragno*, Enrico Brignardello*

* equally contributed to this work.

Free Radic Biol Med, 2022; 178:76-82

The first study in this second section investigates the accumulation of AGEs and their related signaling pathways within a distinct context compared to the previous section, offering a different and more clinically oriented perspective on the role of AGEs in promoting chronic low-grade inflammation.

Adult survivors of childhood Hodgkin lymphoma (HL), despite high survival rates, face increased risks of late-onset complications, particularly cardio-vascular diseases and secondary malignant tumours.

The research focuses on the role of AGEs, oxidative stress, and inflammation in these complications by comparing HL survivors with healthy controls. In particular, in this paper we aimed to:

- evaluate glycoxidation, inflammatory status, and oxidative stress in plasma and peripheral blood mononuclear cells (PBMC) of adult survivors of childhood Hodgkin lymphoma (HL), compared to age- and sex-matched healthy controls.
- investigate the potential role of AGEs accumulation and the activation of their associated signaling pathways (particularly the AGE/RAGE axis) in the onset of late complications in these survivors, focusing on their contribution to chronic inflammation and oxidative stress.

The main findings were:

- ***Higher oxidative stress and inflammation markers in HL survivors*** Increased expression of NADPH oxidase (also confirmed at protein levels in peripheral blood mononuclear cells PBMC) and a higher GSSG/GSH ratio indicated heightened oxidative stress in HL survivors. To confirm these results, we also reported in HL survivors a reduced levels of alpha-tocopherol and impaired catalase activity, highlighting an impaired antioxidant defence. Moreover, elevated plasma levels of inflammatory markers such as C-reactive protein (CRP), interleukin-1 β (IL-1 β), and

interleukin-6 (IL-6), confirmed by an upregulation of NLRP3 inflammasome and NF- κ B signaling pathways in (PBMC) of HL survivors, confirmed a low-grade pro-inflammatory status in this population.

- ***HL survivors showed an higher AGEs Accumulation*** HL survivors exhibited significantly higher plasma levels of AGEs, in particular N ϵ -carboxymethyl-lysine (CML) and methylglyoxal hydroimidazolone (MG-H1).
- ***AGE Receptor Pathway resulted altered in HL survivors*** Hodgkin lymphoma survivors exhibited increased expression of RAGE (receptor for AGEs) alongside decreased expression of AGER1 (AGE receptor 1), indicating an imbalance in the AGE/RAGE axis.

Our results show that, five or more years after the end of cancer therapies, the paediatric HL survivors show an unbalanced oxidative state and low-grade chronic inflammation. HL survivors bear increased plasma levels of AGEs in comparison with age-matched healthy controls. AGE formation occurs not only under conditions of hyperglycemia, but also in the presence of oxidative stress¹⁰¹. It is well known that exposure to ionizing radiations or to certain chemotherapy agents causes an immediate increase in ROS production, generating oxidative stress¹⁰². Therefore, it can be hypothesized that oxidative stress leads to the formation and accumulation of AGEs, which may trigger inflammation, weaken antioxidant defenses, and increase ROS production. This creates a feedback loop where oxidative stress continues even after cancer therapies have ended. In this cycle, AGEs could act as a "metabolic memory," perpetuating the cycle of ROS formation, oxidative stress, and inflammation.

In this study, also the expression of AGE receptors was evaluated: the full-length RAGE expression in PBMCs was higher in HL survivors than in controls. Under conditions of chronic and sustained inflammation, RAGE signalling is upregulated, which may lead to the development and progression of pathological states observed in HL survivors: indeed, cardiovascular diseases and second malignant tumours, which are the main late effects responsible for the excess mortality observed in CCS, are both associated with AGE accumulation, oxidative stress and chronic^{103,104}.

In conclusion, the findings of this study demonstrate that these survivors exhibit markedly elevated levels of AGEs in their plasma, which in turn activate RAGE and trigger a cascade of intracellular signalling pathways that exacerbate oxidative damage and inflammatory responses. This persistent pro-inflammatory and pro-oxidative state, likely induced by prior cancer therapies, may contribute to the early onset of aging-related disorders and increase the risk of cardiovascular disease and secondary malignancies in this vulnerable population.

4.5 STUDY VI

Evaluation of new potential biomarkers of inflammation and premature aging in a cohort of childhood Hodgkin lymphoma survivors

The data here reported are preliminary and still under investigation.

Paediatric Hodgkin lymphoma (HL) is today one of the most curable cancers thanks to the progress of therapeutic protocols. Noteworthy, the populations of childhood and young adult cancer survivors (CAYACS), which constantly increased in the last decades, is susceptible to the development of late effects of cancer therapy with higher risk of secondary malignant tumours and cardiovascular diseases, pathologies typically prevalent in an elderly population. Here we aimed to investigate whether CAYACS are at risk of premature aging^{64,105}, which is characterized by the accumulation of advanced glycation end products (AGEs), together with chronic inflammation and oxidative unbalance, as also reported in *study V*.

The main results obtain in the preliminary phases of this study are:

- ***Accumulation of AGEs differs among the different populations recruited*** Middle aged controls accumulate higher plasmatic levels of AGEs examined. Indeed, as largely reported in the literature, accumulation of advanced glycation end products on nucleotides, lipids and proteins are an inevitable component of the aging process^{106,107}. As we previously demonstrated in *study V*, AGEs can also accumulate in childhood cancer survivors. In this specific context survivors showed an intermediate condition on plasmatic accumulation of CML, CEL and pentosidine without any statistical differences compared to both young and middle-aged controls suggesting that survivors, which were age-matched with the younger controls, accumulated more AGEs than the aforementioned group.

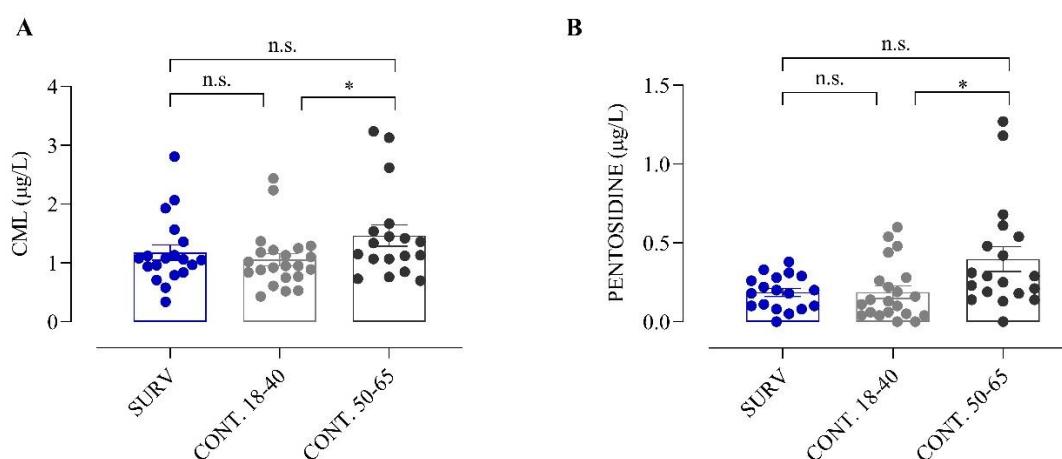


Figure 12 CML (A) and pentosidine (B) levels in plasma of HL survivors and in the two control populations. Data are expressed as scattered dot plot and mean±SEM of n=23/25 individuals per group. * $p < 0,05$.

- **Plasma sRAGE and MMP9 levels confirm an alteration in the AGEs related mechanisms suggesting an over activation of the metalloprotease system.** A specular data distribution was found when the soluble form of the receptor for AGEs (sRAGE), a scavenger form of the full-length receptor, was analysed in plasma. Indeed, a significant reduction of the circulating levels was found in the older control population, while no differences were found between the SURV groups and the two control populations. As well reported in the literature, aging decreases sRAGE in healthy individuals^{108,109}: total sRAGE results from the proteolytic cleavage of RAGE by ADAM10 or through membrane-bound RAGE proteolysis by MMPs. For this reason, we decided to evaluate the plasmatic concentration of the matrix metalloprotease 9 (MMP9). Surprisingly, higher levels of MMP9 were found in the plasma of childhood cancer survivors and this could potentially lead to a higher cleavage and release of sRAGE.

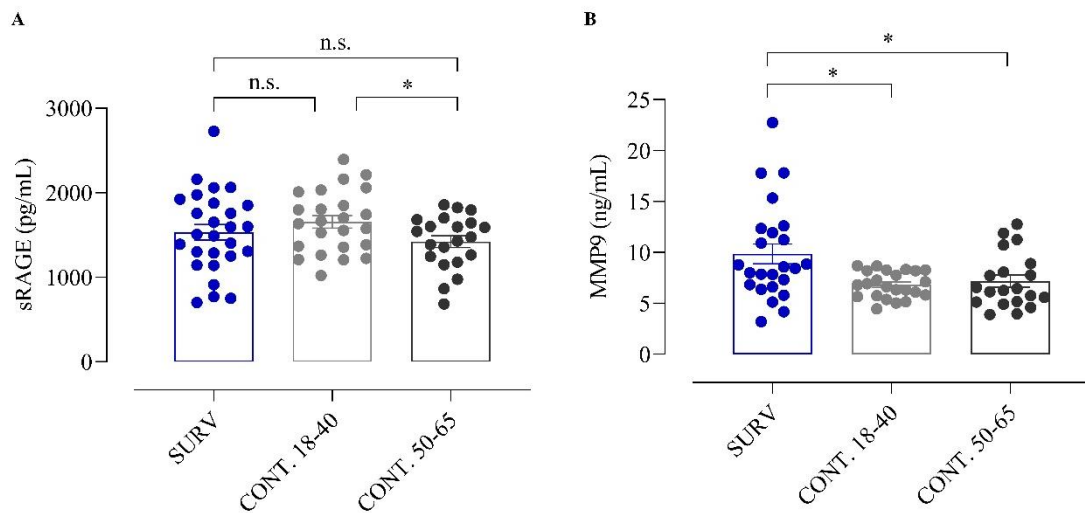


Figure 13 sRAGE (A) and MMP9 (B) levels in plasma of HL survivors and in the two control populations. Data are expressed as scattered dot plot and mean $\pm$ SEM of n=23/25 individuals per group. * $p < 0,05$.

These preliminary data indicate that survivors of paediatric Hodgkin lymphoma exhibit an accumulation of AGEs, potentially leading to sustained activation of intracellular signalling pathways related to oxidative stress and inflammation. Our findings also suggest an over-activation of MMP pathways, particularly with increased release of the MMP9 isoform, which is capable of cleaving and releasing the soluble form of RAGE.

Further studies on the PBMCs are needed to verify that the co-existence of AGEs accumulation, unbalanced oxidative status, and inflammation could play a role in activation of mechanisms by which senescent cells drive-age associated pathologies lead to a premature aging in HL survivors. Indeed, in addition to cardiovascular disease and cancer, childhood cancer survivors are burdened with a wide range of chronic health conditions typically seen in elderly people. This suggests an early onset of physiological aging and/or frailty. Physiological aging is associated with various cellular mechanisms, including cellular

senescence. In CCS, these molecular mechanisms appear to be accelerated due to cancer and exposure to cancer therapies¹¹⁰.

In conclusion, our preliminary findings may suggest that survivors of paediatric Hodgkin lymphoma exhibit molecular signs of accelerated aging, including AGE accumulation, oxidative stress, and inflammation. The over-activation of MMP9 and related pathways may further contribute to the premature aging process in these survivors. Further research is needed to confirm the role of these mechanisms in age-associated pathologies among childhood cancer survivors.

5. DISCUSSION

The results of the different studies developed during the PhD project aim to offer insights on the pathogenic role of AGEs in two different settings: diet-induced metabolic derangements and cancer survivorship. Besides, potential nutritional and pharmacological approaches aimed to counteract AGEs accumulation were also investigated.

In the initial section of this thesis (*study I*), we provided a more comprehensive elucidation of the mechanisms by which various unhealthy dietary habits contribute to the accumulation of AGEs and how they differentially affect peripheral organs, in particular the heart and the skeletal muscle, with a particular focus on the inflammatory state and the associated mitochondrial damage. We also investigated how the affected organs respond and adapt to these conditions, revealing a complex scenario where a diet high in saturated fats and simple sugars significantly increases AGE accumulation, resulting in substantial mitochondrial oxidative stress. Our findings showed that the heart possesses a remarkable ability to adapt to the oxidative stress induced by a high-fat, high-sugar diet, primarily by maintaining mitochondrial function through modified dynamics. To address these abnormalities, we implemented three distinct *in vivo* strategies (*studies II, III and IV*), with a particular focus on mitigating the inflammation triggered by the accumulation of AGEs and the subsequent activation of pro-inflammatory kinases. The first approach was nutritional, involving the incorporation of soluble dietary fibres, specifically fructooligosaccharides (FOS), into the diet. Soluble dietary fibres are largely fermentable by the gut microbiota, mainly in the large intestine¹¹¹. These fibres are widely recognized as health-promoting food components, with significant implications for a range of diseases, including diabetes, cardiovascular disorders, and inflammatory bowel diseases¹¹². While most research to date has focused on general comparisons between low- and high-fibre diets the functional properties of specific dietary fibre subtypes have been largely overlooked in the literature. With our research, we demonstrated for the first time the potentiality of FOS in counteracting AGEs accumulation preventing the pro-inflammatory state and, consequently, metabolic abnormalities. In addition to the nutritional approach, we explored the nutraceutical potential of a natural supplement, zinc-enriched *Spirulina platensis*, aiming to determine whether its known health benefits are connected to the anti-glycative properties of its bioactive components. This investigation allowed us to uncover a previously unrecognized attribute of this blue-green algae, demonstrating its capacity to mitigate glycation processes. By highlighting this novel anti-glycative effect of both FOS and zinc-enriched *Spirulina platensis*, our studies open new avenues for research into the application of nutraceutical strategies in the prevention and management of metabolic diseases. Although the findings on AGEs accumulation and the activation of AGE-dependent pathways in both studies are highly promising, some differences can be observed. The duration of the *in vivo* protocols was relatively similar, but the composition of the diets, particularly the fat content, differed significantly. In *study II*, the fat content was 20%, which did not lead to notable changes in body weight. In contrast, in *study III*, the fat content exceeded 50%, resulting in a pronounced state of obesity that was not reversed by the concomitant administration of zinc-enriched *Spirulina platensis*. Although anti-obesity effects of this supplement have been reported in some preclinical and clinical^{113,114}, we did not observe any impact of Zn-SP supplementation on body weight gain,

illustrating how nutraceutical approaches often yield puzzling and conflicting results. These findings align with scientific reports showing that significant weight changes are typically observed with diets containing more than 40% sucrose, fructose, or fat. Diets with lower percentages of these components generally do not induce significant weight changes, although local metabolic alterations are often detected¹¹⁵. Indeed, even the milder diet used in Study II induced clear local changes, such as the accumulation of chemoattractant chemokines CCL2 and CXCL10, which are known to contribute to T-cell recruitment at sites of inflammation. This suggests that local recruitment is an early event, preceding the release of systemic inflammatory mediators¹¹⁶.

In the framework of diet-induced abnormalities, considering that the activation/regulation of inflammatory pathways is primarily based in a phosphorylation-dependent manner^{117,118}, we aimed to explore the potential of pharmacologically targeting spleen tyrosine kinase (SYK) in the context of diet-induced inflammation (*study IV*). Our preliminary results, presented here for the first time, suggest that fostamatinib, a drug recently approved for selective immune-mediated disorders, may offer a promising opportunity for drug repositioning in the treatment of diet-induced metabolic disturbances.

In the fourth study presented in this thesis, we investigated a pharmacological approach aimed at counteracting the effects of dietary abnormalities, with the objective of drawing hypotheses on the potential differences between the nutraceutical interventions explored in the second and third studies and a treatment based on an already approved drug. The primary distinction observed was that, while neither the nutritional approach with FOS or the nutraceutical supplementation with zinc-enriched *Spirulina platensis*, effectively reduced body weight, the administration of fostamatinib led to a significant reduction after just a few weeks. Although the nutritional and nutraceutical strategies showed promising improvements in other metabolic parameters, particularly in reducing metabolic inflammation, a pharmacological intervention was required to achieve a notable reduction in weight gain. This finding highlights the added potency of pharmacological treatments in addressing more advanced or resistant aspects of metabolic dysfunction, such as obesity, compared to nutritional or nutraceutical approaches alone. Indeed, based on the mounting evidence generated from both basic and clinical research, these approaches are commonly used for suppressing inflammation, improving insulin sensitivity¹¹⁹. Currently, the nutritional or nutraceutical approach to treat diet-induced abnormalities is increasing in use, and in many cases is used by physicians as the first choice in the treatment of patients with borderline values. For these reasons, diet enrichment with fibres or nutraceuticals may represent an excellent opportunity to treat the preliminary conditions not yet showing the pathological signs of obesity, while it is necessary to use a pharmacological approach to counteract the accumulation of body weight.

In the second part of this work, we extended our research questions to a more clinical perspective. Specifically, we conducted an in-depth investigation into the role of Advanced Glycation End Products (AGEs) and chronic inflammation in paediatric Hodgkin Lymphoma survivors, as part of *studies V and VI*. While the initial studies focused on the effects of nutraceutical and pharmacological interventions in metabolic disorders, this phase aimed to broaden our understanding by investigating AGEs accumulation in a distinct clinical context. The rationale for this shift lies in the increasing recognition that cancer survivors, particularly those exposed to aggressive treatments, are at heightened risk for long-term health complications, many of which share mechanisms with metabolic disorders, particularly chronic low-grade inflammation. Chronic inflammation, often exacerbated by chemotherapy and radiation, is a critical factor in these late-onset health conditions¹²⁰. These treatments generate oxidative stress and ROS, leading to DNA damage and the formation of AGEs. We hypothesized that the oxidative stress-induced accumulation of AGEs could contribute to the persistent inflammatory state observed in survivors of paediatric Hodgkin Lymphoma, similar to what we observed in metabolic disorders. Our findings revealed that these cancer survivors exhibited significantly elevated plasma AGEs levels compared to healthy, age-matched controls, suggesting that AGEs accumulation could play a role in their chronic inflammatory state. These results are consistent with the growing body of literature that links anti-cancer therapies to oxidative stress and AGEs formation⁶². Moreover, these findings suggest that AGEs accumulation may activate inflammatory pathways, contributing to long-term risks such as cardiovascular disease (CVD), metabolic disorders and premature aging in post-treatment patients. Indeed, an additional layer to this understanding comes from the concept of cellular senescence, a state where cells lose their capacity to replicate or grow and adopt a senescence-associated secretory phenotype (SASP)¹²¹. The production of SASPs are induced by various factors such as NAD⁺ depletion, increased mtROS production, impaired mitochondrial fission/fusion, and, in particular, compromised autophagy¹²². Indeed, autophagy is also widely accepted to provide a protective role in aging and aging-related disorders, and its decline with age might precipitate the onset of a variety of diseases¹²³. SASP involves the secretion of pro-inflammatory cytokines, chemokines, and growth factors, which can affect both nearby and distant normal cells. This systemic condition, known as *inflammaging*, predisposes individuals to various disorders, including cardiovascular, metabolic, and neurodegenerative diseases, infertility, and osteoporosis. In this study, together with other published evidence, we demonstrated that survivors of childhood and adolescent cancers exhibit increased levels of inflammatory markers. This is further supported by Latoch et al. (2022), who demonstrated that survivors of acute lymphoblastic leukaemia (ALL) exhibit a biological profile consistent with premature aging, characterized by an increased secretion of multiple SASP biomarkers¹²⁴. These findings align with our hypothesis that cancer therapies not only induce oxidative stress but also drive cellular senescence, contributing to the chronic inflammation seen in survivors.

In conclusion, the perspective presented in the second part of this thesis complements the first by highlighting the potential role of advanced glycation end-products (AGEs) as a unifying factor across various pathological conditions. While the initial focus was on the involvement of AGEs in metabolic inflammation, this section extends the investigation to

cancer survivorship, where similar processes of AGE accumulation and inflammation may occur. Therefore, exploring AGEs in clinical contexts beyond metabolic disorders enhances our understanding of how AGEs and associated inflammatory pathways contribute to long-term health risks across diverse disease settings. Furthermore, our experimental findings provide critical insights into the current body of research. AGE accumulation, exacerbated by oxidative stress during cancer treatment, not only drives chronic inflammation but may also activate senescence-associated secretory phenotype (SASP) pathways, thereby amplifying systemic inflammation and contributing to premature aging and other health complications. This highlights the importance of targeting AGEs as a potential therapeutic strategy to mitigate both metabolic complications and the long-term consequences of cancer therapy, including cardiovascular disease, neurodegenerative disorders, and osteoporosis.

6. CONCLUSIONS

The main aim of this research lies in bridging the gap between metabolic disorders and cancer survivorship, with AGEs highlighted as a potential unifying factor linking chronic inflammation, cellular senescence, and long-term health risks

Summarising, this thesis highlights:

- the growing global burden of diet-related health issues, such as obesity and type 2 diabetes, emphasizing the role of chronic low-grade inflammation as a key driver in the progression of these conditions. Our research sheds light on the contribution of AGEs to this inflammatory state, particularly in peripheral organs. We demonstrated the potential of dietary and pharmacological interventions to mitigate AGEs accumulation and inflammation.
- the examination of paediatric Hodgkin Lymphoma survivors revealed a similar inflammatory pattern, linking cancer treatments to elevated AGEs levels and long-term health complications.

While this project could evolve into a broader investigation of AGEs as therapeutic targets, not only aiming to improve metabolic health but also to ameliorate long-term outcomes of cancer-related conditions, it is important to take a more cautious approach in evaluating this future perspective. Indeed, although targeting inflammation in metabolic and oncological diseases is not new^{125,126}, the lack of successful clinical translation of several attempts must be acknowledged. This is partly due to the challenges posed by chronic immunopharmacological modulation, which can have complex consequences, particularly in cancer contexts. Unlike metabolic diseases, where the immune response might be more straightforward, targeting inflammation in cancer survivors could introduce more significant complexities¹²⁷.

Moreover, there are still significant areas that require deeper exploration, especially regarding whether AGEs serve primarily as biomarkers or they can be also targeted pharmacologically. While inflammatory kinases are more readily targetable, the development of drugs targeting advanced glycation end-products (AGEs) presents unique challenges. This is due to the complex nature of AGE formation, which involves multiple pathways, and the fact that AGEs can activate a wide range of biological processes both directly and indirectly. Indeed, understanding how to pharmacologically target AGEs compared to other inflammatory mediators remains a hurdle in therapeutic advancement.

Finally, there is substantial evidence indicating that dietary interventions, often in combination with physical activity, can significantly improve the overall quality of life in cancer survivors¹²⁸. Building on this knowledge, the present thesis offers a potential new avenue of research aimed at exploring the impact of reducing dietary intake of exogenous AGEs. This reduction could be achieved either through decreased consumption of AGE-rich foods or through the adoption of nutritional or nutraceutical strategies. These strategies, which may serve as alternatives or complements to pharmacological approaches, could potentially mitigate the detrimental effects of AGEs that have been observed in the course of this study. By addressing the role of dietary AGEs in cancer survivors, this research could

contribute to the development of novel dietary interventions designed to alleviate AGE-induced alterations, thereby improving long-term health outcomes in this population.

7. FULL-TEXT ARTICLES INCLUDED IN THE THESIS (*study I, II, III, V*)

STUDY I

STUDY II

STUDY III

STUDY V

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