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**Fasting-mimicking diet-based non-toxic combination
therapy in cancer treatment: from molecules to bedside**

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For all people suffering from cancer

“In scientific research, neither the degree of one’s intelligence nor the ability to carry out one’s tasks with thoroughness and precision are factors essential to personal success and fulfilment. More important for the attaining of both ends are total dedication and a tendency to underestimate difficulties, which cause one to tackle problems that other, more critical and acute persons instead opt to avoid.”

“Nella ricerca scientifica né il grado di intelligenza né la capacità di eseguire e portare a termine il compito intrapreso sono fattori essenziali per la riuscita e per la soddisfazione personale. Nell’uno e nell’altro contano maggiormente la totale dedizione e il chiudere gli occhi davanti alle difficoltà: in tal modo possiamo affrontare problemi che altri, più critici e acuti, non affronterebbero.”

Rita Levi Montalcini

ABSTRACT

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Fasting and fasting-mimicking diet (FMD) was shown to delay tumor progression and sensitize a wide range of tumor types to the toxic effect of chemotherapy, through a mechanism which can involve the lowering of IGF-1 and glucose levels. Here, I evaluate FMD-based non-toxic interventions for the treatment of KRAS mutant cancer, focusing on colorectal cancer (CRC).

KRAS activating mutations are predictors of poor prognosis and resistance to standard and targeted anti-cancer treatments, making the patient's survival rate very poor. Recently, a pharmacological dose of vitamin C has been proposed to be effective in the treatment of KRAS mutant colorectal cancer (CRC), however its mechanism of action remains controversial and its efficacy limited. A growing body of evidence indicates that the increased ROS levels and disruption of iron metabolism characterizing tumor cells, are responsible for vitamin C-mediated toxicity. Free labile iron, by reacting with hydrogen peroxide generated through vitamin C oxidation, leads to the formation of hydroxyl radical (Fenton reaction), which causes oxidative damages and cell death.

In my thesis, I show that vitamin C alone has a relatively mild toxic effect against CRC and I discovered that this is caused in part by the up-regulation of the stress-inducible protein heme-oxygenase-1 (HO-1). FMD can cause a major enhancement in vitamin C toxicity in KRAS mutant CRC both *in vitro* and *in vivo*. The mechanism underlying this effect involves, at least in part, the FMD-dependent downregulation of HO-1. I show that FMD, by reverting the HO-1 induction mediated by vitamin C, is able to reduce ferritin level, leading to an increase in free ferrous ions (Fe^{2+}). The increase of Fe^{2+} , together with

a dramatic enhancement of ROS generation, leads to DNA damage and cell death, probably through Fenton reaction.

Collectively, my findings show that FMD represents a safe therapeutic intervention, able to potentiate vitamin C anti-cancer effect. In addition, I provide evidence indicating that FMD and vitamin C combo therapy enhances oxaliplatin efficacy in KRAS-driven CRC mouse models, thus representing a promising therapeutic option, which can be hopefully translated into the clinic.

ABBREVIATIONS

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ROS	Reactive oxygen species
SOD	Superoxide dismutase
MMR	Mismatch repair
NER	Nucleotide-excision repair
BER	Base-excision repair
DSBR	Double-strand break repair
OXPHOS	Oxidative phosphorylation
CR	Caloric Restriction
GH	Growth hormone
GHBP	Growth hormone binding protein
TIL	Tumor infiltrating lymphocytes
NK	Natural killer
LS	Laron syndrome
FMD	Fasting-mimicking diet
IF	Intermitted fasting
PF	Prolonged fasting
STS	Short –Term Starvation
DSR	Differential stress resistance
DSS	Differential stress sensitization
GAPs	GTPases-activating proteins
GEF	Guanine exchange factors
CRC	Colorectal cancer
CIN	Chromosomal instability

MSI	Microsatellite instability
LOH	Loss of heterozygosity
PFS	Progression-free survival
TCA	Tricarboxylic acid cycle
GSH	Glutathione
NAC	N-acetyl cysteine
LIP	Labile iron pool
DHA	Dehydroascorbic acid
LC-MS/MS	Liquid chromatography - mass spectrometry/ mass spectrometry
SILAC	Stable isotope labeling with amino acids in cell culture
HFMD	Human fasting-mimicking diet
CAT	Catalase
ZnPP	Zinc protoporphyrin
OXP	Oxaliplatin
5-FU	5-fluorouracil
BMI	Body mass index
CT	Computed tomography
PET	Positron emission tomography
XELOX	Capecitabine and oxaliplatin
FDG	¹⁸ F-fluoro-2-deoxy-D-glucose
XELIRI	Capecitabine and irinotecan

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INTRODUCTION

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1. Aging and age-related diseases

Aging is a complex process characterized by accumulation of damages at the molecular, cellular and organ level, which leads to increased vulnerability to diseases and ultimately death (Kirkwood, 2005; Longo et al., 2008). Oxidative stress, DNA damage, cellular senescence, nutrient and growth signalling deregulation are the major alterations associated with aging (Figure 1) (Lieber and Karanjawala, 2004; Kenyon, 2005; López-Otín et al., 2013).

Some of the major phenotypic consequences of aging are age-related diseases, which are commonly described as degenerative pathological conditions characterized by alterations and failure of tissue structure and function (Campisi, 2005). Age-related pathologies include a wide range of complex diseases, among which cardiovascular diseases, type 2 diabetes, neurological disorders, cognitive decline and cancer (Fabbri et al., 2015).

Even if in the last century life span has increased, this has not been associated with an improvement in healthy life expectancy, which is defined as “*health-span*” (Hung et al., 2011). In fact, more than 70% of people over 65, experience at least two chronic conditions (Hung et al., 2011). It has been estimated that by 2050, the number of people over 80 years will triple with a consequent increase in frailty, defined as a poor health status due to age-associated decline (Fontana et al., 2014; Xue, 2011). Thus, the understanding of the aging process and its regulatory pathways is fundamental to identify possible novel targets to slow aging, to prevent age-related diseases and consequently to extend lifespan and health-span (Longo et al., 2015).

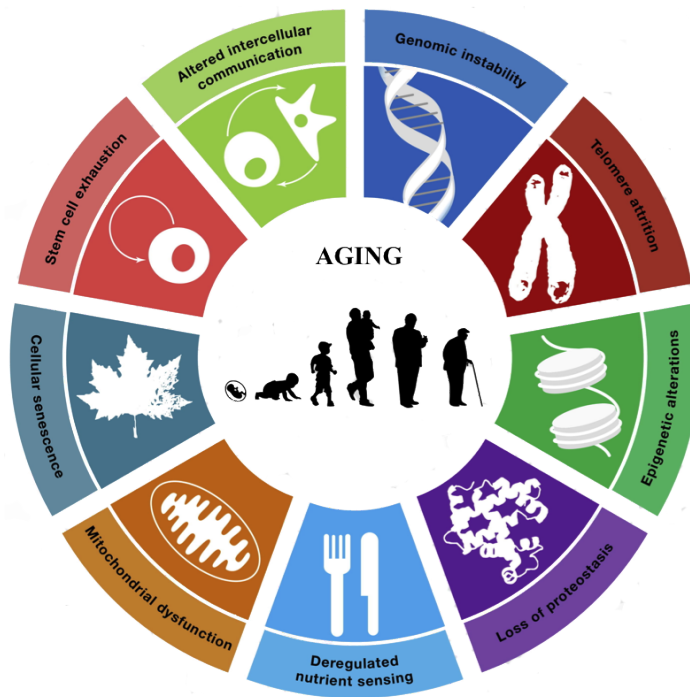


Figure 1. The hallmarks of aging. Nine hallmarks characterize the aging process: genomic instability, telomere attrition, cellular senescence, mitochondrial dysfunction, loss of proteostasis, deregulated nutrient sensing, epigenetic alterations, altered intracellular communication and stem cell exhaustion (adapted from López-Otín et al., *Cell*, 2013).

1.1 Causes of Aging

In the last two decades, major advances have begun to explain why aging and death occur and which are the mechanisms controlling their rate. A large body of studies identified conserved genes that regulate aging and generate species-specific lifespan (Guarente and Kenyon, 2000; Kirkwood and Austad, 2000; Finch and Ruvkun, 2001).

According to *evolutionary theory*, aging is regulated by evolutionary-conserved genes that evolved in response to environmental pressure, such as environmental stress including heat and toxins that can lead to cell damage or death. As a consequence, aging occurs for those organisms in which the force of natural selection is reduced or minimized because they have reached an age in which they are expected to have generated a sufficient number of

offspring (Williams, 1957). Thus, aging can be defined as a consequence of the weakening of natural selection, and age-related diseases represent the phenotype for those who persist (Campisi, 2005). Fundamental concept of the evolutionary theory is the “*antagonistic pleiotropy*” proposed by Williams in 1957, which tries to explain why organisms age. Antagonistic pleiotropy states that genes which are good and beneficial at young age, are detrimental at late age and are themselves aging-promoting genes (Williams, 1957). Cancer may represent an example of this concept. Apoptosis and cellular senescence are two selected mechanisms that evolution selects for, to avoid the onset of cancer at young age by eliminating damaged cells or impairing their proliferation, respectively. However, at later ages, both of these mechanisms may contribute to the aging process (Campisi, 2005).

In 1977, Kirkwood proposed the *disposable soma theory*, which is based on the concept that organisms have limited energy that needs to be allocated between maintenance (repair of damages) and reproduction (Darwinian fitness) (Kirkwood, 1977). This theory takes also in consideration the trade-offs in allocating the energy, in order to reach the optimization of the resources. The necessity of compromises, and thus the inability to devote all the energy to maintenance, ultimately causes damage accumulation and aging (Kirkwood, 1977; Kirkwood, 2005).

Whereas all the previously described theories consider aging as a stochastic event, the identification of mutations able to extend lifespan in yeast, worms, flies and mice sustains the possibility of a “programmed longevity” (Longo et al., 2005). For instance, during period of caloric restriction, which is one the most potent tool to extend lifespan, organisms activate a “protection mode” programme, in which they maintain a hypo-metabolism, activate stress-resistance pathways and decrease fertility (Longo et al., 2005; Wei et al., 2009). Therefore, the *programmed longevity theory* proposes that aging is caused by an impairment of the repair and protection program, which ultimately leads to accumulation of damages (Longo et al., 2005).

Further studies on *Saccharomyces cerevisiae* also support an altruistic view of aging (the *programmed and altruistic aging theory*) according to which, under certain conditions, aging and death have an altruistic purpose for the group, by providing benefits, such as population stabilization, genetic diversity and adaptation (Longo et al., 2005). In support of an altruistic role of aging, chronologically aging studies in yeast show that the majority of the population dies to select better-adapted mutants (Fabrizio et al., 2004). For instance, mutations in superoxide dismutase 1 (*SOD1*) gene decrease cellular protection to hydrogen peroxide, consequently increasing DNA mutation rate and leading to early death. Nutrients released by death cells feed the remaining viable cells, which are better adapted to the new environment (Fabrizio et al., 2004; Longo et al., 2005). Evidence showing that also some animals die immediately after reproduction, raises the possibility that also higher Eukaryotes can undergo an altruistic programmed aging (Finch, 1990; Longo et al., 2005).

1.2 Molecular basis of Aging

Different theories have been proposed to explain how aging occurs at molecular level: i) *somatic mutation theory*; ii) *telomere loss theory*; iii) *mitochondrial theory*; iv) *free radical theory*; v) *altered proteins theory*.

According to the *somatic mutation theory*, the age-dependent accumulation of somatic mutations represents the major cause of tissue and organ failure characterizing the aging process (Promislow, 1994; Kirkwood, 2005; Kennedy et al., 2012). This theory is supported by studies showing that defects in DNA repair machinery lead to premature aging (Vogel et al., 1999; Li and Comai, 2000). Accordingly, activation of DNA damage repair enzymes, such as Poly (ADP-ribose) polymerase, correlates with longer lifespan in different species (Grube and Burkle, 1992).

The *telomere loss theory* explains aging through the decline of cellular division ability (Kim et al., 2002; Campisi, 2005). Telomeres, DNA sequence repeats and proteins which cap the end of linear chromosomes, shorten with each cell division in most of somatic cells. This process is due to the lack of telomerase activity, an enzyme physiologically expressed in mammalian germline and early embryonic cells, which ensures the maintenance of telomeric length by adding telomeric DNA repeats (Kim et al., 2002). As a consequence of telomere shortening, cells can undergo a finite number of cell divisions, known as the Hayflick limit, entering in a cell state defined as cellular senescence (Hayflick, 1965). A large body of evidence shows that senescent cells accumulate with aging (Dimri et al., 1995; Ding et al., 2001; Krtolica et al., 2001). Their resistance to apoptosis and the senescence-induced functional changes ultimately cause their accumulation and a progressive alteration of tissues, which contribute to the aging process (Campisi, 2005).

The identification of the connection between aging and the accumulation of mutations in mitochondrial DNA gives the basis for the *mitochondrial theory* (Wallace, 1999). Indeed, several studies show that high levels of mitochondrial DNA damage lead to impairment in ATP-production and tissue failure (Cottrell et al., 2001; Taylor et al., 2003).

According to the *free radical theory*, reactive oxygen species (ROS), generated by cellular metabolism, represent the major players in promoting aging by damaging macromolecules over time (Harman, 1956; Balaban et al., 2005). Despite the observation that ROS accumulate with age, recent studies make their role highly controversial. In fact, it has been found that overexpression of anti-oxidant enzymes is not able to extend lifespan in mice (Cochemé et al., 2011; Van Remmen et al., 2003; Zhang et al., 2009). Furthermore, increased ROS levels, as a consequence of *SOD* deletion, are able to even prolong survival in *Caenorhabditis elegans* (Van Raamsdonk and Hekimi, 2009). Indeed, a growing body of evidence suggests that ROS can assume a dual role, by promoting cellular adaptation in

response to stress stimuli and by causing damages when accumulate over a certain threshold with aging (Sena and Chandel, 2012; López-Otín et al., 2013).

Lastly, the *altered proteins theory* finds an age-related accumulation of damaged proteins whose turnover does not occur properly (Terman and Brunk, 2004). Protein homeostasis or “proteostasis” involves a range of different mechanisms that allow the correct protein folding and the degradation of misfolded ones (Koga et al., 2011). Studies show that loss of proteostasis strongly contributes to the development of age-related disorders (Powers et al., 2009).

Despite these theories provide different molecular explanation of aging processes, all of them are based on a fundamental pillar: DNA and cellular damage accumulation and the consequent loss of genomic and proteomic stability and integrity (López-Otín et al., 2013).

1.3 Cancer: an age-related disease

Aging is the major risk factor to develop cancer. This is due to an age-dependent progressive accumulation of somatic mutations, epigenetic gene silencing, telomere dysfunction, deregulated metabolism and altered microenvironment (Figure 2) (DePinho, 2000; Hanahan and Weinberg, 2011). However, differently from all the other age-related diseases which are mainly degenerative, cancer represents a disease of both degeneration and acquired function due to the ability of tumor cell to hyper-proliferate, escape from cell death and spread to distant organs (Campisi, 2005; Hanahan and Weinberg, 2011).

The most fundamental feature of cancer cell is the ability to sustain chronic proliferation (Hanahan and Weinberg, 2011). In fact, differently from normal cells that respond to tightly controlled release of growth-signals, tumor cells harbour mutations which allow a constitutive activation of pro-growth signalling and inhibition of growth inhibitory pathways (Hanahan and Weinberg, 2011). Oncogenes, tumor suppressor genes and

stability genes are three classes of genes, which are required to be altered in order to initiate tumorigenesis (Vogelstein and Kinzler, 2004).



Figure 2. The hallmarks of cancer. Ten hallmarks describing cancer cells. In addition to the previous six identified features (sustaining tumor proliferative signalling, evading growth suppressors, enabling replicative immortality, resisting cell death, inducing angiogenesis and activating invasion and metastasis), four new additional abilities were considered fundamental hallmarks of cancer: deregulating cellular energetics, tumor-promoting inflammation, avoiding immune-destruction, genome instability and mutation (adapted from Hanahan and Weinberg, *Cell*, 2011).

Oncogenes, which encode for proteins involved in cell division, growth, motility and nutrient uptake, harbour mutations able to make the gene product constitutively active, such as chromosomal translocations, gene amplification or intragenic mutations. Tumor suppressor genes, which encode for proteins involved in cell cycle control, growth inhibition and cell death induction, carry missense mutations, deletions, insertions or epigenetic silencing able to reduce the activity of gene products, which can block or prevent cancer generation (Vogelstein and Kinzler, 2004). The third class of genes comprises those involved in DNA repair machinery such as mismatch repair (MMR),

nucleotide-excision repair (NER), base-excision repair (BER) and double-strand break repair (DSBR); or genes involved in the control of mitotic recombination and chromosomal segregation. When these genes are inactivated, several other genes can be mutated at a high rate. Among these, only oncogenes and tumor suppressors have the potentiality to confer a selective proliferative advantage to the cell, therefore generating a clonal expansion. Multiple mutations, and thus multiple clonal expansions, are required to allow neoplastic transformation and tumor progression (Hoeijmakers, 2001; Vogelstein and Kinzler, 2004).

The chronic and uncontrolled proliferation characterizing cancer cells, is not only the result of deregulation in growth signalling, but also of a metabolic reprogramming (Hanahan and Weinberg, 2011). Tumors strongly rely on glucose uptake and lactate production, a phenomenon known as “Warburg effect”, which describes a metabolic switch from oxidative phosphorylation (OXPHOS) to glycolysis, even in the presence of oxygen (aerobic glycolysis) (Warburg, 1956a, Warburg, 1956b; Kroemer and Pouyssegur, 2008; Vander Heiden et al., 2009). A large body of studies show that, in addition to the production of ATP from glycolysis, glycolytic metabolic intermediates are required for proliferation and growth of cancer cells (Lunt and Vander Heiden, 2011). In particular, cancer cells use these metabolic intermediates to refuel anabolic reactions for nucleotide, amino acids and lipid biosynthesis, which are fundamental to sustain tumor cell growth (Lunt and Vander Heiden, 2011). Several studies show that tumor metabolic reprogramming is driven by growth signal stimuli and oncogene activation; for instance, phosphatidylinositol-3 kinase cascade (PI3K/AKT signalling) controls both cell growth and glucose metabolism, by up-regulating glucose uptake and glycolysis, and dispatching carbon sources in biosynthetic pathways (Buzzai et al., 2005; Ward and Thompson, 2012). In addition, emerging evidence sustains the pivotal role of the tumor microenvironment in sustaining cancer growth (Hanahan and Weinberg, 2011). Angiogenesis is one of the major processes promoting cancer survival by guaranteeing nutrients and oxygen to tumor cells

(Hanahan and Weinberg, 2011). It has been shown that in the tumor microenvironment, angiogenesis is chronically activated as a consequence of an “angiogenic switch”, which promotes neo-vasculature formation (Hanahan and Folkman, 1996). The release of pro-angiogenic molecules by cancer cells is further accompanied by the secretion of pro-inflammatory cytokines, which recruit lymphocytes and macrophages to the tumor bed.

Studies have shown the dual role of the immune system in both antagonizing and promoting tumor progression (DeNardo et al., 2010). In particular, immune-surveillance mechanisms allow the recognition and elimination of neoplastic cells by CD4⁺ and CD8⁺ tumor infiltrating lymphocytes (TIL) and natural killer (NK) cells. However, selection of cancer cells able to escape death, through mechanisms of immune-subversion, increases tumorigenesis and result in poor prognosis in patients (Zitvogel et al., 2006). Moreover, infiltrated pro-inflammatory cells can assume a pro-tumorigenic role by sustaining proliferation, stimulating angiogenesis and facilitating invasion and metastatic dissemination (DeNardo et al., 2010; Egeblad et al., 2010; Hanahan and Weinberg, 2011).

2. Dietary interventions to slow aging

Caloric restriction (CR), considered as a reduction of calorie intake without malnutrition, is able to extend lifespan in different organisms, including yeast, flies, rodents and monkeys (Weindruch and Walford, 1982; Weindruch et al., 1986; Wei et al., 2008; Anderson et al., 2009; Grandison et al., 2009; Fontana et al., 2010; Colman et al., 2014). These effects can be achieved by a reduction from 10 to 50% of caloric intake in mammals (Fontana et al., 2010). Studies in mice and monkeys found that caloric restricted animals live longer and show a lower incidence of age-related diseases (Anderson et al., 2009). In humans, a caloric restricted diet slows metabolism and decreases systemic

oxidative damage, leading to a reduction of risk factors for diabetes, cardiovascular diseases and cancer (Fontana and Klein, 2007; Redman et al., 2018). A similar effect of caloric restriction on longevity and health span is observed upon inactivation of nutrient and growth pathways (Figure 3).

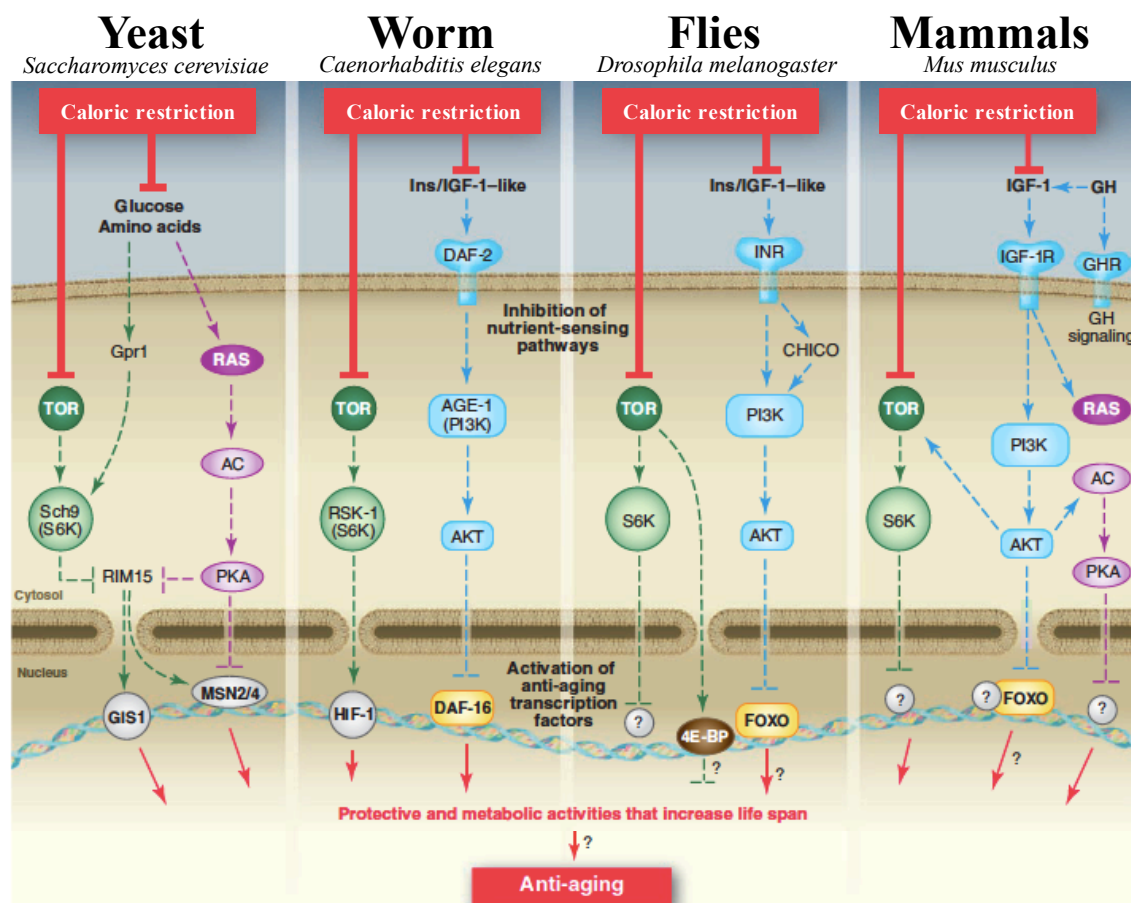


Figure 3. Anti-aging effect of caloric restriction (CR). Schematic representation of the CR-dependent down-regulation of evolutionary conserved nutrient-signalling pathways. In yeast two major nutrient-sensing signalling have been identified to promote aging: 1) the target of rapamycin (TOR) and the serine-threonine kinase Sch9a pathway; 2) the RAS, adenylate cyclase (AC) and protein kinase A (PKA) pathway. The pro-aging role of Tor/S6K results to be conserved also in worms, flies and mammals, whereas AC/PKA is conserved in yeast and mammals. In worms, flies and mammals the Insulin/IGF-1 signalling is also involved in controlling the aging process. CR down-regulates the aforementioned pathways and induces a protective transcriptional program. (adapted from Fontana et al., *Science*, 2010).

Since these pathways are down-regulated by caloric restriction, mutations in genes involved in these signalling cascades can mimic its effect. Studies in yeast identified two

major nutrient-sensing signalling pathways: 1) the target of rapamycin (TOR) and the serine-threonine kinase Sch9a pathway; 2) the Ras, adenylate cyclase (AC) and protein kinase A (PKA) pathway (Smith et al., 1998; Fabrizio et al., 2001; Kaeberlein, 2005; Wei et al., 2009).

A reduced activity of these two crucial signalling pathways is able to extend both replicative (number of daughter cells generated by a single mother cell) and chronological (survival of a population of non-dividing cells) yeast lifespan by activating stress resistance and protective programme, including the manganese-dependent superoxide dismutase (Mn-SOD), which scavenges superoxide free radicals, and Msn2/Msn4 transcription factors that are involved in protective mechanisms (Fabrizio et al., 2001; Kaeberlein et al., 2005; Wei et al., 2009).

The nematode worm *Caenorhabditis elegans* was among the first multicellular organisms in which unknown mutations, later identified, were shown to promote longevity (Johnson, 1990). It has been found that insulin/insulin-like growth factor signalling pathway (IIS) and the consequent activation of the transcription factor DAF-16 are able to extend worm life span (Johnson, 1990; Kenyon et al., 1993; Gems et al., 1998). As in yeast, the inactivation of TOR signalling also contributes to increased lifespan (Hansen et al., 2008). Similar mechanisms were also identified in the fruit fly *Drosophila melanogaster*, supporting the evolutionary conserved role of these “longevity pathways” (Clancy, 2001).

Longevity studies in yeast, worms and flies have been crucial to understand the role of these pathways in higher organisms, in particular rodents. As in lower Eukaryotes and in invertebrates, mutations in nutrient-pathways increase life expectancy also in mice (Fontana et al., 2010). Pharmacological inhibition of the mTor pathway by rapamycin or deletion of ribosomal S6 protein kinase (S6K1) is able to extend mouse lifespan and reduce age-related pathologies (Harrison et al., 2009; Selman et al., 2009). Furthermore, studies found that mutations in growth hormone (GH) and insulin-like growth factor-1 (IGF-1) signalling extend rodent lifespan and health-span (Brown-Borg et al., 1996;

Holzenberger et al., 2003). On the contrary, GH overexpression in transgenic mouse model leads to premature-aging (Steger et al., 1993).

GH is a pituitary gland-secreted hormone and its action mediates the somatic growth and body size (Bartke, 2005; Brown-Borg, 2007). Upon GH stimulus, liver cells secrete IGF-1, which exerts its action on cartilage, bones and muscle by binding to its receptor (IGF-1R) and thus promoting cell division and growth (Bartke, 2005; Brown-Borg, 2007). Mice carrying homozygous mutations in the pituitary specific transcription factors *prop-1* or *pit-1*, known as Snell and Ames mice respectively, show deficiency in GH, thyroid stimulating hormone (TSH), prolactin and IGF-1 levels. As a consequence, they are dwarf but they live 65% longer compared to the wild type counterparts (Brown-Borg et al., 1996; Flurkey et al., 2002). It has been shown that reduced levels of GH and IGF-1 are associated to higher expression of antioxidant enzymes and stress resistance pathways such as those involving superoxide dismutase (SOD), catalase, heat shock protein, whereas GH or IGF-1 administration reverts these protective effects (Brown-Borg and Rakoczy, 2000; Brown-Borg et al., 2001; Longo and Fabrizio, 2002). In the same way, the lack of GH binding protein (GH-BP) extends mice lifespan up to 50% and decreases the incidence of cognitive decline, insulin resistance and neoplastic transformation (Coschigano et al., 2003, Ikeno et al., 2009). Moreover, dwarf mice with high plasma level of GH but deficiencies in IGF-1, show an increase in life expectancy, supporting the fundamental role of IGF-1 in mediating the lifespan extension in mice lacking GH, GHR and GHRBP (Coschigano et al., 2000).

The GH/IGF-1 axis has been also the object of studies in humans; in fact, the human Laron syndrome (LS; OMIM entry: #262500) is caused by mutations in GHR. These patients are characterized by high GH level but a severe deficiency in circulating IGF-1; moreover, they are short in stature, and can be obese (Laron, 1999). Studies of an Ecuadorian cohort of LS patients show that these individuals are protected by age-related pathologies in particular cancer and type 2 diabetes. However, differently from model organisms mutated in GHR, they do not live 50% longer than normal, which may be explained by their poor

diet, obesity and high percentage of deaths for non-age-related causes such as accidents and alcohol toxicity (Guevara-Aguirre et al., 2011).

Despite the role of GH/IGF-1 inhibition in mimicking the effect of CR in extending life span and in reducing age-related diseases, the two strategies may not be mediated by identical mechanisms. In fact, CR is able to further extend lifespan of certain dwarf mice, possibly by avoiding side effects caused by GH impairment, including fat accumulation and other problems due to obesity (Abdu et al., 2001; Bartke, 2001; Longo and Finch, 2003).

2.1 Caloric restriction and age-related cancer

A large body of studies performed in several model organisms and also in humans support the role of high level of IGF-1 in promoting mutations and neoplastic lesions, making elevated IGF-1 a risk factor for developing cancer (Kennedy, 2003; Ikeno et al., 2009; Guevara-Aguirre et al., 2011; Levine et al., 2014). Furthermore, epidemiologic analysis show that high IGF-1 level is associated with an increased risk of different tumor types such as colorectal, breast, prostate and lung cancers (Chan et al., 2000; Giovannucci et al., 2000; Yu and Rohan, 2000).

Long-term CR is able to reduce serum IGF-1 level by 30-40% in mouse models, whereas in humans a reduction of circulating IGF-1 is not observed unless protein intake is reduced (Sonntag et al. 1999; Hursting et al., 2003; Fontana et al., 2008). Therefore, these findings suggest that the lowering of IGF-1 level is fundamental for the CR-mediated protection from cancer development (Longo and Fontana, 2010).

Several studies show that CR without malnutrition is able to decrease the incidence and delay the onset of spontaneous and chemically induced cancer in different rodent models (Tannenbaum and Silverstone, 1949; Klurfeld et al., 1989; Albanes et al., 1987; Thompson

et al., 2003). Moreover, studies in rhesus monkeys show that 30% CR is able to reduce cancer incidence by 50% (Colman et al., 2009).

The protective effect of CR and low IGF-1 level against cancer is explained by different mechanisms:

i) increased resistance to oxidative damages

studies found that dermal fibroblasts from long-lived Ames mice (*pit-1* mutant, characterized by low IGF-1 level) result to be resistant to toxic agents such as hydrogen peroxide *in vitro* (Longo and Finch, 2003; Salmon et al., 2005).

ii) reduced somatic mutation frequency

it has been shown that hepatocytes from long-lived Ames mice undergo more readily apoptosis than wild type counterpart when challenged with oxidative insults (Kennedy, 2003). Thus, these findings indicate that the lowering of IGF-1 level promotes various strategies in different tissues in order to eliminate more efficiently damaged cells and avoid the onset of neoplastic lesions (Longo and Finch, 2003; Garcia et al., 2008).

iii) reduction of genome instability

this mechanism possibly occurs by the IGF-1 dependent down-regulation of RAS pro-oncogenic signalling or phosphatidylinositol-3 kinase cascade (PI3K/AKT/mTor/S6k pathway) (Longo and Fontana, 2010; Longo et al., 2008). Studies from yeast to mammals reveal that the connection between genome instability and oncogenic pathways is a process dependent on cell division, anti-oxidant defence and error-prone DNA repair machineries (Longo et al., 2008). In *Saccharomyces cerevisiae*, the mutation frequency is reduced upon deletion of the oncogene homologues SCH9 and RAS2 or upon overexpression of antioxidant enzymes, whereas the constitutive activation of RAS2 increases the level of oxidative stress and the mutation rate (Fabrizio et al., 2004). Also in mammalian cells, it has been found that activation of RAS enhances genome instability by altering the replication fork progression (Di Micco et al., 2006).

Furthermore, studies found that the mutational status of oncogenic signalling determines the dependency to IGF-1 and thus the sensitivity to CR. In particular, cancer cells harbouring activating mutation in PI3K signalling result to be resistant to CR and in culture they are able to grow in an IGF-1 independent way (Kalaany and Sabatini, 2009). The replacement of the mutant allele with the wild type allele, as well as PI3K pharmacological inhibition, is able to restore CR sensitivity, supporting the fundamental role of IGF-1 signalling in mediating CR anti-cancer effect (Kalaany and Sabatini, 2009).

2.2 Fasting and Fasting-Mimicking Diet (FMD)

Fasting is defined as a condition of no or minimal food ingestion that can last from 1 day or less to weeks. Differently from CR, which is characterized by a chronic decrease of caloric intake by 20-40% without altering the meal frequency, fasting includes water-only consumption or very low-calorie intake (less than 200 kcal/day), for limited periods. Several studies indicate that fasting is able to extend lifespan in multiple model organisms (Figure 4) (Longo and Mattson, 2014).

In *Escherichia coli* the switching from a nutrient-rich broth to a calorie-free one, extends survival by four-times (Gonidakis et al., 2010). In yeast, the switching from a standard glucose and ethanol-rich medium to a water-only medium increases yeast chronological lifespan by 2-fold and enhances resistance to multiple types of stress (Longo et al., 1997; Wei et al., 2008).

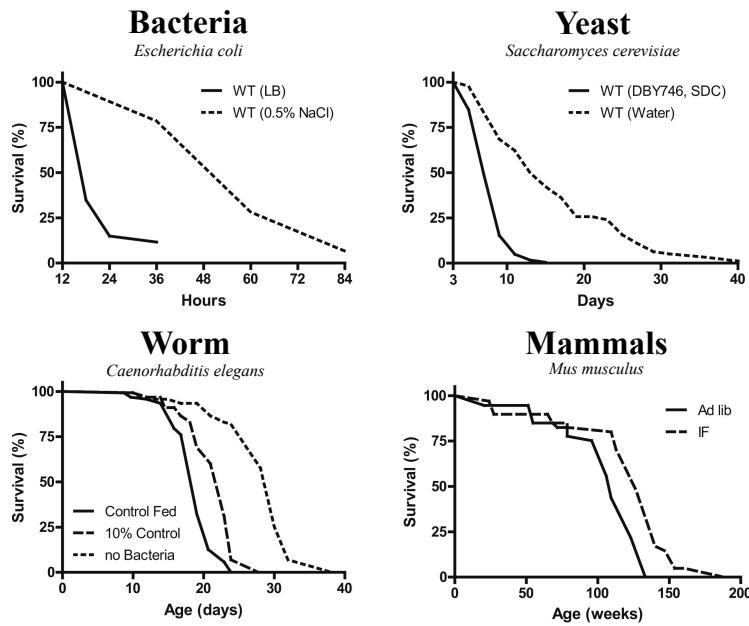


Figure 4. Fasting extends lifespan in different model organisms. Lifespan extension induced by calorie free medium (0.5% NaCl) in bacteria, water medium in yeast, 90% reduction or complete removal of bacterial food in worms, and alternate days of water-only fasting in mice (adapted from Longo and Mattson, *Cell Metab*, 2014).

The mechanism behind this effect involves the down-regulation of Tor/Sch9 and Ras/AC/PKA pathways and the consequent activation of a protective transcriptional program, which includes Msn2, Msn4 and Gis1 stress-responsive transcription factors, SOD and heat shock proteins (Fontana et al., 2010). Also in *Caenorhabditis elegans*, fasting periods, which consist in feeding the worm with little or no bacteria, result in lifespan extension through a mechanism which involves the aforementioned stress resistance transcription factor DAF-16 (Lee et al., 2006).

Differently from CR, in mammals fasting causes a broader spectrum of alterations and a complete metabolic switch (Longo and Mattson, 2014). In the first few hours of fasting, blood glucose level remains constant by using the glycogen reservoir present in the liver. When glycogen is depleted, amino acids, derived from muscle proteolysis, and glycerol, derived from triglycerides, fuel gluconeogenesis, which become necessary to generate glucose for tissues. In prolonged periods of fasting, Acetyl-CoA derived from fatty acid β -

oxidation is utilized to generate ketone bodies (β -hydroxybutyrate, acetoacetate and acetone), which become the major energy source for the brain. In humans, this phase represents an adaptive stage and can last even weeks. After fatty acid depletion, muscles degradation becomes the only source of intermediates to fuel gluconeogenesis. This final stage can be sustained for a short period and, if prolonged, leads to the death of the animal (Wang et al., 2006; Longo and Mattson, 2014).

Different forms of fasting have been studied. Intermittent fasting (IF) lasts 24 hours with 1 or more days of refeeding between each cycle, whereas prolonged fasting (PF), usually lasts 2 or more days and each cycle is followed by at least 1 week of refeeding to allow bodyweight recovery (Longo and Mattson, 2014). In rodents, the metabolic and growth factors alterations as well as the effect on longevity and health span, result to be different according to the form of fasting and also to the age at which fasting cycles start (Mattson et al., 2017). It has been found that IF every other day prolongs life expectancy by 2-fold in young rats, whereas when IF cycles start at middle-ages, lifespan is increased by 30-40% with preservation of motor, sensory and cognitive function in the elderly (Goodrick et al., 1982; Singh et al., 2015). Moreover, it has been found that rats undergoing IF for 1 month showed improved glucose tolerance, which result to be impaired when IF was prolonged to 8 months (Cerqueira et al., 2011). In addition, IF exerts detrimental effects on glucose metabolism in hypercholesterolemic mice, suggesting that IF could have a variable impact on health in different circumstances (Dorighello et al., 2014).

A large body of studies have shown that fasting is able to delay age related diseases, by potentiating cellular resistance and decreasing pro-growth signalling, in particular affecting GH/IGF-1 axis (Fontana et al., 2010; Guevara-Aguirre et al., 2011; Lee and Longo., 2011; Cheng et al., 2014; Longo and Mattson, 2014). In mice 24-72 hours of fasting cause a 41% reduction of blood glucose level, decrease IGF-1 level by 70%, decrease the IGF-1 binding protein-3 (IGFBP-3) by 42% and dramatically increase the IGF-1 signalling inhibitor IGF-1 binding protein-1 (IGFBP-1) (Lee et al., 2010). Also in humans, 3-5 days of fasting

strongly reduce glucose and circulating IGF-1 level (Merimee et al., 1982; Isley et al., 1983; Maccario et al., 2001).

Several studies indicate that PF is feasible and safe for humans, however minor side effects have been reported such as headache, nausea, light-headedness, anaemia and weakness especially in the elderly and frail subjects (Drenick et al., 1964; Thomson et al., 1966; Lee and Longo, 2011). Thus, water-only fasting, but also forms of fasting which have to be repeated every other day or twice a week, remain a challenging option for the majority of population. For this reason, our laboratories have recently identified a monthly or bi-monthly diet which mimic the metabolic effect generated by fasting (Brandhorst et al., 2015).

Fasting-Mimicking Diet (FMD) is a low-caloric diet, which contains low levels of protein and sugar, and as fasting, it is able to improve health span by reducing risk factors for age-related pathologies (Brandhorst et al., 2015). Studies in rodents found that 4 days of FMD are able to reduce blood glucose level by 40%, insulin level by 10-fold, IGF-1 level by 45% and increase IGFBP-1 by 8-fold, similarly to fasting (Table 1). Moreover, FMD increases ketone bodies level by 10-fold, indicating that this diet regimen is able to induce the metabolic switch observed during fasting. Notably, all these markers return to levels similar to those of ad libitum fed mice in one week after refeeding (Brandhorst et al., 2015).

	Fasting (72 hours)			FMD (96 hours)	
	<i>Effect</i>	<i>Change</i>	<i>Reference</i>	<i>Effect</i>	<i>Change</i>
Blood glucose	↓	~45%		↓	~40%
Ketone bodies	↑	~10-fold	*	↑	~10-fold
IGF-I	↓	~40- 70%		↓	~45%
IGFBP-1	↑	~7- 11-fold		↑	~8-fold

Table 1. Fasting and Fasting-Mimicking Diet (FMD) cause similar alterations in metabolic markers. Mice undergoing FMD (96 hours) show a reduction in blood glucose level, IGF-1 level, an increase in IGFBP-1 and ketone bodies similar to those observed during 72 hours of fasting. * 24 hours (adapted from Brandhorst et al., *Cell Metab.*, 2015).

Bi-monthly FMD cycles, started in middle-aged mice, extend median lifespan and show broad beneficial effects on health-span by causing weight and visceral fat loss, by reducing inflammation, cancer incidence and delaying tumor onset (Brandhorst et al., 2015). Furthermore, FMD regimen reduces multiple organ size and promotes their regeneration upon refeeding (Brandhorst et al., 2015). We have also shown that FMD cycles are able to: *i)* revert “*immunosenescence*”, by promoting immune system regeneration and rejuvenation; *ii)* prevent the age-dependent decline in motor and cognitive functions; *iii)* promote neurogenesis, possibly through the reduction of IGF-1 levels and PKA signalling (Cheng et al., 2014; Brandhorst et al., 2015).

In two recent works, our laboratory has shown that in healthy humans, 5 days of FMD per month result to be safe and well tolerated. In healthy individuals, FMD reduces bodyweight, waist circumference, total body and trunk fat (Brandhorst et al., 2015, Wei et al., 2017). At the end of each FMD cycle blood glucose and IGF-1 levels are reduced by 11.3% and 24%, respectively, and differently from studies in rodents, after the third cycle their levels remain lower than baseline even after resuming regular diet (Brandhorst et al., 2015; Wei et al., 2017). FMD cycles have stronger beneficial effects in subjects classified as at-risk for age-related pathologies compared to subjects classified as low risk. In fact, in at-risk subjects, monthly FMD cycles for 3 months are able to significantly reduce blood

pressure, fasting glucose, IGF-1 level, triglycerides, total and low-density lipoprotein cholesterol and C-reactive protein (CRP) (Wei et al., 2017).

In summary, our work indicates that periodic FMD cycles are effective in reducing risk factors for age-related pathologies. Studies in mouse models and current clinical trials raise the possibility of applying FMD not only as preventive tool but also as a potential therapeutic option for age-related diseases, in particular for cancer, diabetes and autoimmunities (Di Biase et al., 2016; Choi et al., 2016; Cheng et al., 2017; Wei et al., 2017).

2.3 Fasting, FMD and Cancer

It has been shown that CR is able to decrease risk factors for cancer and to delay tumor progression in different mouse models, through a mechanism which may involve IGF-1 level reduction (Longo and Fontana, 2010). However, a caloric-restricted regimen, only causes minor changes in glucose, IGF-1 and other factors and it would have limited efficacy in both protection of normal cells and sensitization of cancer cells to toxic agents (Kalaany and Sabatini, 2009; Lee and Longo, 2011). Furthermore, chronic CR would not be useful in the clinic since it would cause a progressive loss of weight, muscle mass, and most likely immune function and the ability to fight infections and withstand the challenge of cancer therapy (Racette et al., 2006; Kristan, 2008; Lee and Longo, 2011). In contrast, fasting for a limited time (4-5 days) can represent a more powerful and feasible option for oncological patients.

Our laboratory showed that an *in vitro* condition mimicking fasting (low glucose and low serum levels), referred as Short-Term Starvation (STS), is able to protect normal cells but not a wide range of cancer cells from ROS and ROS-inducing agents, such as hydrogen peroxide or different chemotherapeutic drugs (Raffaghello et al., 2008).

This phenomenon is described as differential stress resistance (DSR). Our *in vitro* and *in vivo* studies show that fasting-mediated reduction of IGF-1 level is, at least in part, responsible of the DSR effect. In particular, IGF-1 restoration during fasting periods is able to revert the protective effect of fasting from chemo-toxicity (Lee et al., 2010).

Moreover, liver IGF-1 deficient (Lid) mice (characterized by an 80% reduction in circulating IGF-1 level followed by a conditional hepatic *igf-1* gene knockout) show a higher survival rate compared to wild type mice when challenged with different chemotherapy drugs, such as etoposide, cyclophosphamide and doxorubicin (Lee et al., 2010). Thus, the reduction in IGF-1 level and the consequent decreases in growth signalling pathways, such as RAS and AKT cascades, enable normal cells to enter a protection mode, by inducing cell cycle arrest and activating genes of repair and protection, among which FOXO transcription factor, SOD and DNA repair machinery genes (Longo and Fabrizio, 2002; Longo et al., 2008; Raffaghello et al., 2008; Lee et al., 2012). In addition, we have also shown that glucose reduction caused by fasting is also involved in DSR process (Di Biase et al., 2017). In particular, we have found that fasting-mediated decrease in circulating glucose protects cardiomyocytes from doxorubicin toxicity through a mechanism involving the activation of the mammalian Msn2/4 ortholog early growth response protein 1 (EGR1) (Di Biase et al., 2017). Accordingly, glucose restoration during fasting is able to revert the cardioprotective effect (Di Biase et al., 2017).

In cancer cells the activation of oncogenes and the loss of function of tumor suppressor genes make pro-growth signalling constantly active, thus preventing the switching to a protective mode (Raffaghello et al., 2008; Lee et al., 2010; Lee et al., 2012).

In yeast, deletion in SCH9 and RAS2 genes enhances the starvation-induced resistance to oxidative stress compared to cells expressing the constitutively active oncogene homolog RAS^{val19} (Raffaghello et al., 2008). The overexpression of the oncogene-like RAS^{val19} not only reverses the fasting-mediated protection, but also enhances the sensitivity to oxidative

stress compared to wild type yeast cells, supporting the possibility that fasting can make cancer cells more susceptible to toxic agents (Raffaghello et al., 2008). In line with these results in yeast, studies on mammalian cells show that *in vitro* STS is able to sensitize a wide range of cancer cell types to doxorubicin and cyclophosphamide (Lee et al., 2012). In the same way, 48 to 72 hours of fasting enhance their toxicity to cancer cells in mouse models (Figure 5) (Raffaghello et al., 2008; Lee et al., 2012). This phenomenon is described as differential stress sensitization (DSS).

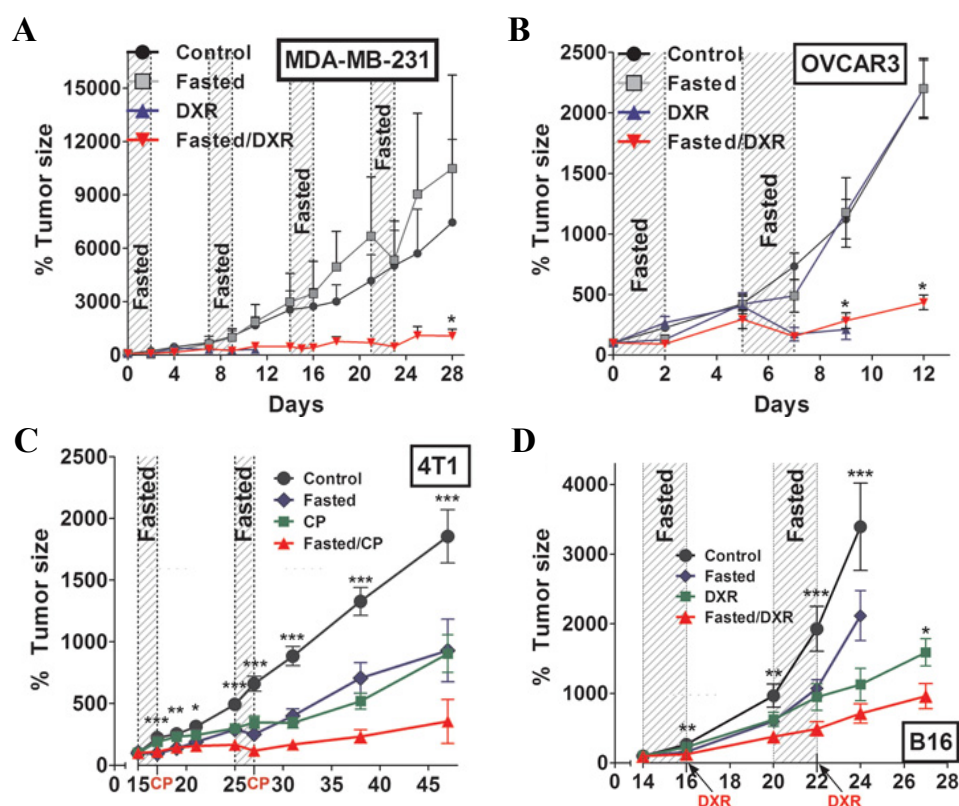


Figure 5. Fasting cycles sensitize tumors to chemotherapy in xenograft and allograft mouse models. Fasting cycles potentiate doxorubicin (DXR) or cyclophosphamide (CP) effect in delaying tumor growth in xenograft mouse model of breast (A) and ovarian (B) cancers, and on allograft model of murine breast cancer (C) and murine melanoma (D) (adapted from Lee et al., *Sci Transl Med*, 2012).

In syngeneic mouse models of murine melanoma, glioma, breast and colorectal cancers and in xenograft of human breast and ovarian cancers, 2 cycles of 48-hours fasting are as effective as chemotherapy in retarding tumor growth. Importantly, the best therapeutic

outcome was achieved when fasting cycles were combined with chemotherapy (Lee et al., 2012; Bianchi et al., 2015). In metastatic mouse model of breast cancer and melanoma, fasting cycles potentiate the effect of chemotherapy alone by reducing metastases formation and extending survival (Lee et al., 2012). Moreover, we showed that fasting cycles combined with doxorubicin and cisplatin promote cancer-free long-term survival in 25% of mice bearing metastatic neuroblastoma (Lee et al., 2012). In addition, the protection from chemo-toxicity conferred by fasting may allow the use of higher doses and multiple cycles of chemotherapy in a safe and feasible way, improving the patient therapeutic outcome (Lee et al., 2012).

The molecular mechanism responsible for these effects involves at least in part the reduction in IGF-1 level, in fact its restoration is able to revert cancer cell sensitization to chemo-toxicity, supporting the key role of IGF-1 also in DSS (Lee et al., 2012).

Furthermore, oxidative stress and ROS production could also contribute to the synergistic effect of fasting and chemotherapy. In particular, fasting alone causes an increase in ROS levels and DNA damage, which in turn make cancer cells more susceptible to the oxidative insults exerted by chemotherapy (Lee et al., 2012). Recent studies showed that fasting-mediated ROS increase is partially caused by an alteration in glucose metabolism (Bianchi et al., 2015). We showed that fasting is able to reduce glucose level, glutaminolysis and to enhance oxidative phosphorylation, generating an “anti-Warburg effect” possibly through the inhibition of PI3K/AKT pro-growing pathway (Bianchi et al. 2015). STS, by up-regulating complex I and IV of respiratory chain, without affecting complex II, increases oxygen consumption rate but decreases ATP synthesis, finally leading to a boost in ROS levels and oxidative stress (Bianchi et al. 2015).

However, our recent studies indicate that the molecular mechanisms responsible for fasting-mediated DSS are not only tumor-dependent but also tumor-independent. Our previous works show that fasting and FMD are able to promote immune system regeneration, thus arising the possibility that FMD could exert its anti-cancer effect also by

potentiating immune system surveillance (Cheng et al., 2014; Brandhorst et al., 2015; Di Biase et al., 2016). Recently, we found that FMD in combination with doxorubicin promotes CD8⁺ tumor-infiltrating lymphocytes (TIL) accumulation in murine breast tumor, through a mechanism, which partially involves the down-regulation of Heme-Oxygenase 1 (HO-1) (Di Biase et al., 2016). Moreover, CD8⁺ TIL depletion is sufficient to reverse FMD anti-cancer effect, supporting the key role of TIL in mediating DSS (Di Biase et al., 2016).

3. RAS mutant cancer

One of the most frequently mutated oncogene family in human cancers is represented by *RAS* genes (*HRAS*, *NRAS*, *KRAS*) (Cox et al., 2014; according to COSMIC; *catalogue of somatic mutation in cancer*). After the first detection in human tumors in 1982, cancer genome sequencing reveals RAS mutations in the most lethal cancers: lung, pancreatic and colorectal cancer, with an incidence of 20%, 71% and 42% respectively (Stephen et al., 2014).

Several studies have shown that RAS mutations appear early during tumor progression and they are required, together with mutations in other oncogenes and tumor suppressor genes, to sustain tumorigenesis (Hingorani et al., 2005; Ji et al., 2007; Haigis et al., 2008). In addition, its activation is crucial for tumor maintenance, while its silencing is able to impair tumor growth in both *in vitro* and *in vivo* model of RAS-driven tumors (Brummelkamp et al., 2002; Collins et al., 2012).

RAS mutant tumors are associated with a poor prognosis (Figure 6), due to their unresponsiveness to the majority of standard and targeted therapies, for this reason a major effort has been devoted to identify specific RAS inhibitors. However, all the past efforts

have failed in achieving this, creating the idea that the target is “undruggable” (Lièvre et al., 2006; Cox et al., 2014; Verissimo et al., 2016).

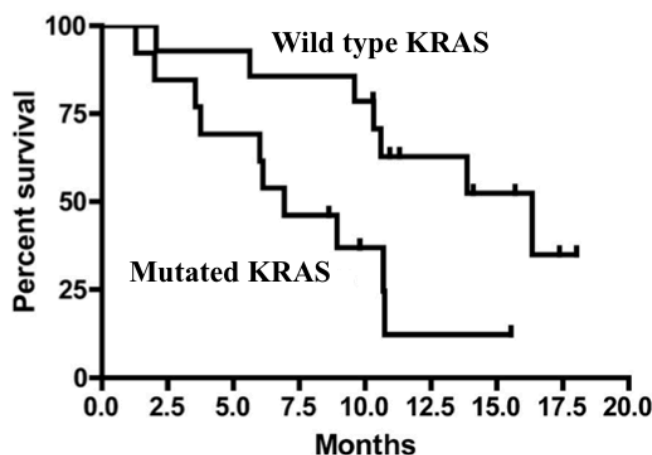


Figure 6. Patients bearing mutated KRAS cancer show a lower overall survival compared with those carrying wild type KRAS tumors. Mutated KRAS is associated with poor prognosis. The plot shows the overall survival of colorectal cancer patients carrying wild type or mutated KRAS colorectal cancer. Patients bearing mutated KRAS colorectal cancer result to be unresponsive to therapy, thus showing a decrease in the overall survival compared with patients bearing wild type KRAS (adapted from Lievre et al., *Cancer Res*, 2006).

3.1 The history of RAS

RAS genes were initially identified from oncogenic viruses able to cause sarcoma in infected animals and induce neoplastic transformation in immortalized fibroblast *in vitro* (Cox and Der, 2010). In 1973, Scolnick and colleagues discovered that the oncogenic potential of the viruses was the result of the transduction of normal rat sequences into the viral genome, which converts normal genes into oncogenes (Scolnick and Parks, 1974). These genes were then named *RAS*, from RAt Sarcomas, and they were distinguished one from the other by the name of their discoverers, thus *H-ras* from Harvey and *K-ras* from Kirsten (Harvey, 1964; Kirsten and Mayer, 1967). These pioneering studies were followed by the discovery that RAS genes were able to transform NIH/3T3 immortalized fibroblast in culture (Shih et al., 1981; Der et al., 1982; Parada et al., 1982). Then, mutant *HRAS*,

KRAS, and also a third *RAS* gene, *NRAS*, were identified in human cancer cell lines and in patient tumors, but not in normal tissues, supporting the key role of mutant RAS in tumorigenesis and confirming what observed in cell lines (Hall et al., 1983; Santos et al., 1984). Mutations in codon 12, 13 and 61 were identified as causative of RAS constitutive activation in human cancers (Capon et al., 1983; Bos et al., 1987; Rodenhuis et al., 1987). Interestingly, genome sequencing data reveal that *HRAS* is the least frequently mutated (3%), followed by *NRAS* (8%), whereas *KRAS* result to be the most frequent one (21%) in human cancers (Cox and Der, 2010). However, not all the mutations in the different RAS isoforms have the same outcome, and specific therapies need to be formulated for each of them (Cox et al., 2014). Several studies tried to elucidate possible mechanisms responsible for the different frequency of *RAS* mutations. Despite RAS proteins are highly conserved during evolution, some studies suggest that KRAS has specific properties which confer additional advantages to tumorigenic processes; in particular it has been reported that KRAS4B splicing variant, differently from HRAS, NRAS and KRAS4A, is able to confer stem cell properties to specific cell types (Quinlan and Settleman, 2008; Cox and Der, 2010). The importance of KRAS is also highlighted by the lethality of its depletion during embryogenesis, while mice lacking HRAS or NRAS are viable (Johnson et al., 1997).

A large body of studies characterized *RAS* genes showing that they encode for 21kDa proteins, associated to plasma membrane and are able to bind to GTP and GDP (Scolnick et al., 1979; Shih et al., 1979; Willingham et al., 1980).

RAS proteins are small GTPases and represent the first characterized members of a wider superfamily of small GTPases, which includes RAS-related protein families: RHO, RAB, ARF and RAN (Colicelli, 2004). Small GTPases alternate from an active conformational state, when they are bound to GTP, to an inactive conformational state, when they are GDP-bound (Bourne et al., 1991). The cycling from the inactive to the active state is regulated and accelerated by GTPases-activating proteins (RASGAPs), which stimulate

GTP hydrolysis, and guanine exchange factors (RASGEFs) that catalyse the release of GDP and the replacement with GTP (Figure 7) (Bos et al., 2007; Cox et al., 2014).

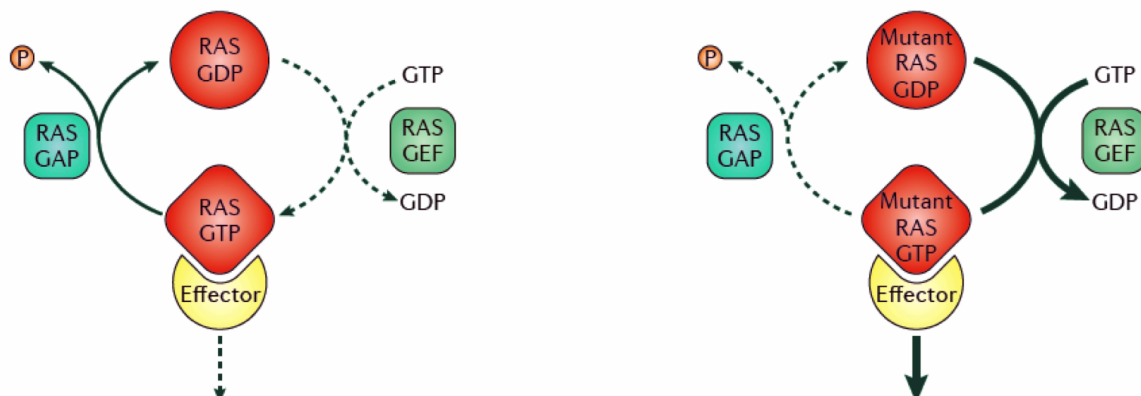


Figure 7. RAS GTP-GDP cycle. RAS proteins are small GTPases, which alternate from an active conformational state, when they are bound to GTP, to an inactive conformational state, when they are GDP-bound. The cycling from the inactive to the active state is regulated and accelerated by GTPases-activating proteins (RASGAPs), which stimulate GTP hydrolysis, and guanine exchange factors (RASGEFs) that catalyse the release of GDP and the replacement with GTP (left panel). RAS mutations impair the intrinsic and GAP-dependent GTP hydrolysis, making RAS constitutively active (right panel) (adapted from Cox et al., *Nat Rev Drug Discov*, 2014).

In physiological condition, growth factor stimuli activate cells surface receptor tyrosine kinases that, through the action of adaptor proteins, allow the activation and relocalization to the plasma membrane of RASGEFs, which leads to GDP-GTP exchange (Cox and Der, 2010). The observation that stimulation of epidermal growth factor receptor (EGFR) results in RAS activation, made EGF cascade one of the first object of study to characterize RAS signalling (Kamata and Feramisco, 1984). Upon EGF stimulus, the activation of EGFR kinase activity leads to the binding to the adaptor protein Grb2, which contains two binding domains: a SH2 domain able to bind to the tyrosine phosphorylated sequence of the intracellular portion of the receptor; and an SH3 domain which acts as an adaptor protein and allows the localization of the RASGEF Sos-1 near RAS (Rozakis-Adcock et al., 1993; Li et al., 1993).

The majority of RAS mutations impair intrinsic and GAP-dependent GTP hydrolysis, thus making RAS constitutively active (Cox et al., 2014). Once activated, RAS exerts its action on a wide range of downstream effectors. Pioneering studies identified proteins containing RAS binding domain or RAS association domain, which allow the binding of the effectors to the GTP-bound RAS (Cox and Der, 2010). RAF/MEK/ERK and the PI3K/AKT/mTOR cascades were initially identified as the major signalling pathways activated by RAS, through the direct interaction of RAS with RAF and PI3K, respectively (Wood et al., 1992; Gallego et al., 1992; Vojtek et al., 1993; Rodriguez-Viciana et al., 1994). In addition, the RAS-related protein RAL and RAL-GEF, TIAM1 (a GEF of the Rho GTPases), and phospholipase C- ϵ , have been identified as potential new effectors of RAS signalling (Hofer et al., 1994; Kelley et al., 2001; Lambert et al., 2002).

A large body of studies have shown that RAS activity is not only regulated by the cycling of GTP and GDP, but also by a spatial and temporal control of RAS localization (Cox and Der 2010). In order to be correctly associated to the membrane and exert their activity, RAS proteins are modified in the “*variable*” domain by farnesylation at the residue Cys186 of the C-terminal region and palmitoylation, which occur in the hypervariable region of HRAS, NRAS and KRAS4A or in the polybasic domain of KRAS4B (Willumsen et al., 1984a; Willumsen et al., 1984b; Hancock et al., 1990). In addition, other post-translational modifications were discovered to be linked to RAS activity, such as C-terminal phosphorylation, mono- and di-ubiquitination (Bivona et al., 2006; Jura et al., 2006; Lim et al., 2009). In summary, all these modifications regulate RAS localization and also its interaction and activation of effectors, such as Raf and ERK (Leevers et al., 1994; Jura et al., 2006).

3.2 Colorectal cancer

Colorectal cancer (CRC) is one of the most widespread and lethal cancers in the western world (Ferlay et al., 2015; Siegel et al., 2017). Despite an overall reduction in CRC incidence and mortality, there is an urgent need to increase prevention and improve lifestyle by reducing risk factors such as bodyweight, smoking, alcohol and meat consumption (Siegel et al., 2017).

CRC starts as an adenomatous benign polyp, but then, by acquiring a series of mutations in oncogenes and tumor suppressor genes, it develops into an invasive carcinoma (Figure 8) (Fearon and Vogelstein, 1990; Markowitz and Bertagnolli, 2009).

The initiating event in CRC formation is the mutation in the Adenomatous Polyposis Coli (APC) tumor suppressor gene, which leads to the activation of Wnt signalling and the acquisition of a hyperproliferative phenotype (Morin et al., 1997; MacDonald et al., 2009). In normal condition, APC degrades β -catenin and inhibits its nuclear localization, whereas upon its loss, β -catenin is accumulated into the nucleus, where it acts as co-activator of T-cell factor-lymphocyte enhancer factor family (TCF-LEF) that lead to the transcription of Wnt responsive genes (Morin et al., 1997; MacDonald et al., 2009).

Next, different oncogenes play a crucial role in advanced stages of CRC progression. *KRAS* mutations are reported in 42% of cases, while *BRAF* mutations are found in 13% of cases (Bos et al., 1987; Davies et al., 2002; Rajagopalan et al., 2002). Another key step during CRC progression is the mutation in *TP53*, which often marks the transition from a large adenoma to an invasive carcinoma, followed by the inactivation of a third tumor suppressor gene *TGFBR2*, which impair the transforming growth factor beta (TGF β) signalling (Baker et al., 1989; Baker et al., 1990; Markowitz et al., 1995; Grady et al., 1999).

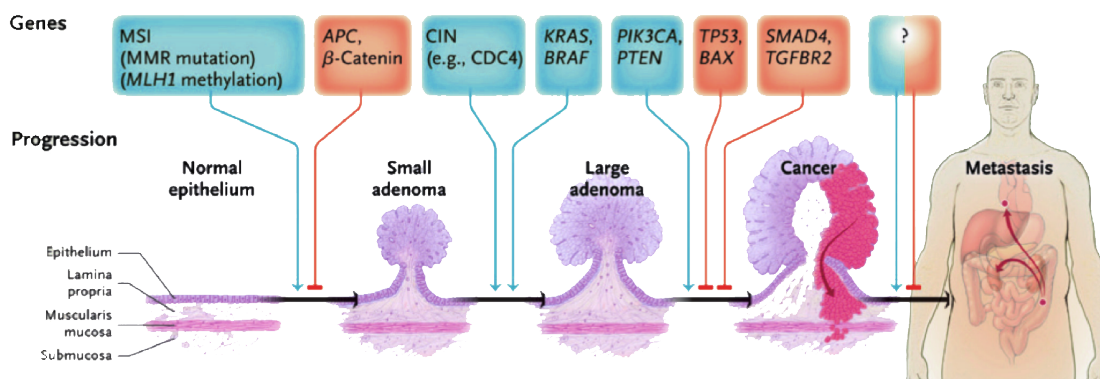


Figure 8. CRC progression. CRC cancer progression starts with the transition from a normal epithelium to a small adenoma, as a consequence of mutations in *APC* or *CTNNB1* (β -catenin), and/or mutations in mismatch repair genes, which lead to errors during replication. The next steps, which cause the progression from a small to a large adenoma and finally to an invasive carcinoma, are characterized by the acquisition of activating mutations in oncogenes, such as *KRAS*, *BRAF* or *PIK3CA*, and inactivating mutations in tumor suppressors, among which *TP53*, *BAX*, *SMAD4* and *TGFBR2* (adapted from Markowitz and Bertagnolli, *N Engl J Med*, 2009).

CRC genetic instability can occur through two different mechanisms: chromosomal instability (CIN) or microsatellite instability (MSI) (Lengauer et al., 1997).

The majority of sporadic CRC are characterized by CIN as a consequence of inactivation of genes involved in chromosomal stability maintenance, and show aneuploidy and loss of heterozygosity (LOH) (Lengauer et al., 1997). Whereas, 15% of sporadic CRC are nearly diploid and arise through a different process, which involves defects in mismatch repair machinery (Lengauer et al., 1997). Inactivation of mismatch-repair genes results in errors during the replication of repetitive DNA sequences, leading to the presence of unrepaired strand slippage and consequently the formation of new alleles, a phenomenon known as MSI (Thibodeau et al., 1993; Markowitz and Bertagnolli, 2009). Tumor suppressors that contain repetitive sequences, such as *TGFBR2*, *TGF β* and BCL-2 associated protein (BAX), are found inactivated in MSI colorectal tumors (Markowitz and Bertagnolli, 2009).

The major intervention for CRC treatment is surgery, followed by chemotherapy, radiotherapy and targeted-therapy, according to CRC stages and genetic features (André et al., 2004; Brenner et al., 2014). In the clinic, CRC is classified in different stages on the basis of the degree of invasion. From stage I to stage II disease, cancer is limited in the colonic area, and after surgery the survival rates 5-year post diagnosis are 90% and 75%, respectively (Brenner et al., 2014). When the tumor invades lymph nodes (stage III), after surgery, patients are treated with standard chemotherapy, such as 5-fluorouracil or oxaliplatin, which extends the survival to 60% (Markowitz et al., 2002). Finally, when the disease spreads in distant sites, mainly targeting the liver (stage IV), the prognosis is very poor, with a 5-year survival of 12% in patients with stage IV disease (Markowitz and Bertagnolli, 2009; Brenner et al., 2014).

Targeted-therapies represent a novel beneficial option for CRC patients showing resistance to conventional chemotherapy (Mendelsohn and Baselga, 2006). In particular, anti-EGFR monoclonal antibodies Cetuximab and Panitumumab extend progression-free survival (PFS) in patients with metastatic CRC, refractory to standard therapies (Cunningham et al., 2004; Van Cutsem et al., 2007). However, anti-EGFR targeted therapies show limited efficacy in individuals harbouring KRAS mutant tumors, thus making only a subset of patients eligible to this intervention (Lievre et al., 2006; Amado et al., 2008; Karapetis et al., 2008; Van Emburgh et al., 2016). Importantly, also patients that initially respond to the therapy, later develop resistance, in particular through the emergence of RAS mutations, occurring in the majority of cases, and mutations in EGFR extracellular domain, which impair the binding of the monoclonal antibody (Bardelli and Siena, 2010; Van Emburgh et al., 2016). Thus, increasing efforts are required to identify novel therapies, which can be effective also in patients bearing KRAS mutant CRC.

3.3 Therapies for RAS-mutant cancers

A large body of studies have shown that RAS-mutant cancers result to be refractory to standard and targeted-therapies, thus supporting the urgent need of new specific treatments (Lievre et al., 2006; Cox et al., 2014; Verissimo et al., 2016).

Several attempts have been made to identify drugs able to selectively interfere with RAS signalling (Figure 9). *Low affinity inhibitors* were classified as compounds that, by a non-covalent binding to RAS, inhibit the nucleotide exchange, or impair the formation of complexes with effectors, such as RAF. However, the low potency and the poor selectivity, make them unsuitable for clinical use (Taveras et al., 1997; Herrmann et al., 1998). The same limitations were faced by other drugs, such as *GEF inhibitors*, which comprise compounds able to bind to RAS in the GDP-bound form and inhibit its activation. An example is the synthetic alpha helix that mimics SOS1 structure and it is able to interfere with RAS-SOS1 complex formation (Patgiri et al., 2011). Other studies focus on the use of molecules able to covalently bind to the specific mutant form of RAS G12C, which is commonly found in lung cancer. It has been shown that these compounds inhibit the Sos1-mediated RAS activation and impair the RAS binding to RAF, making it possible to identify small molecules able to interfere with each specific RAS mutations (Ostrem et al., 2013). However, efforts are required to reach a sufficient selectivity for each mutation (Cox et al., 2014). Another possibility of RAS targeting is represented by inhibition in RAS farnesylation, palmitoylation and phosphorylation, which can impair the correct localization to plasma membrane and alter RAS pro-growing functions (Berndt et al., 2011; Laheru et al., 2012; Dekker et al., 2010; Bivona et al., 2006). However, the major limitation of these approaches is represented by the possibility of off-target effects and the consequent toxicity to normal tissues (Cox et al., 2014). In recent years, inhibitors of the two main RAS effector pathways (RAF/MEK/ERK and PI3K/AKT/mTOR) have been the most extensively studied (Gulhati et al., 2012; Yeh et al., 2007; Engelman et al., 2008;

Sarker et al., 2015). Supported by the high mutation frequency of these signalling cascades in human tumors, inhibitors of RAF and MEK1/2 were developed and tested in pre-clinical and clinical models (Davies et al., 2002; Yeh et al., 2007). Unfortunately, their inhibition leads to the activation of compensatory mechanisms able to limit treatment efficacy (Rinehart et al., 2004; Sun et al., 2014; Manchado et al., 2016). For this reason, several studies are focusing on the identification of resistance pathways, in order to formulate combinatorial approaches able to sensitize cancer cells to RAS effector inhibitors (Engelman et al., 2008; Sun et al., 2014; Manchado et al., 2016; Verissimo et al., 2016). Recently, “*synthetic lethality*” represents another promising approach utilized to identify effective interventions for RAS mutant cancers (Kaelin, 2009). Two genes are defined as synthetically lethal when mutation in one of them is compatible with viability, but mutations in both lead to cell death (Kaelin, 2009). To this purpose, RNA interfering-screens are used to identify potential targets in RAS mutant tumors (Luo et al., 2009; Barbie et al., 2009; Sun et al., 2014). Importantly, since normal cells lack mutant RAS, this treatment should be selectively “*lethal*” to cancer cells (Kaelin, 2009). Despite several technical challenges this approach has to face, it is providing additional candidates for the treatment of RAS mutant tumors.

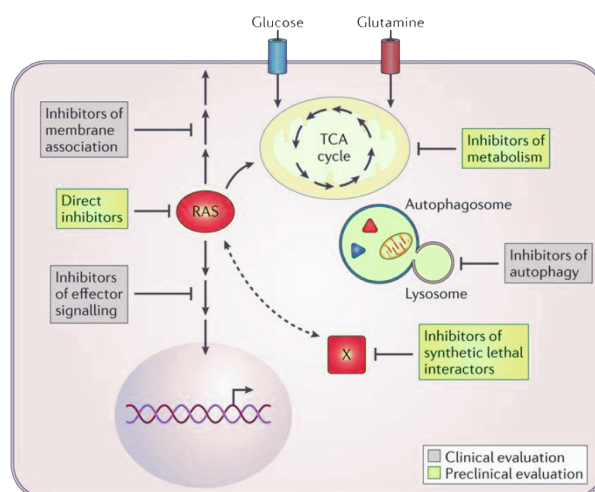


Figure 9. Inhibitors against RAS pathway. Different inhibitors have been object of studies to target RAS signaling, among which direct inhibitors, inhibitors of RAS-association to the membrane, inhibitors of RAS effectors, inhibitors of metabolism and finally inhibitors of synthetic lethal interactors (adapted from Cox et al., *Nat Rev Drug Discov*, 2014).

3.4 Metabolism of KRAS mutant cancers

As discussed above, oncogenes drive metabolic reprogramming in tumor cells (Buzzai et al., 2005; Ward and Thompson, 2012). Several studies show that KRAS mutations are able to promote a metabolic rewiring, in order to sustain cell proliferation and growth (Figure 10) (Kimmelman, 2015).

One of the key strategies utilized by KRAS-driven tumors to meet their metabolic needs is autophagy, a process of “*cellular self-eating*” that allows the degradation and recycling of cellular components (Yang et al., 2011; Commisso et al., 2013). By generating intermediates that refuel metabolism, autophagy is important to sustain KRAS tumor growth (Yang et al., 2011). Moreover, KRAS mutant cancers are able to engulf extracellular proteins through a process known as micropinocytosis, which allows the uptake of extracellular albumin as amino acidic source to fuel tricarboxylic acid cycle (TCA) (Commisso et al., 2013).

Several studies support the fundamental role of glucose in sustaining metabolism of KRAS-driven cancer (Kimmelman, 2015). In particular, it has been found that KRAS mutant cancers upregulate glucose-transporter-1 (GLUT-1), generating an increased glucose uptake, glycolysis rate and lactate production (Yun et al., 2009). In addition, studies in pancreatic models reveal that glycolytic intermediates are dispatched to the hexosamine biosynthetic pathway (HBP), to generate glycosylation precursors, and to the non-oxidative arm of the pentose phosphate pathway (PPP) to sustain nucleotide biosynthesis (Ying et al., 2012).

Beyond glucose metabolism, KRAS tumors result to be also addicted to glutamine, which sustains their growth by refuelling anabolic reactions (Gaglio et al., 2011; Son et al., 2013). In particular, it has been found that pancreatic cancer upregulates glutamine influx through a pathway which leads to the production of pyruvate and increased NADPH/NADP⁺ ratio,

a cofactor fundamental to maintain reduced-glutathione (GSH) pool and redox homeostasis (Son et al., 2013).

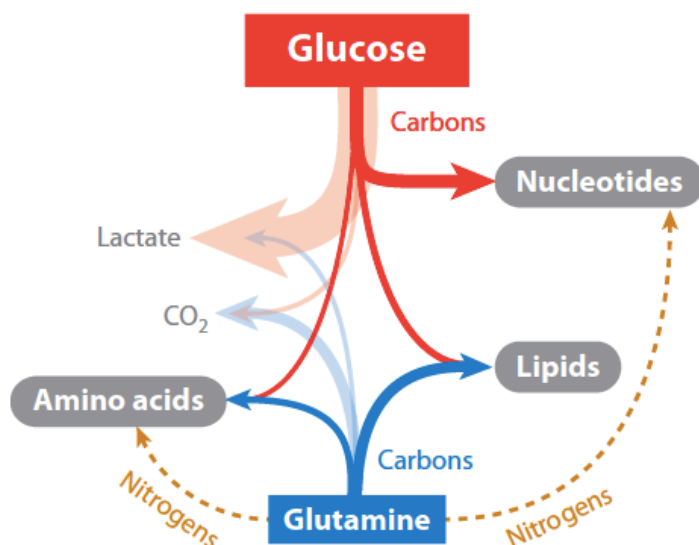


Figure 10. Metabolic reprogramming in RAS-driven tumors. The scheme shows the metabolic requirements for RAS-driven cancers. Glucose and glutamine are two fundamental sources of carbons and nitrogens to produce lactate, CO₂ and refuel anabolic reactions (nucleotide, amino acid and lipid biosynthesis) for sustaining proliferation and tumor growth (adapted from Lunt and Vander Heiden, *Annu. Rev. Cell Dev. Biol.*, 2011).

Oxidative stress in KRAS-driven tumors

In contrast to the first hypothesis postulated by Warburg, tumor cell mitochondria are functional, however their metabolism is reprogrammed in order to meet cancer cell needs of macromolecules biosynthesis (Ward and Thompson, 2012). A consequence of mitochondrial activity is the production of ROS, which are byproducts of oxygen metabolism, such as hydroxyl radical ($\bullet\text{OH}$), superoxide anion ($\text{O}_2^{\bullet-}$) and the non-radical molecule hydrogen peroxide (H_2O_2) (Muller et al., 2004; Murphy, 2009; Reczek and Chandel, 2017).

A large body of studies have revealed that, differently from normal cells, cancer cells show a high degree of oxidative stress (Szatrowski and Nathan., 1991; Aykin-Burns et al., 2009). In particular, ROS act as mitogenic signaling molecules which result crucial to sustain and regulate cancer cell proliferation, in fact by causing lipid, protein and DNA damage, they

can promote genomic instability and tumorigenic processes (Weinberg et al., 2010; Sabharwal and Schumacker, 2014). Moreover, experiments showing that mitochondrial anti-oxidants limit the proliferation rate of KRAS tumor cells, strongly sustain the fundamental role of ROS for the growth of KRAS-driven tumors (Weinberg et al., 2010). In addition, it has been reported that oncogenic RAS activation is able to enhance ROS production and lead to their accumulation through a Nox4 dependent mechanism (Ogrunc et al., 2014).

However, a balance of ROS levels is crucial for the survival of cancer cells (Figure 11), for this reason, the generation of NADPH through the non-oxidative pentose phosphate pathway and the reduced-glutathione (GSH) pool, are necessary for KRAS-driven tumors to maintain cellular redox homeostasis (Son et al., 2013; Kimmelman, 2015). Indeed, an unbalanced ROS generation, by exacerbating their production or decreasing anti-oxidant defences, could be lethal for cancer cells, thus representing an important therapeutic opportunity (Trachootham et al., 2006; Galadari et al., 2017).

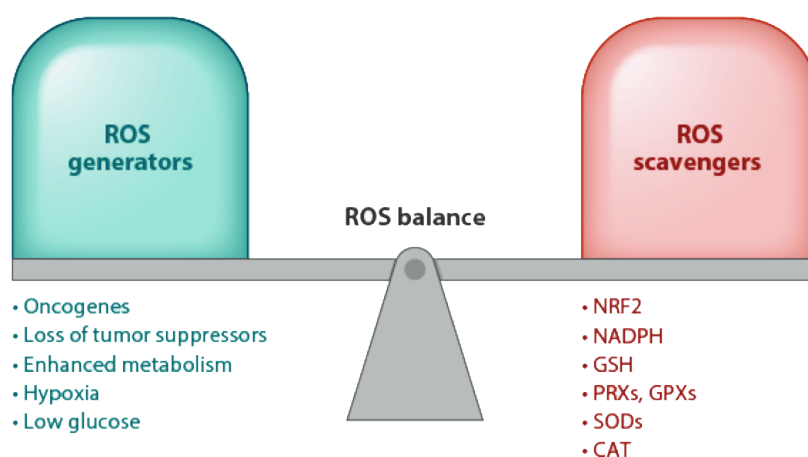


Figure 11. ROS homeostasis in tumors. Cancers are characterized by high level of ROS, which sustain tumorigenesis. However, cancer cells need to maintain a balance between ROS generators (such as oncogene activation, loss of tumor suppressors, increased metabolic activity, glucose deprivation, hypoxia) and ROS scavenging activity (anti-oxidant enzymes, the stress-resistance transcription factor NRF2, glutathione), in order to avoid a ROS-overload, which can lead to cell death (adapted from Reczek and Chandel, *Annu. Rev. Cancer Biol.*, 2017).

Another mechanism through which KRAS sustains redox homeostasis is by up-regulating the nuclear factor-erythroid 2-related factor-2 (NRF2) (Tao et al., 2014; Satoh et al., 2013; Hamada et al., 2017). NRF2 is a transcription factor, which plays a crucial role in the oxidative stress response by inducing the expression of genes containing an Anti-oxidant-Responsive Element (ARE) among which: glutamate-cysteine ligase (GCLC/GCLM), thioredoxin reductase 1 (TXNRD1), aldo-keto reductase (AKR), glutathione S-transferase (GST), multidrug resistance-associated protein 2 (MRP2), NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HMOX1) (Kensler et al., 2007; Nguyen et al., 2009). By protecting tumor cells from oxidative damages, it has been found that NRF2 expression is responsible for KRAS-driven chemoresistance (Tao et al., 2014). Indeed, in a mouse lung cancer model, it has been shown that NRF2 inhibition is able to limit therapy-resistance mechanisms and to sensitize tumor cells to chemotherapy efficacy (Tao et al., 2014).

KRAS-driven cancer and iron metabolism

Several studies have revealed that intracellular ROS are able to alter iron metabolism and increase the unbound iron form, known as “*labile iron pool*” (LIP) (Caltagirone et al., 2001; Ibrahim et al., 2013). In particular, this mechanism involves superoxide, the major reactive oxygen species, which is capable of releasing iron from protein complexes (Ibrahim et al., 2013). In addition, iron itself can in turn generate ROS, through the Fenton reaction chemistry, causing lipid, protein and DNA damage, which ultimately sustain tumorigenesis (Torti and Torti, 2013). Several studies demonstrated that cancer cells are characterized by a higher iron content than normal cells, which contributes to the iron-mediated oxidative stress (Brookes, 2006; Torti and Torti, 2013; Xue and Shah, 2013). In addition, emerging evidence reveals that LIP could be increased further in RAS mutated cancer, raising the possibility that RAS signalling can alter iron metabolism, as found for other oncogenes such as c-myc and E1A (Kakhlon et al., 2002; Yang and

Stockwell, 2008; Tsuji et al., 1995; Wu et al., 1999). In fact, it has been shown that RAS oncogenic activation modulates iron metabolism by upregulating iron-uptake proteins, such as transferrin receptor (Trf1), and downregulating iron export and storage proteins, in particular ferritin, finally increasing the LIP (Kakhlon et al., 2002; Yang and Stockwell, 2008). Interestingly, high LIP can render RAS mutant cancer cells more susceptible to oxidative stress, making iron metabolism an attractive target for cancer treatment (Schoenfeld et al., 2017; Torti and Torti, 2013).

Heme oxygenase-1

Heme oxygenase-1 (HO-1), also known as heat-shock protein 32 (HSP32), represents the only way through which cells catabolize heme. It has a broad anti-apoptotic and anti-inflammatory activity on epithelial, endothelial and muscle cells and it mediates the anti-inflammatory action of monocyte and macrophages (Lee et al., 1996; Peyton et al., 2002; Otterbein et al., 2003). There are two HO isoforms encoded by two different genes: HO-2, is constitutively expressed in several tissues, at high level in the brain, at low level in the testis, distal nephrons, liver and gut; whereas, HO-1 expression is induced upon stress stimuli, such as ROS, nitric oxide (NO), UV irradiation, heat shock but also by heme itself (Maines et al, 1986; Cruse and Maines, 1988; Muñoz-Sánchez and Cháñez-Cárdenas, 2014; Was et al., 2010). HO-1 catabolizes heme in carbon oxide (CO), biliverdin and free iron (Figure 12) (Otterbein et al., 2003). CO has anti-inflammatory action and it can mimic HO-1 effect in suppressing pro-inflammatory responses in mouse models; biliverdin, which is then rapidly converted in bilirubin, shows anti-oxidant properties and anti-inflammatory activity in mice; lastly, free iron leads to the expression of ferritin and an iron efflux ATPase pump, which in turn exert a protective function by sequestering and removing free iron, respectively, and avoiding potential oxidative stress (Sato et al., 2001; Stocker et al., 1987; Ferris et al., 1999). It has been reported that ferritin is able to substitute HO-1 in protecting endothelial cells from oxidative damage, supporting the key

role of ferritin in the anti-oxidant action of HO-1, at least in certain biological settings (Balla et al., 1992). In addition, it has been shown that HO-1 deficient fibroblasts accumulate free iron, leading to free radical formation through the Fenton chemistry reactions, with consequent oxidative damages and cell death. In contrast, HO-1 overexpression, by decreasing the free iron pool through the induction of ferritin and iron efflux pumps, results to have a cytoprotective role (Ferris et al., 1999; Gonzales et al., 2002). These preliminary studies were crucial to understand the role of HO-1 in cellular iron homeostasis.

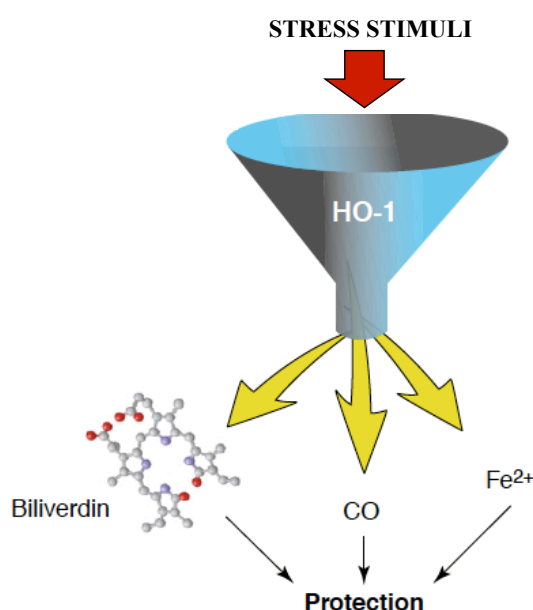


Figure 12. Heme oxygenase-1 (HO-1) mediates protection from stress stimuli. HO-1 expression is induced upon stress stimuli such as ROS, nitric oxide, UV irradiation, heat shock and heme. By catabolizing heme in carbon oxide (CO), biliverdin and free iron (Fe^{2+}), HO-1 mediates protection against oxidative damages, through different mechanisms. CO shows anti-inflammatory properties; biliverdin, which is rapidly converted into bilirubin, exerts anti-oxidant and anti-inflammatory action and finally, free iron stimulates the expression of ferritin avoiding potential oxidative damage (adapted from Otterbein et al., *Trends Immunol*, 2003).

A growing body of evidence indicates that HO-1 plays a key role in tumorigenesis, through different pro-survival mechanisms (Was et al., 2010; Jozkowicz et al., 2007). It has been shown that HO-1 level is higher in several tumor types (melanoma, brain/prostate/pancreatic cancers), than in healthy tissues (Torisu-Itakura et al., 2000; Hara

et al., 1996; Sacca et al., 2007; Berberat et al., 2005). Moreover, its expression is even higher in response to chemotherapy and radiotherapy, in fact, HO-1 overexpression shows a cytoprotective and anti-apoptotic role in different types of cancer, such as colorectal, gastric and lung cancers, making its induction a mechanism of therapy resistance (Was et al., 2010; Busserolles et al., 2006; Liu et al., 2004; Kim et al., 2008; Berberat et al., 2005). In agreement with this, recently, it has been shown that high level of HO-1 expression represents a poor prognostic factor in breast cancer patients undergoing chemotherapy (Muliaditan et al., 2018).

Supporting the pro-tumorigenic role of HO-1, several studies reveal that its pharmacological or genetic inhibition leads to a decrease in cell proliferation and tumor growth both *in vitro* and in *in vivo* mouse models, making this protein an intriguing possible target for cancer therapy (Kondo et al., 2007; Hirai et al., 2006; Bergé et al., 2010).

Several mediators could contribute to the cytoprotective action of HO-1 in cancer cells: the removal of free heme, which acts as a pro-oxidant for the cell; the anti-oxidant properties of bilirubin; the activation of p38 MAPK cascade mediated by CO; and finally, ferritin that, by sequestering free iron, reduces tumor cell susceptibility to oxidative insults (Was et al., 2010).

Moreover, it has been shown that HO-1 promotes angiogenesis, metastasis formation and mediates the immunosuppressive function of regulatory T cells in different tumors (Sunamura et al., 2003; Choi et al., 2005; El Andaloussi and Lesniak, 2007).

4. Pharmacological doses of vitamin C in cancer treatment

The use of high-dose vitamin C (ascorbic acid) as anti-cancer treatment was proposed for the first time by Pauling and Cameron (Cameron and Pauling, 1976). In 1976 and 1978, they published two controlled retrospective studies showing that advanced stage cancer patients, undergoing 10 g ascorbate/day intravenously for 10 days and then orally, show a mean survival time three and four times higher than the controls, respectively (Cameron and Pauling, 1976; Cameron and Pauling, 1978). They hypothesized that vitamin C, by stabilizing extracellular matrix through the inhibition of matrix-degradative enzymes and the enhancement of collagen synthesis, encapsulates tumors avoiding cancer growth and metastasis formation (Cameron and Pauling, 1973; Cameron et al., 1979). However, these studies were object of criticism, since they were neither randomized nor placebo-controlled (Comroe, 1978; DiPalma and McMichael, 1979). Later, two-randomized clinical trials, which use orally administered vitamin C, failed to demonstrate any beneficial effect on cancer patient survival (Creagan et al., 1979; Moertel et al., 1985). These contradictory outcomes are partially explained by the different administration route; in fact, pharmacokinetic studies show that orally administered vitamin C can't achieve concentration higher than 50-100 μM , whereas intravenous administration allows to bypass the gastric barrier, reaching millimolar concentrations (Padayatty et al., 2010; Chen et al., 2007; Chen et al., 2008, Stephenson et al., 2013). For this reason, there is an increasing interest in studying vitamin C mechanism of action and its therapeutic potential, either as single agent or in combination with chemotherapy, through several pre-clinical and clinical trials (Hoffer et al., 2008; Monti et al., 2012; Welsh et al., 2013; Ma et al., 2014; Schoenfeld et al., 2017; NCT02344355; NCT03146962; NCT02420314; NCT01752491).

4.1 Vitamin C biochemical properties

Vitamin C was discovered for its antiscorbutic activity, and isolated for the first time by Szent-Györgyi in 1932 (Svirbely and Szent-Györgyi, 1932). Vitamin C can be synthesized through the hexuronic acidic pathway in the kidney of amphibians and reptiles, whereas in mammals it is synthesized in the liver. However, humans, other primates, guinea pig and a few bat species, lack the enzyme required for the last reaction of vitamin C biosynthesis (L-gulonolactone oxidase); for this reason, vitamin C needs to be introduced by food, in particular through fruits and vegetables (Bánhegyi et al., 1997).

Absorption of dietary vitamin C occurs in the enterocytes of small intestine via sodium dependent active transporters (SVCT1 and SVCT2) up to 500 mg; for higher doses, diffusion transport also contributes to its uptake (Takanaga et al., 2004; Verrax and Calderon, 2008). However, in other compartments, such as in erythrocytes and in tumor microenvironment, ascorbic acid needs to be oxidized in dehydroascorbic acid (DHA) to enter in the cell through facilitative glucose transporters GLUTs (Vera et al., 1993; Montel-Hagen et al., 2008; Agus et al., 1999). Once in the cell, DHA is reduced back to ascorbic acid by GSH or glutaredoxin or thioredoxin reductase, in order to be accumulated intracellularly against a concentration gradient preventing its efflux (Winkler et al., 1994; Park and Levine, 1996; May et al., 1997).

It has been shown that vitamin C possesses both anti-oxidant and pro-oxidant properties (Carr and Frei, 1999). At physiological concentrations (40-80 μM in plasma), ascorbic acid acts as an anti-oxidant, by undergoing two consecutive one-electron oxidations to form ascorbyl radical (one-electron oxidation product) and DHA (two-electron oxidation product) (Figure 13). Ascorbyl radical is unreactive and can be reduced again in ascorbate (Buettner and Jurkiewicz, 1996; Carr and Frei, 1999; Du et al., 2012). Through these reactions, vitamin C acts as scavenger protecting cells from oxidative damages (Carr and Frei, 1999; Du et al., 2012).

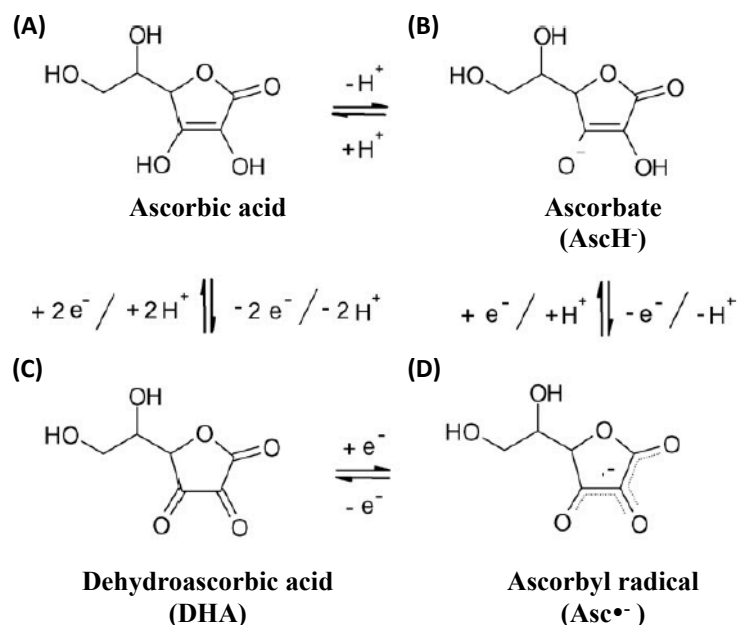


Figure 13. Different states of vitamin C. Ascorbic acid (A) undergoes two consecutive one-electron oxidations to form dehydroascorbic acid (DHA) (C). Alternatively, ascorbate (B), which is the prevalent form of ascorbic acid at physiological pH, releases one electron and one proton to form ascorbyl radical (D). Ascorbyl radical, due to resonance stabilization of the unpaired electron, is unreactive and it can be reduced back to ascorbate or further oxidized to DHA. These features render ascorbic acid an excellent anti-oxidant, able to reduce the majority of radicals such as hydroxyl radical and superoxide anion (adapted from Verrax and Calderon, *Biochem. Pharmacol*, 2008).

However, ascorbate can exert a pro-oxidant function in presence of transition metal ions, such as iron and copper. In particular, as show in figure 14, ascorbate reduces ferric iron (Fe³⁺) to ferrous iron (Fe²⁺), while generating ascorbyl radical (Asc^{•-}). Fe²⁺ reduces molecular oxygen (O₂) into superoxide radical (O₂^{•-}), which in turn dismutates to hydrogen peroxide (H₂O₂) and O₂. Through the Fenton reaction chemistry, Fe²⁺ reacts with H₂O₂ generating the highly damaging hydroxyl radicals (HO[•]), and Fe³⁺, which can react back with ascorbate, sustaining the formation of highly reactive species (Buettner and Jurkiewicz, 1996; Carr and Frei, 1999; Du et al., 2012).

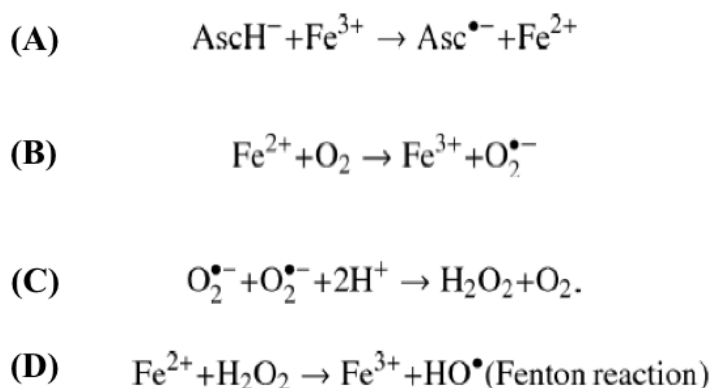


Figure 14. Pro-oxidant function of vitamin C. In the presence of catalytic metals, ascorbate (AscH⁻) exerts a pro-oxidant function. **(A)** Ascorbate reduces ferric iron (Fe³⁺) into ferrous iron (Fe²⁺), while generating ascorbyl radical (Asc^{•-}). **(B)** Fe²⁺ reduces molecular oxygen (O₂) into superoxide radical (O₂^{•-}), which in turn generates hydrogen peroxide (H₂O₂) and molecular oxygen (O₂) **(C)**. **(D)** Fe²⁺ reacts with H₂O₂ generating hydroxyl radical (HO[•]) and Fe³⁺ (Fenton reaction), that can react back with ascorbate (adapted from Du et al., *Biochim. Biophysica Acta*, 2012).

4.2 Vitamin C selective cytotoxicity to cancer cells and its mechanism of action

A large body of studies have consistently demonstrated that high-dose vitamin C selectively kills a wide range of cancer cells, while leaving normal cells unaffected, both *in vitro* and *in vivo* studies (Chen et al., 2008; Ma et al., 2014; Yun et al., 2015; Schoenfeld et al., 2017). In fact, in mouse models of pancreatic, ovarian, colorectal, lung cancer and glioblastoma, parenteral administration of vitamin C (4 g/kg twice a day, every day) results to be effective in delaying tumor progression, without toxicity on normal tissues (Chen et al., 2008; Ma et al., 2014; Yun et al., 2015; Schoenfeld et al., 2017). In addition, a growing body of clinical studies indicates that high dose vitamin C administered intravenously is safe and well tolerated in cancer patients even in the presence of chemotherapy and radio-

chemotherapy (Padayatty et al., 2010; Hoffer et al., 2008; Stephenson et al., 2013; Ma et al., 2014; Schoenfeld et al., 2017).

Until now only two pathological conditions are reported to be incompatible with pharmacological vitamin C administration.

Patients with glucose-6-phosphate dehydrogenase (G6PDD) deficiency: G6PDD deficiency leads to impaired production of NADPH, which is crucial for maintaining reduced-GSH pool and mediating oxidative stress-protection (Cappellini and Fiorelli, 2008). This inefficient anti-oxidative protection leads to radical formation upon vitamin C administration, ultimately causing haemolysis of red blood cells (Campbell et al., 1975; Rees et al., 1993; Chen et al., 2005).

Patients with renal dysfunction: it has been reported that upon high-dose vitamin C administration, patients with renal dysfunction or kidney stone history suffer from oxalate nephropathy, a condition caused by the crystallization of oxalate (an end product of vitamin C oxidation) (Robitaille et al., 2009). However, individuals with normal renal function do not show an increased risk of oxalate crystallization (Robitaille et al., 2009).

With the exception of these pathological conditions, the selective toxicity for tumor cells, but not for normal tissues, has prompted a growing interest in using vitamin C for cancer treatment either as single therapy or combined with chemotherapy and radiotherapy (Ma et al., 2014; Schoenfeld et al., 2017).

Several studies show that the mechanism through which vitamin C mediates its cytotoxicity is by acting as pro-drug for hydrogen peroxide delivery to tissues (Figure 15) (Chen et al., 2005; Chen et al., 2007; Chen et al., 2008; Du et al., 2010; Ma et al., 2014).

The selective toxicity of vitamin C for cancer cells is partially explained by the low level of anti-oxidant enzymes (catalase, glutathione peroxidase, peroxiredoxins) and high level of ROS, which characterizes tumors but not normal cells (Liu et al., 2004; Oberley, 2005; Du et al., 2012; Doskey et al., 2016). In support of this, it has been found that ascorbate radicals and hydrogen peroxide are detectable in tumor extracellular fluids, but not in the

blood, where red blood cells have efficient system of removal (Chen et al., 2005; Chen et al., 2007).

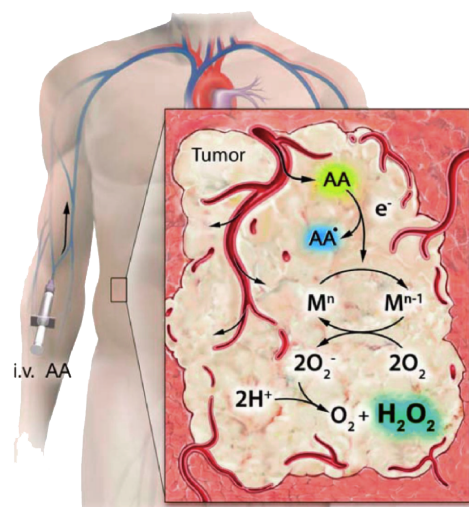


Figure 15. High-doses of vitamin C deliver hydrogen peroxide to the tumor. High-dose vitamin C (Ascorbate; AA) (0.3-20 mM) acts as a pro-drug to generate hydrogen peroxide (H_2O_2), which is selectively toxic to tumors. To reach pharmacological doses of vitamin C, intravenous administration is required in order to bypass the gastric barrier. $\text{AA}^\bullet$: ascorbyl radical; Mn : unknown metalloprotein catalyst (adapted from Chen et al., *Proc Natl Acad Sci*, 2008).

Recently, Yun and colleagues showed that KRAS and BRAF-driven cancers result to be more susceptible to vitamin C toxicity than KRAS and BRAF wild type tumors (Yun et al., 2015). In fact, high-dose vitamin C is able to delay tumor growth in xenograft model of KRAS mutant CRC cell lines, and to reduce the number of polyps in genetically engineered mouse model of KRAS-driven CRC, leaving unaffected the progression of KRAS wild type CRC (Yun et al., 2015).

As discussed in previous paragraphs, KRAS-driven tumors strongly rely on glycolysis and overexpress the glucose transporter GLUT1, which leads to a higher uptake of the ascorbic acid-reduced form DHA. Accordingly, GLUT1 inhibition reduces DHA uptake in KRAS mutant tumors, and its overexpression enhances DHA uptake in KRAS wild type cancer. However, GLUT-1 overexpression does not sensitize KRAS wild type cells to vitamin C toxicity, suggesting that oncogene-mediated metabolic reprogramming is required (Yun et

al., 2015). The mechanism underlying the selective toxicity in KRAS-driven tumors involves an unbalanced increase in ROS, as a consequence of GSH and NADPH depletion due to the intracellular DHA reduction to vitamin C (Yun et al., 2015). Consequently, ROS accumulation inhibits glycolysis through inactivation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which results to be lethal in KRAS and BRAF highly glycolytic cancer cells, ultimately causing an energetic crisis and cell death (Yun et al., 2015). In support of this model, anti-oxidants, such as N-acetyl cysteine (NAC), were able to revert vitamin C cytotoxic effect both *in vitro* and *in vivo* (Yun et al., 2015).

Schoenfeld and colleagues have recently published a comprehensive work which finally elucidates the molecular mechanism responsible for vitamin C selective toxicity to cancer cells (Schoenfeld et al., 2017). Their work demonstrates that the ROS-mediated disruption of iron metabolism and the consequent increase in LIP, which characterize cancer cells but not normal cells, are responsible for vitamin C selective toxicity (Schoenfeld et al., 2017). The increased level of free iron enhances vitamin C oxidation and H₂O₂ production, which in turn further fuel LIP by disrupting, at least in part, Fe-S cluster-containing proteins (Du et al., 2015a; Schoenfeld et al., 2017). High level of H₂O₂ and free iron exacerbate Fenton reaction chemistry, generating hydroxyl radicals and leading to oxidative damages and cell death (Du et al., 2015a; Schoenfeld et al., 2017). In support of this model, iron chelation by desferrioxamine (DFO), reverses vitamin C cytotoxic effect (Du et al., 2015a; Schoenfeld et al., 2017). Interestingly, deletion of *SOD2* is able to sensitize a lung cancer cell line, which is slightly susceptible to vitamin C, by enhancing superoxide production and increasing LIP (Schoenfeld et al., 2017).

Given the high content of ROS and free iron previously described in RAS mutant cancer, this model could partially explain why KRAS-driven tumors result to be more sensitive to vitamin C toxicity (Yun et al., 2015; Schoenfeld et al., 2017).

MATERIALS AND METHODS

MATERIALS AND METHODS

Cell lines and culture conditions

HCT116, HT29, NCI-H23 and PC-3 cells were obtained from NCI 60 panel; CT26.WT, CCD-84CoN and BJ cells were purchased from ATCC; DLD1 cell line was purchased from DSMZ; NCI-H727, PANC-1, Cov362 and SW48 cells were purchased from ECACC. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Cat. #: 10566) supplemented with 10% FBS (Biowest, Cat. #: S1810), 1% non-essential amino acids (Biowest, Cat. #: X-0557), and 1% penicillin/streptomycin (Biowest, Cat. # L0022). All cells were tested for mycoplasma contamination routinely. Cells were maintained in a humidified, 5% CO₂ atmosphere at 37 °C.

For FMD-like condition experiments, cells were grown in DMEM medium without glucose (DMEM no glucose, Life Technologies, Cat. #: 11966025) supplemented with 0.5 g/l glucose (Sigma-Aldrich, Cat. #: G8769) and 1% FBS, referred as Short-Term starvation medium (STS). For mimicking standard condition, cells were grown in DMEM medium without glucose (DMEM no glucose, Life Technologies, Cat. #: 11966025) supplemented with 1 g/l glucose (Sigma-Aldrich, Cat. #: G8769) and 10% FBS, referred as control medium (CTR).

Reagent preparations

Oxaliplatin

Oxaliplatin was kindly provided by the IEO hospital pharmacy (Milan). The stock solution (5mg/ml) was dissolved in solution for injections (water, tartaric acid, sodium hydroxide).

Vitamin C

Sodium ascorbate was purchased from Sigma-Aldrich (Cat. #: A4034) and was dissolved in sterile saline. Stock solutions of 20 mg /ml or 240 mg/ml were prepared for *in vitro* and *in vivo* experiments, respectively. Vitamin C was freshly prepared each time before use.

Glutathione

Reduced glutathione (GSH) was purchased from Sigma-Aldrich (Cat. #: G6013) and dissolved in sterile water to a final concentration of 32.5 mM (stock solution). Stock solutions were stored at -20°C.

N-acetyl cysteine

N-acetyl cysteine (NAC) was purchased from Sigma-Aldrich (Cat. #: A9165) and dissolved in sterile water to a final concentration of 100 mM (stock solution). Stock solutions were freshly prepared for each experiment.

Desferrioxamine

Desferrioxamine (DFO) was purchased from Sigma-Aldrich (Cat. #: D9533) and dissolved in sterile deionized water to final concentration of 40 mg/ml. Stock solutions were stored at -20°C.

Hemin

Hemin was purchased from Sigma-Aldrich (Cat. #: 51280) and dissolved in 1.4 M NH₄OH (Sigma-Aldrich, Cat. #: 221228) to final concentration of 25 mg/ml (stock solution). Stock solutions were stored at + 4°C.

Zinc protoporphyrin

Zinc protoporphyrin (ZnPP) was purchased from Sigma-Aldrich (Cat. #: 282820) and dissolved in DMSO to final concentration of 25 mg/ml. Stock solutions were stored at -20°C.

Catalase

Catalase (Cat) from bovine liver (2000-5000 U/ml) was purchased from Sigma-Aldrich (Cat. #: C1345) and dissolved in 50 mM potassium phosphate buffer to a final concentration of 5000 U/ml. Stock solutions were freshly prepared for each experiment.

MnTMPyP

MnTMPyP (superoxide dismutase mimetic) was purchased from Merck Millipore (Cat. #: 475872) and dissolved in sterile deionized water to a final concentration of 0.697 mM. Stock solutions were stored at -20°C.

Viability assays

MTT assay

The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a quantitative colorimetric assay used to determine cell viability (Mosmann, 1983). MTT is characterized by a yellow colour, and upon its reduction to formazan by mitochondrial dehydrogenases, turns into a purple molecule, which has an absorbance peak at 570 nm. The absorbance is proportional to the amount of formazan produced, thus giving a measure of living cells, since the reaction only occurs in living and metabolically active cells (Mosmann, 1983).

To evaluate viability, cells were seeded into a 96-well plate (2000 cells per well) in CTR medium. 24 hours later, cells were rinsed twice in 1X PBS, and CTR or STS medium was added. After 24 hours, media were refreshed to ensure that glucose and serum levels were not completely exhausted and cells were treated with 40 µM oxaliplatin or vehicle for

further 24 hours. At the end of the experiment, medium was removed and cells were incubated with 100 μ l of MTT (Sigma-Aldrich, Cat. #: M2128) solution (0.5 mg/ml, dissolved in medium) per well, for 3 hours at 37°C in the dark. Next, 100 μ l of lysis buffer (10% SDS, HCl 0.01M) were added to each well to dissolve formazan crystals and plates were incubated overnight at 37°C. Absorbance was recorded at the wavelength of 570 nm using a microplate reader (Infinite M200 TECAN). The absorbance values of media background were subtracted from the absorbance of each sample. No-treated samples were used as reference values (100% survival) to normalize the absorbance of treated samples.

Erythrosin B exclusion assay

Erythrosin B is a vital dye that is impermeable to biological membranes, thus staining only unviable cells with disintegrated membranes (Kim et al., 2016).

To measure cell death, cells were seeded in 12-well plates at a concentration of 34000 cells per well in CTR medium. The next day, cells were rinsed twice in 1X PBS and CTR or STS medium was added. After 24 hours, media were refreshed to ensure that glucose and serum levels were not completely exhausted and cells were treated with 40 μ M oxaliplatin or vehicle for further 24 hours. At the end of the experiment, cells were harvested by trypsinization, centrifuged and resuspended in the respective media for a final concentration of 1×10^6 cells per ml. Cell death was measured by erythrosine B exclusion assay. Cell suspension was diluted 1:1 with erythrosin B 0.1% in PBS (Sigma-Aldrich, Cat. #: 200964), then cells were counted in a Bürker chamber, and percentage of cell death was calculated as the number of Erythrosin B-positive cells with respect to the total number of cells.

Muse cell analyzer

The Muse cell analyser is a fluorescent-based microcapillary cytometer for single cell analysis (Merck Millipore). The Muse viability assay kit (Merck Millipore, Cat. #: MCH100102) uses a single reagent containing 2 fluorescent dyes, which intercalates DNA molecules. One dye is cell permeable, and stains all nucleated cells, allowing to discriminate cells from debris; whereas the second dye stains only cells with compromised membrane, giving a measurement of cells that are dead or are dying.

For cell death measurement, cells were seeded in 12-well plates at a concentration between 20'000 to 80'000 cells according to the cell line, so that at the moment of vitamin C treatment, cells reach 40% of confluence. 24 hours after seeding, cells were rinsed twice in PBS and then grown in CTR or STS medium. After 24 hours, media were refreshed to ensure that glucose and serum levels were not completely exhausted, and after medium pH stabilization at 37 °C and 5% CO₂ atmosphere, cells were treated with 350 µM vitamin C or vehicle (deionized water) for the next 24 hours. For experiments with anti-oxidant agents, cells were treated with 5 mM glutathione, and 5 mM N-acetyl cysteine, together with vitamin C. For experiments with desferrioxamine, 500 µM desferrioxamine was added 6 hours before vitamin C treatment for HCT116 cells or 12 hours for DLD1 and CT26.WT. After the specific incubation time, cells were washed twice in PBS to evaluate only the intracellular effect of DFO, and then, CTR or STS fresh medium and vitamin C were added for the next 24 hours for HCT116 and DLD1 cells, and 9 hours for CT26.WT. For hydrogen peroxide scavenging experiments, cells were treated with catalase from bovine liver (50 U/ml) 1 minute before vitamin C was provided as previously described (Schoenfeld et al., 2017) or with MnTMPyP, 2 hours before vitamin C administration. For HO-1 activation experiments, cells were treated with hemin at a concentration of 20 µM, 3 hours before vitamin C was provided. For HO-1 inhibition experiments, cells were treated with zinc protoporphyrin (ZnPP) at a concentration of 20 µM, 3 hours before

vitamin C. At the end of the experiment cells were harvested by trypsinization, centrifuged and resuspended in the respective media for a final concentration of 1×10^6 cells per ml. Cell suspension and Muse viability reagent are mixed in 1:10 ratio and after 5 minutes of incubation in the dark, viability was analysed by Muse cell analyser. Data are expressed as percentage of dead cells.

Reactive oxygen species measurement

Intracellular reactive-oxygen species (ROS) were measured using the CellROX oxidative stress reagents (Life Technologies, Cat. #: 10422).

CellROX fluorogenic probe is designed to measure reactive-oxygen species (ROS) in live cells. The probe is cell permeable and in reduced state it is no or weakly fluorescent, whereas upon oxidation it shows a fluorogenic signal. CellROX probe exhibits a fluorescence excitation at 640 nm and fluorescent emission at 665 nm (deep red).

For ROS measurement, cells were seeded in 10 cm petri dish (4×10^5 cells in CTR medium). After 24 hours cells were rinsed twice in 1X PBS and CTR or STS medium was added; 24 hours later, cells were trypsinized, resuspended in their respective media, and treated with 1 mM sodium ascorbate and 1 μ M CellROX deep red reagent in the dark, for 30 minutes at 37°C and 5% CO₂. Then, fluorescence was immediately analyzed by flow cytometry (Attune NxT flow cytometer). Data were processed by Kaluza analysis software (Beckman coulter, version 2.0). Data were expressed as fold change of the median fluorescent intensity (MFI) of each treated sample versus the MFI of control sample.

Intracellular ferrous iron detection

Intracellular ferrous ions were measured using Iron Assay Kit (Cat. #: ab83366). Ferrous ions (Fe^{2+}), but not ferric ions (Fe^{3+}) specifically reacts with ferene-S (an iron chromogen) producing a stable colored complex, whose absorbance is measured at 593 nm.

For Fe^{2+} measurement experiment, cells were seeded in 10 cm petri dish (4×10^5 cells) in CTR medium. After 24 hours cells were rinsed twice in 1X PBS and CTR or STS medium was added; 24 hours later, medium was refreshed and cells were treated with 350 μM vitamin C or vehicle for 3 hours. Next, cells were lysed in iron assay buffer and intracellular ferrous iron level was measured according to manufacturer protocol. Absorbance was recorded at the wavelength of 593 nm using a microplate reader (Infinite M200 TECAN). Data are expressed as fold change versus control sample.

RNA interference with siRNA oligonucleotides

RNA interference was carried out on HCT116 using Lipofectamine RNAiMAX (Invitrogen, Cat. #: 13778150) following supplier's protocol. HCT116 cells were seeded the day before transfection and transfected at 50-60% confluence with the indicated siRNA oligonucleotides (25 nM). The following oligonucleotides were used: ON-TARGETplus human *HO-1* siRNA (pool of four siRNA) and ON-TARGETplus non-targeting pool (as a negative control) (Dharmacon). Knockdown efficiency was assessed by western blot analysis.

Protein extraction and Western blot analysis

Cells were washed twice in ice-cold PBS and lysates were prepared in RIPA lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholic acid, 1 mM EDTA) supplemented with protease and phosphatase inhibitors (protease inhibitor cocktail set III EDTA-free, Calbiochem, Cat. #: S39134; PhosStop, Roche). Samples were sonicated (bioruptor plus, diagenode) and centrifuged at 16.1 g for 30 minutes at 4 °C.

Tumor tissues were collected and snap frozen in liquid nitrogen immediately after mice were sacrificed, and stored in -80 °C until use. For protein extraction, tumors were homogenized with Tissue lyser II (Qiagen) in RIPA buffer supplemented with protease and phosphatase inhibitors and then ultra-centrifuged (45000 rpm using a MLA-130 Beckman rotor) for 1 hour. Protein concentrations were determined by BCA assay (Thermo Fisher Scientific, Cat. #: 23225). Proteins were diluted in 1X Laemmli sample buffer, boiled at 95°C for 5 minutes and 30 µg of total proteins were separated by using SDS–PAGE and analyzed by western blotting by standard procedures. After protein transfer, the nitrocellulose membranes with 0.22 µm pore size (GE Healthcare Life Sciences, Fisher Scientific) were blocked by incubation with 5% non-fat dry milk in 1X TBS-Tween (0.1%) for 1 hour at room temperature. Membranes were incubated overnight at 4 °C with gentle shaking with the following primary antibodies: HO-1 (1:1000, Enzo Life Science, Cat. #: ADI-SPA894), FTH (1:1000, Cell Signaling, Cat. #: 3998), AMPK (1:1000, Cell Signaling, Cat. #: 2532), phospho- threonine 172 AMPK (1:1000, Cell Signaling, Cat. #: 2535), eIF2α (1:1000, Cell signaling, Cat. #: 5324), phospho serine 51 eIF2α (1:1000, Cell signaling, Cat. #: 3597), AKT (1:1000, Cell Signaling, Cat. #: 9272), phospho-serine 473 AKT (1:1000, Cell Signaling, Cat. #: 9271), H2AX (1:4000, Abcam, Cat. #: ab11175), phospho serine 139 H2AX (1:5000, Merck Millipore, Cat. #: 05636), Vinculin (1:10000, Sigma-Aldrich, Cat. #: V9131), β-actin (1:3000, Sigma, Cat. #: A2066).

Next, membranes were washed 3 times (5 minutes each) with TBS-Tween (0.1%) and then incubated for 1 hour at room temperature with the following secondary antibodies linked to horseradish peroxidase (Biorad): anti-Mouse (1:3000, Cat. #: 170-6516) and anti-Rabbit (1:3000, Cat. #: 170-6515). After 3 washes in TBS-Tween, immunostained bands were detected under a ChemiDoc imaging system (Biorad) using the chemiluminescent method (super signal west PICO and super signal west DURA, Thermo Fisher, Cat. #: 34577, Cat. #: 34075). Bands intensity was quantified with NIH Image J software (version 1.48v).

RNA extraction, RT-PCR and qRT-PCR

Total RNA was isolated using the miRNeasy Mini Kit (QIAGEN, #217004) according to the manufacturer's instructions. Next, 1 µg of purified RNA was retro-transcribed by using SuperScript Vilo cDNA synthesis kit (Invitrogen, #11754050). Resulting cDNA (1/20 v/v) was analyzed by real-time polymerase reaction (qRT-PCR) using TaqMan MBG probes with FAM reporter dyes. Human target gene primers for heme-oxygenase-1 (HMOX1:Hs01110250_m1, ThermoFisher Scientific) were utilized. Target transcript levels were normalized to those of a reference gene (GAPDH: hs99999905_m1, ThermoFisher Scientific).

***In vivo* procedures**

Mouse models

The animals were housed under specific pathogen-free conditions with 12 hours day/light cycles. All experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and were approved by the Italian Ministry of Health.

For xenograft experiments, 8-weeks old female NOD scid gamma (NSG, Charles River) were subcutaneously injected with 2×10^6 HCT116 cells (NCI 60 panel) resuspended in 100 μ l of PBS. For experiment with chemotherapy, 12-weeks old NOD scid gamma (NSG, Charles River) were subcutaneously injected. For syngeneic model, 8-weeks old female Balbc/Ola Hsd mice (Envigo) were subcutaneously injected with 3×10^5 CT26.WT (ATCC) cells resuspended in 100 μ l of PBS.

When tumors were palpable (approximately 7 days after inoculation), mice were randomly divided in the different experimental groups. Body weights were recorded daily, and tumor volumes were measured every 2-3 days by a digital vernier caliper according to the following equation: tumor volume (mm^3) = (length x width²) x 0.5, where the length and width are expressed in millimetres. At the end of the experiments, mice were euthanized by using CO₂.

Animal diets and treatments

Mice were fed *ad libitum* with irradiated VRFI (P) diet (Charles River) containing 3,89 kcal/g of gross energy.

Our fasting-mimicking diet (FMD) diet is based on a nutritional screen that identified ingredients that allow nourishment during periods of low calorie consumption (Brandhorst et al., 2015). The FMD diet consists of two different components designated as day 1 diet and days 2–4 diet. Day 1 diet contains 7.67 kJ/g (provided 50% of normal daily intake; 0.46 kJ/g protein, 2.2 kJ/g carbohydrate, 5.00 kJ/g fat); the day 2–4 diet contains 1.48 kJ/g (provided at 10% of normal daily intake; 0.01 kJ/g protein/fat, 1.47 kJ/g carbohydrates). Before FMD diet was supplied, mice were transferred in fresh cages to avoid residual chow feeding and coprophagy. Mouse weight was monitored daily and during FMD cycle weight loss did not exceed 20%.

For experiments on tumor growth, mice were fed with standard rodent diet or underwent FMD cycles. FMD was reduced to 3 days because the faster rate of body weight loss of

these mouse strains. In experiments with NSG mice undergoing chemotherapy, the second FMD cycle was reduced to 2 days because of the faster bodyweight loss due to chemotherapy. Before FMD cycle was repeated, mice completely recovered their original bodyweight.

For vitamin C experiments, mice undergoing standard feeding or at the last day of the first FMD cycle started to be treated with vitamin C (4 g/kg in saline) via intraperitoneal injection twice a day, every day until the end of the experiment. At least 6-8 hours have elapsed between the two administrations in each day.

For chemotherapy experiments, mice undergoing standard feeding or at the second day of the first FMD cycle started to be treated with vitamin C twice a day, and during the last day of each FMD cycle (24 hours before refeeding), mice were treated with oxaliplatin (10 mg/kg) via intraperitoneal injection. When mice were treated with chemotherapy, vitamin C injection was skipped. At least 8-9 hours have elapsed between oxaliplatin and vitamin C administrations.

Patients

Data of patient #1 were collected at the San Martino hospital (Genoa, Italy), whereas data of patient #2 and #3 were collected at the Istituto Nazionale dei Tumori (Milan, Italy). Bodyweight, blood and urinary parameters were evaluated with standard procedure before, at the end and 21-28 days after each FMD cycles for patient #2 and #3. Body composition of patient #1 was measured by dual energy X-ray absorptiometry (DEXA).

Human FMD

The human FMD is a plant-based, high nourishment diet designed to generate fasting-like effects, containing vegetable soups, energy bars, energy drinks, chip snacks, chamomile flower tea and a vegetable supplement formula tablet. The human FMD is a 5

days regimen: day 1 of the diet provides 4600 kJ (11% protein, 46% fats, 43% carbohydrate), day 2-5 supplies 3000 kJ (9% protein, 44% fats, 47% carbohydrate) per day.

Statistical analysis

GraphPad Prism v6.0c was used for the analysis of the data and graphic representations. Comparisons between groups were performed with two-sided unpaired Student's *t*-test and p values ≤ 0.05 were considered significant.

One-way ANOVA analysis was used for comparison among multiple groups for mouse experiments. One-way ANOVA analysis was followed by Tukey's test post analysis. P values ≤ 0.05 were considered significant.

For clinical data analysis, Wilcoxon matched-pairs signed rank test was utilized.

For the quantification of signals (western blot bands), NIH Image J software (version 1.48v) was utilized.

All data are represented as mean $\pm$ SEM of at least three independent experiments.

RESULTS

RESULTS

Chapter 1

Fasting-Mimicking diet potentiates vitamin C efficacy in KRAS mutant colorectal cancer

1.1 KRAS mutant CRC cells are sensitive to pharmacological vitamin C toxicity

A growing body of studies showed that high dose vitamin C is selectively toxic to cancer cells, without affecting normal tissues; thus, representing an important opportunity for a safe and well tolerated anti-cancer treatment (Schoenfeld et al., 2017). Moreover, it has been found that KRAS mutant cancers show a higher susceptibility to pharmacological doses of vitamin C compared to KRAS wild type tumors; however, its therapeutic potential result to be limited (Yun et al., 2015; Padayatty et al., 2010; Ma et al., 2014).

The goal of my thesis, was to identify an intervention that could cause a major potentiation of the toxicity of vitamin C to cancer cells without causing toxicity to normal cells. I used as an *in vitro* model system the human KRAS mutant (G13D) CRC cell line HCT116.

To test vitamin C efficacy, HCT116 cells were grown in complete medium, which mimic physiological level of glucose and serum (1 g/L glucose, 10% serum), indicated as control (CTR) medium. When cells reach 40% confluency, they were treated with

pharmacological dose of vitamin C (≥ 0.3 mM) for 24 hours. As shown in Figure 1, in my experimental condition, high-dose vitamin C was able to induce cell death, as reported in previous published findings (Yun et al., 2015) (Figure 1).

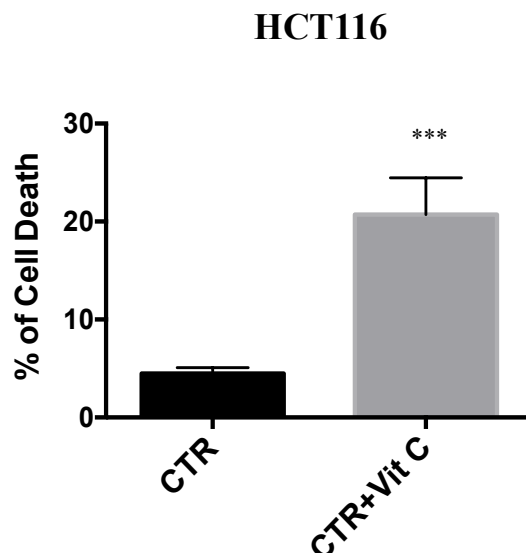


Figure 1. Pharmacological vitamin C induces cell death in HCT116. HCT116 cells were grown in control (CTR) condition medium and were treated with vitamin C (Vit C; 350 μ M) or vehicle for 24 hours. Percentage of cell death was assessed by Muse Cell Viability analyser ($n \geq 5$ biological replicates). Data are represented as mean $\pm$ SEM (two-tailed unpaired t -test; *** p value ≤ 0.001).

1.2 *In vitro* Short-Term Starvation (STS) sensitizes KRAS mutant cancer cells to pharmacological vitamin C toxicity

One of the main goal of our laboratory is to identify novel potential non-toxic interventions, which could be effective for the treatment of different types of cancer, including KRAS-driven tumors. In my thesis project, I aimed at investigating whether our recently developed FMD diet could potentiate the anti-cancer effect of vitamin C in these highly aggressive tumors.

The efficacy of FMD was assessed *in vitro* through a low-glucose and low-serum cell-growing medium, referred as short-term starvation condition (STS), which mimics the

glucose and growth factor reduction mediated by fasting/FMD in *in vivo* physiological setting.

To this purpose, human (HCT116, DLD-1) and murine (CT26.WT) KRAS mutant CRC cell lines grown in CTR or STS condition, were treated with pharmacological dose vitamin C (≥ 0.3 mM) for 24 hours. In my experimental system, I found that KRAS mutant CRC cells grown in CTR medium showed a modest sensitivity to vitamin C (Figure 2). However, when cells were grown in STS condition, 24 hours before and during treatment, vitamin C-cytotoxicity underwent a major enhancement leading to a dramatic increase in cell death (nearly 100%) in all the analysed cell lines (Figure 2).

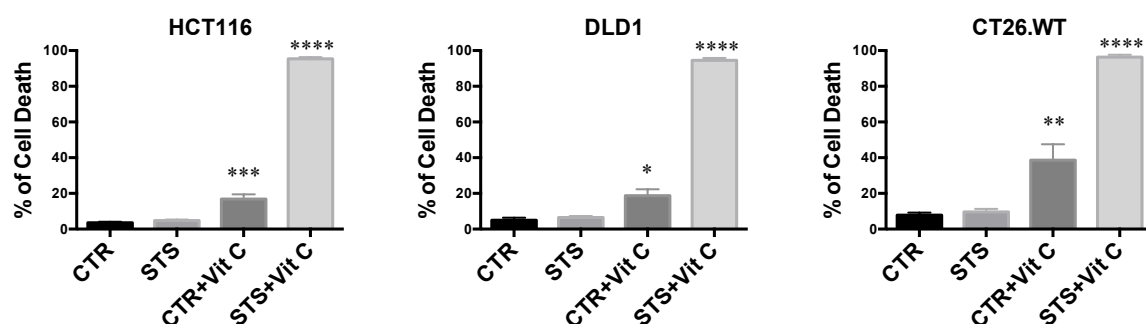


Figure 2. Synergistic toxicity of STS and vitamin C in KRAS mutant colorectal cancer cells. KRAS mutant colorectal cancer cells (HCT116, DLD1, CT26.WT) were grown in CTR or STS conditions for a total of 48 hours. At 24 hours, cells were treated with vitamin C (Vit C; 350 μ M) or vehicle for further 24 hours. At 48 hours, viability was assessed by Muse Cell viability analyser ($n \geq 5$ biological replicates). Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test, ***p* value ≤ 0.01 , *****p* value ≤ 0.0001).

Next, to investigate whether the STS-mediated sensitization to vitamin C toxicity is peculiar of KRAS-driven tumors, I extended my analysis to KRAS mutant cell lines from different tumor types, including lung cancer (H23 and H27) and pancreatic cancer (PANC1) (Figure 3). Notably, the dramatic cell death increase upon combination of STS condition and vitamin C treatment was confirmed in all KRAS mutant cell lines tested, whereas vitamin C treatment alone showed only modest results, with the exception of PANC1 pancreatic cells line (Figure 3).

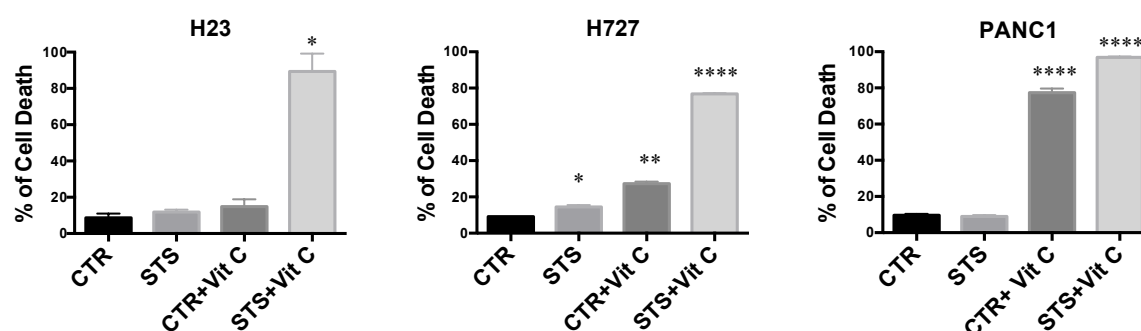


Figure 3. Synergistic toxicity of STS and vitamin C in KRAS mutant lung and pancreatic cancer cells. KRAS mutant, lung cancer (H23, H727) and pancreatic cancer (PANC1), were grown in CTR or STS conditions for a total of 48 hours. At 24 hours, cells were treated with vitamin C (Vit C; 350 μ M) or vehicle for further 24 hours. At 48 hours, viability was assessed by Muse Cell viability analyser ($n \geq 3$ biological replicates). Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test; **p* value ≤ 0.01 , ***p* value ≤ 0.01 , *****p* value ≤ 0.0001).

Importantly, KRAS wild type cell lines derived from colorectal cancer (SW48, HT29), prostate cancer (PC-3), and ovarian cancer (COV362), were not sensitive to vitamin C toxicity, either as single agent, or in combination with STS condition (Figure 4).

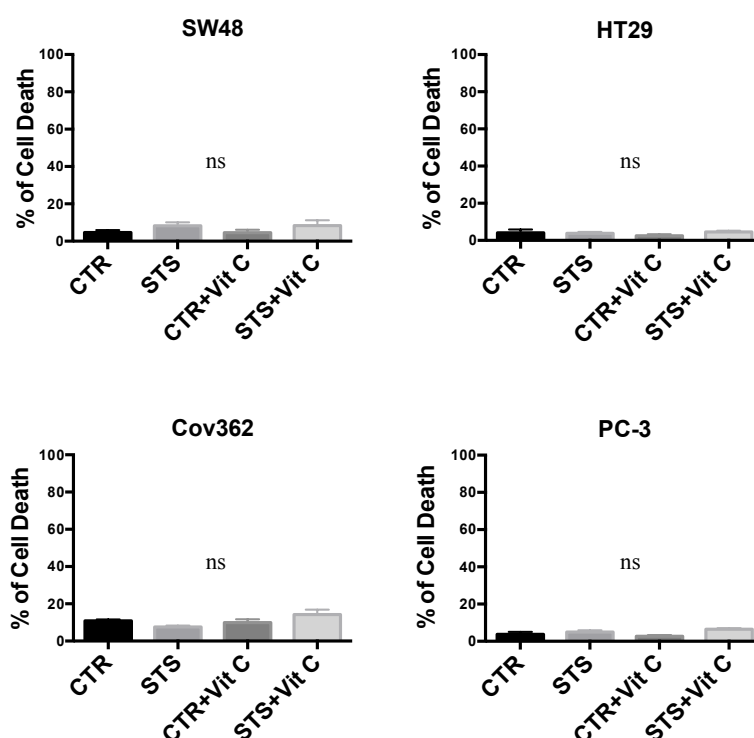


Figure 4. STS and vitamin C combinatorial treatment is not effective in KRAS wild type cancer cells. Figure legend next page.

Figure 4. STS and vitamin C combinatorial treatment is not effective in KRAS wild type cancer cells. KRAS wild type colorectal cancer (SW48, HT29), ovary cancer (Cov362) and prostate cancer (PC-3) cells were grown in CTR or STS conditions for a total of 48 hours. At 24 hours, cells were treated with vitamin C (350 μ M) or vehicle for further 24 hours. At 48 hours, viability was assessed by Muse Cell viability analyser ($n \geq 3$ biological replicates). Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test; ns, not significant).

Finally, in order to evaluate whether this combinatorial treatment could be toxic for normal cells, I tested normal colon (CCD841CoN) and fibroblast (BJ) cell lines. As show in Figure 5, STS and vitamin C or their combination did not induce cell death in CCD841CoN and BJ fibroblasts (Figure 5).

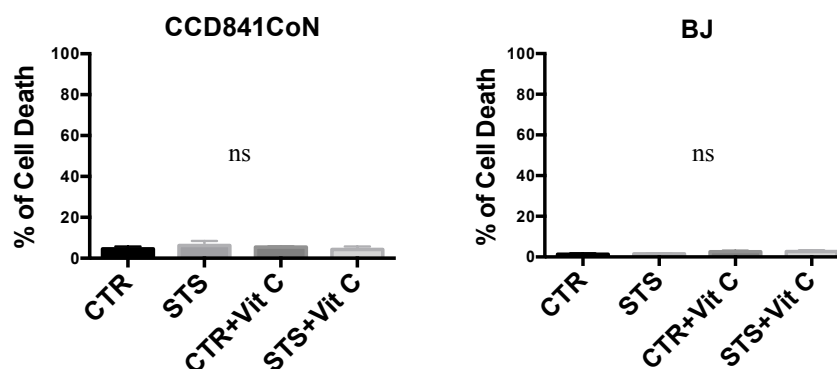


Figure 5. STS and vitamin C combinatorial treatment is not toxic for normal cells. Normal colon cells (CCD841CoN) and normal fibroblasts (BJ) were grown in CTR or STS conditions for a total of 48 hours. At 24 hours, cells were treated with vitamin C (Vit C; 350 μ M) or vehicle for further 24 hours. At 48 hours, viability was assessed by Muse Cell viability analyser ($n \geq 3$ biological replicates). Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test; ns, not significant).

1.3 FMD or fasting cycles delay tumor progression and enhance vitamin C anti-cancer effect in KRAS mutant CRC mouse models

Supported by my *in vitro* results, I investigated whether fasting or FMD cycles could enhance vitamin C toxicity also in mouse models of KRAS mutant colorectal cancer. FMD is a low calories, protein and sugar but high unsaturated fat diet, which is able, as fasting, to delay tumor progression and sensitize cancer cells to chemotherapy (Brandhorst et al., 2015; Di Biase et al., 2016).

First, I tested the effect of complete fasting in combination with high dose vitamin C on human KRAS-driven colorectal cancer cells *in vivo*. For this purpose, nod scid mice (NSG) bearing HCT116 subcutaneous tumors, were fed *ad libitum* with standard rodent diet or fasted (water only) for two days every week.

It has been already described that the parenteral administration mediated by intraperitoneal injection, allows to reach high-dose vitamin C (3 mM), which is necessary to obtain the anti-cancer effect (Chen et al., 2007; Chen et al., 2008). Thus, mice were treated with saline or high dose vitamin C (4 g/kg) intraperitoneally (i.p.). twice a day, every day, as previously described (Chen et al., 2007; Chen et al., 2008).

My results show that three cycles of fasting were as effective as pharmacological vitamin C in reducing tumor progression, however when fasting cycles were combined with vitamin C the anti-cancer effect was significantly enhanced (Figure 6).

Human CRC (HCT116)

Day 23

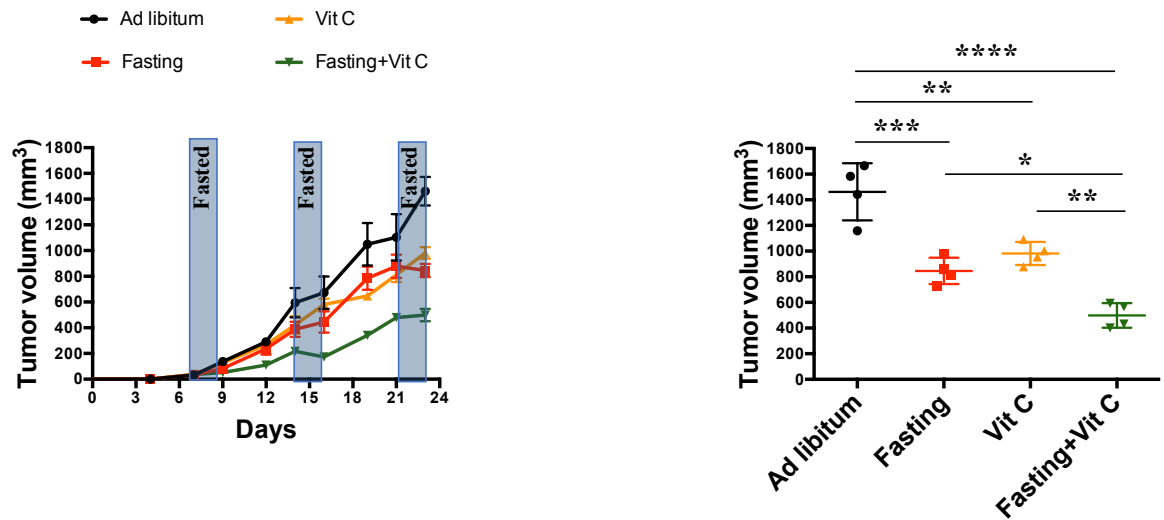


Figure 6. Fasting cycles enhances vitamin C toxicity in reducing tumor progression in HCT116 xenograft. 8-weeks old female NOD scid gamma (NSG) mice were subcutaneously injected with HCT116 cells and subjected to 3 cycles of fasting (2 days, water only) alone or in combination with high dose vitamin C (Vit C; 4 g/kg twice a day, i.p.). Tumor volumes at multiple time points (left panel) and before mice were euthanized (right panel) are reported (n = 4 per group). Data are represented as mean $\pm$ SEM. One-way Anova (Tukey's post-analysis test) was performed (*p value $\leq$ 0.05, **p value $\leq$ 0.01, ***p value $\leq$ 0.001, ****p value $\leq$ 0.0001).

However, fasting remains a challenging option for cancer patients, thus encouraging the pre-clinical and clinical use of FMD, which represents a more feasible therapeutic intervention (Brandhorst et al., 2015; Wei et al., 2017).

Thus, I tested whether FMD is as effective as fasting in sensitizing KRAS CRC cells to vitamin C toxicity. Interestingly, I found that, as for water-only fasting, 3-days of a FMD every week, delayed tumor progression to the same extent as high-dose vitamin C in HCT116 tumor bearing mice (Figure 7). Notably, three cycles of FMD combined with daily treatment of pharmacological vitamin C, showed the best therapeutic outcome in reducing tumor progression by 3-fold compared to mice fed *ad libitum*, and by 2-fold compared to mice undergoing FMD or vitamin C (Figure 7).

Human CRC (HCT116)

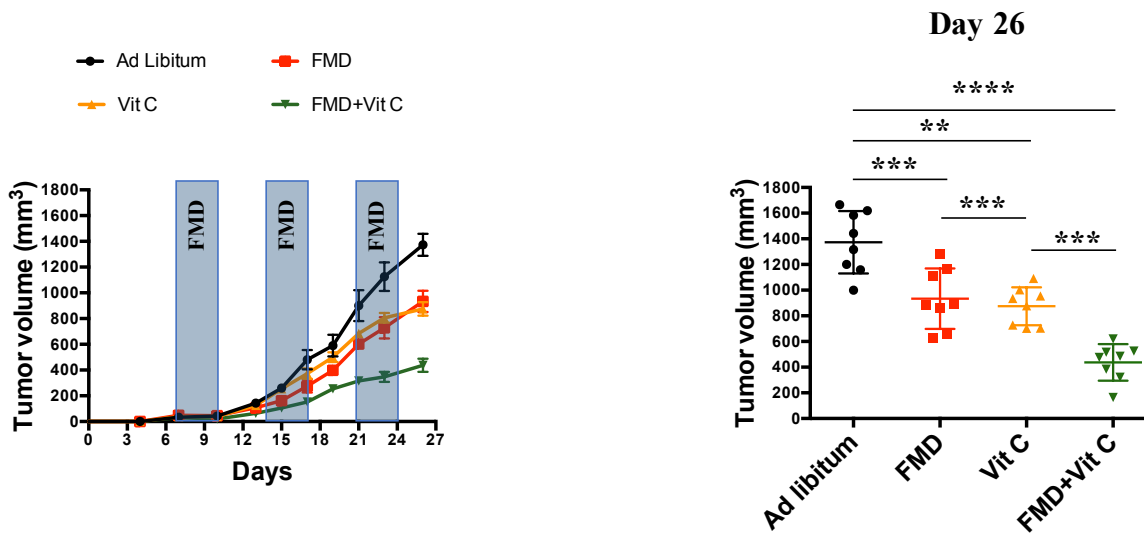


Figure 7. A Fasting-Mimicking Diet (FMD) enhances vitamin C toxicity to reduce tumor progression in HCT116 xenograft. 8-weeks old female NOD scid gamma (NSG) mice were subcutaneously injected with HCT116 cells and subjected to 3 cycles of FMD alone or in combination with high dose vitamin C (Vit C; 4 g/kg twice a day, i.p.). Tumor volumes at multiple time points (left panel) and before mice were euthanized (right panel) are reported (n = 8 per group). Data are represented as mean $\pm$ SEM. One-way Anova (Tukey's post-analysis test) was performed (**p value $\leq$ 0.01, ****p value $\leq$ 0.0001).

Next, I assessed the effect of FMD and vitamin C combo treatment also in a syngeneic mouse model of KRAS-driven CRC. In mice bearing CT26.WT allograft, FMD and vitamin C combinatorial treatment showed a higher efficacy than FMD or vitamin C as single intervention, by reducing tumor volume by 6-fold compared to mice fed ad libitum, 2.6-fold compared to mice undergoing FMD and 3.4-fold compared to mice receiving vitamin C as single treatment (Figure 8).

Importantly, the finding that FMD and vitamin C combinatorial therapy is effective in both immunocompromised and immunocompetent mouse models, suggests that at least part of the observed effect is not dependent on the immune system but it involves a tumor specific component.

Murine CRC (CT26.WT)

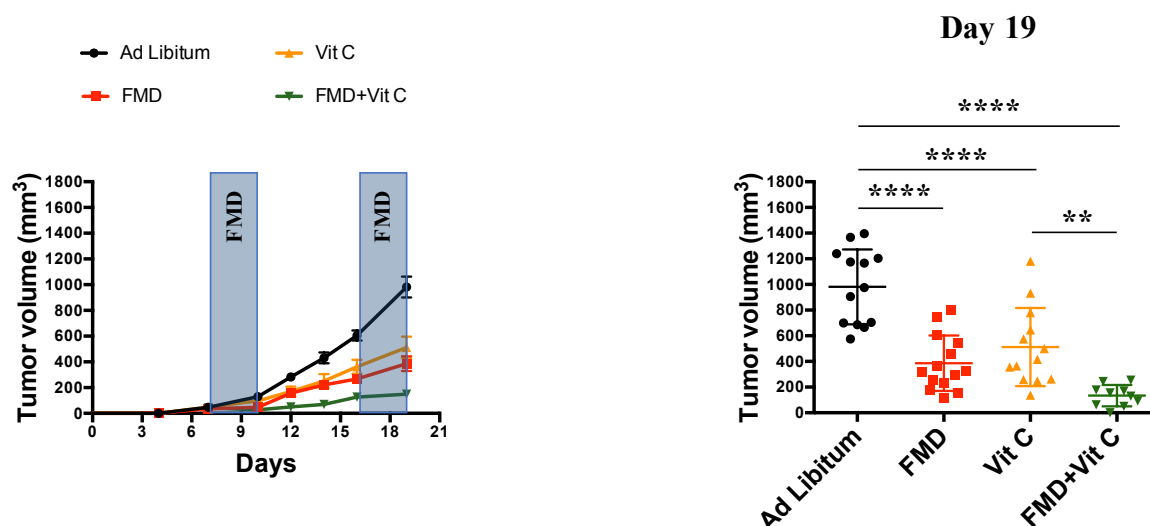
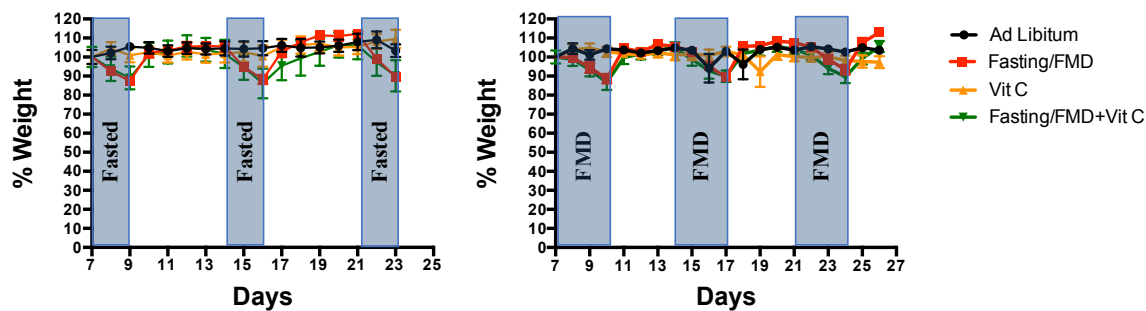


Figure 8. A Fasting-Mimicking Diet (FMD) enhances vitamin C toxicity in reducing tumor progression in CT26.WT allograft. 8-week old female Balb/c mice were subcutaneously injected with CT26.WT cells and subjected to 2 cycles of FMD alone or in combination with high dose vitamin C (Vit C; 4 g/kg twice a day, i.p.). Tumor volumes at multiple time points (left panel) and before mice were euthanized (right panel) are reported (n = 10-14 per group). Data are represented as mean $\pm$ SEM. One-way Anova (Tukey's post-analysis test) was performed (*p value $\leq$ 0.05, **p value $\leq$ 0.01, ****p value $\leq$ 0.0001).

Furthermore, FMD and vitamin C combinatorial treatment resulted to be safe and well tolerated in both mouse strains, as indicated by mouse bodyweight (Figure 9). During each FMD cycle, mice did not lose more than 20% of their initial weight, which is immediately recovered upon refeeding (Figure 9). Cotreatment with vitamin C did not enhance FMD-dependent bodyweight loss and did not impair its recovery after each FMD cycle (Figure 9).

A Nod scid gamma (NSG) strain



B Balb/c strain

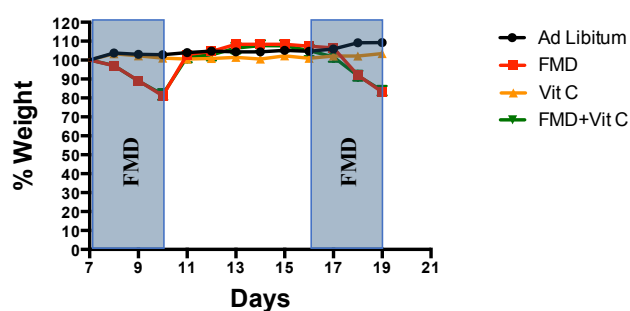


Figure 9. FMD and vitamin C is safe and well tolerated by two different mouse strains. (A) Bodyweight of nod scid mice (NSG) bearing HCT116 derived tumors undergoing 2-days complete fasting (water only) or 3-days FMD alone or in combination with vitamin C (Vit C; 4g/kg twice a day, i.p.). **(B)** Bodyweight of Balb/c OlaHsd mice bearing CT26.WT-derived tumors undergoing 3-days FMD alone or in combination with vitamin C (Vit C; 4 g/kg twice a day, i.p.). Bodyweight was recorded daily and it is indicated as percentage of weight at day 7.

Chapter 2

FMD-mediated sensitization to pharmacological vitamin C acts through ROS generation

2.1 STS and vitamin C combo treatment induces marker of metabolic stress

Our work has previously shown that STS sensitizes CRC cells to chemotherapy-associated oxidative damage in part by exerting an “anti-Warburg” effect (Bianchi et al., 2015). Glycolysis and glutaminolysis reduction, together with the enhancement of OXPHOS uncoupled from energy production, lead to the inhibition of ATP synthesis and an increase in ROS levels (Bianchi et al., 2015).

To explore whether energetic impairment and oxidative stress could be involved in STS-mediated cell death induction upon vitamin C treatment, I evaluated the phosphorylation level of the AMP-activated protein kinase (AMPK). AMPK represents a sensor of energy levels, which is activated by phosphorylation upon reduction of ATP/AMP ratio (Hardie et al., 2012). Interestingly, I found that STS in combination with vitamin C, strongly up-regulated AMPK phosphorylation level, indicating a possible presence of energetic stress (Figure 10). Importantly, in addition to adenylate pool alteration, AMPK results to be activated in response to pro-oxidant conditions, such as increases of ROS levels (Cardaci et al., 2012). It has been shown that sustained AMPK activation induces cell death through, at least in part, the downregulation of AKT activity (Tzatsos and Tsiachlis, 2007).

Phospho-AMPK Protein level (HCT116)

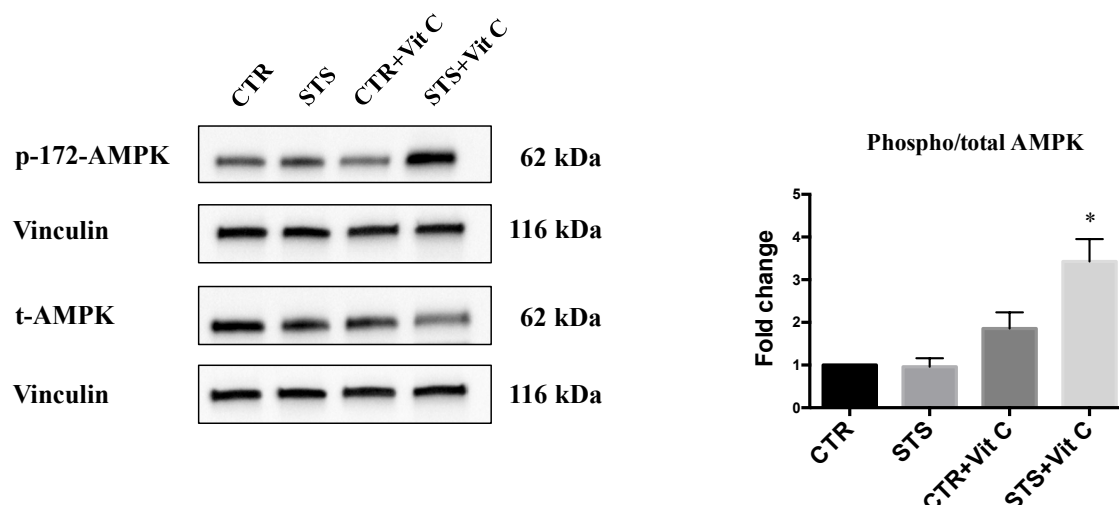


Figure 10. STS and Vitamin C combo treatment up-regulates AMPK phosphorylation level. HCT116 cells were grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M). AMPK and phospho-AMPK (threonine 172) protein expression levels were measured by western blotting. Vinculin as loading control. Representative bands of three biological replicates are shown (quantification on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed (**p* value $\leq$ 0.05).

AKT is a serine-threonine kinase involved in the control of different biological processes, including metabolism, proliferation and survival, in response to several growth factors (Manning and Cantley, 2007). Importantly, we have previously shown that STS exerts its effects by affecting pro-growing signalling pathways, such as PI3K/AKT pathway, which in turn contribute to metabolic reprogramming (Bianchi et al., 2015).

AKT activity, measured by its phosphorylation level, was downregulated upon STS. More importantly, when combined with vitamin C, this effect was potentiated even further (Figure 11).

Phospho-AKT Protein level (HCT116)

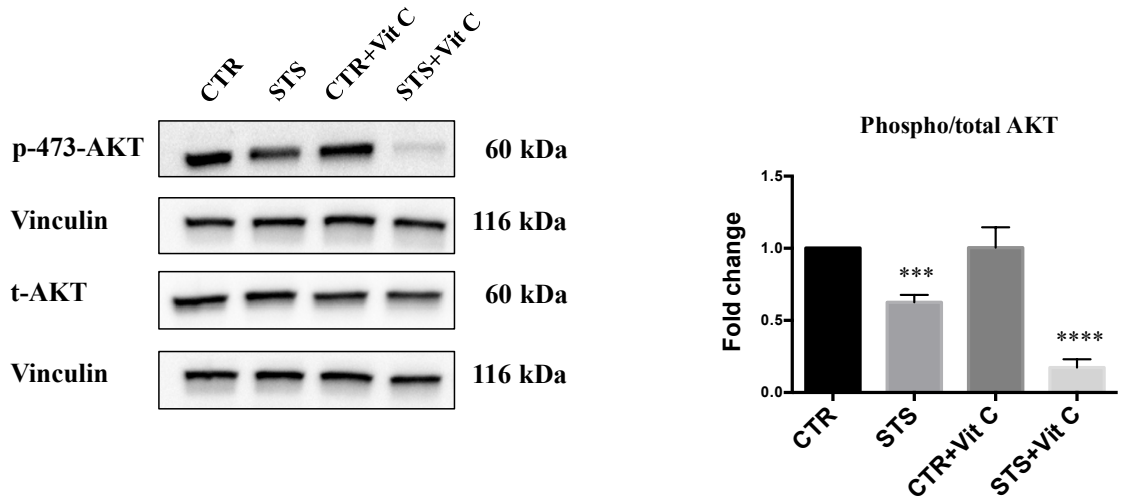


Figure 11. STS and Vitamin C combo treatment down-regulates AKT phosphorylation level.

HCT116 cells were grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M). AKT and phospho-AKT (serine 473) protein expression level were measured by western blotting. Vinculin as loading control. Representative bands of three biological replicates are shown (quantification on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed, (***)*p* value $\leq$ 0.001, (****)*p* value $\leq$ 0.0001).

In agreement with a reduction of the pro-growth PI3K/AKT pathway, STS and vitamin C combo treatment significantly induced the phosphorylation level of the eukaryotic initiation factor 2 alpha (eIF2 α) (Figure 12), which is a marker of global protein synthesis inhibition (Kimball, 1999).

Phospho-eIF2 α Protein level (HCT116)

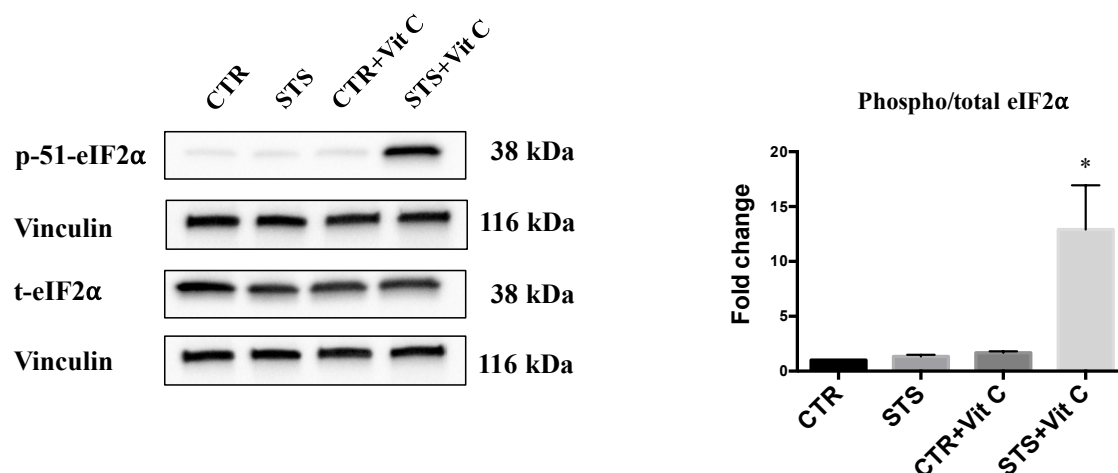


Figure 12. STS and Vitamin C combo treatment up-regulates eIF2 α phosphorylation level. HCT116 cells were grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M). eIF2 α and phospho-eIF2 α (serine 51) protein expression level were measured by western blotting. Representative bands of three biological replicates are shown (quantification on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired Student's *t*-test was performed (**p* value $\leq$ 0.05, ***p* value $\leq$ 0.01).

Taken together, these results indicate that the combinatorial treatment causes a dramatic cell death induction in part by inhibiting crucial processes for viability.

2.2 Oxidative stress is the causative factor for FMD and vitamin C combo treatment toxicity in KRAS mutant CRC cells

The metabolic stress induction, indicated by the alteration of AMPK and AKT levels, prompted me to investigate whether ROS generation could be involved. ROS, which include H₂O₂ and superoxide (O₂^{•-}), are generated as by-products of normal metabolisms. However, excessive ROS production, known as oxidative stress, have detrimental effects on cellular biomolecules, including DNA, lipids and proteins (Reczek and Chandel, 2017).

The observation that STS and vitamin C combo treatment strongly induced DNA damage, measured by the phosphorylation level of the histone H2AX, further support the hypothesis that oxidative stress participates in mediating the cytotoxic effect (Figure 13).

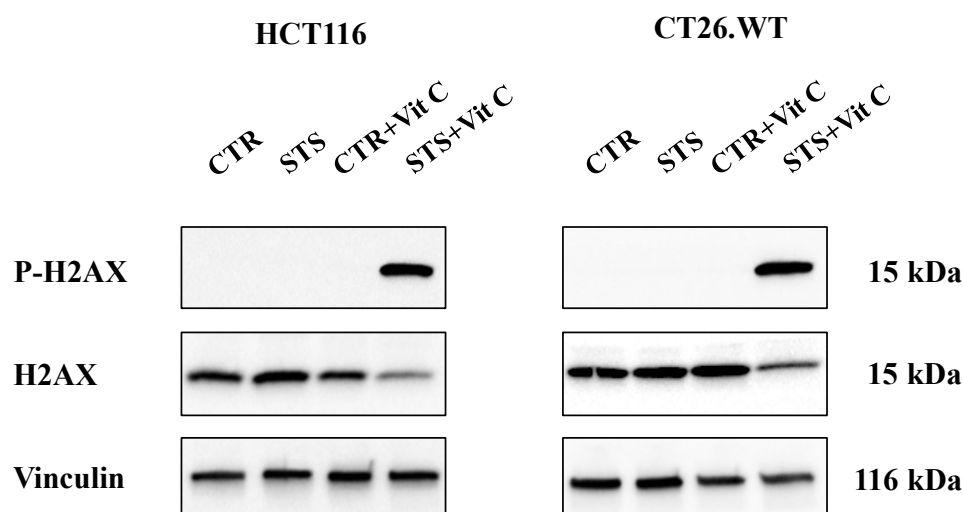


Figure 13. STS and vitamin C co-treatment induces phosphorylation of histone H2AX. Phosphorylated histone H2AX and total level of H2AX in HCT116 and CT26.WT grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M), were measured by western blotting, representative bands of three independent experiments are shown. Vinculin as loading control.

To explore this hypothesis, ROS levels were measured as indicated by the oxidation of the fluorogenic probe CellROX, in HCT116 cells grown in CTR or STS condition. As show in figure 14, STS was able to increase ROS level by nearly 2-fold compared to CTR condition (Figure 14). However, I did not observe induction of DNA damage marker phospho-H2AX or cell death in STS condition as single intervention, thus indicating that the increased ROS level are handled by KRAS mutant cells (Figure 13). In order to respond to oxidative stress and prevent oxidative damages normal and cancer cells express a large array of anti-oxidant enzymes (Kimmelman, 2015).

In collaboration with Dr. Paolo Soffientini (IFOM, Milan), we performed a SILAC (stable isotope labeling with amino acids in cell culture) coupled to LC-MS/MS (Liquid chromatography - mass spectrometry/ mass spectrometry) analysis on HCT116 cells grown

in CTR or STS condition (Appendix, Figure 1). Briefly, HCT116 cells that have incorporated light or heavy isotope labelled arginine and lysine, were cultured in CTR and STS medium. After labelling, light and heavy isotope labelled proteins are mixed and analysed by LC-MS/MS. The relative abundance of proteins in each condition was measured from the relative intensity of the light and heavy peptides.

We checked the expression of different antioxidant enzymes in HCT116 cells grown in an FMD-like condition and we observed an increase in the expression of GSTO1, PRDX2, PRDX5, APOM, MGST1, NQO1 and SOD1 (Appendix table 1), which may suggest a response to the increase in ROS levels. Gene ontology analysis on proteins up-regulated in STS condition reveals also a significative enrichment in molecular processes involved in anti-oxidant response (oxidoreductase activity, acting on CH-CH group of donors [GO:0016628], $p = 0,001496$; oxidoreductase activity, acting on CH-OH group of donors [GO:0016616], $p = 0,005658$; oxidoreductase activity, acting on the aldehyde or oxo group of donors [GO:0016620], $p = 0,03018$).

These findings, together with the extensively demonstrated role of vitamin C pro-oxidant action (Chen et al., 2008; Yun et al., 2015), raise the possibility that a high level of oxidative stress may be involved in FMD-mediated sensitization to vitamin C.

To explore this hypothesis, ROS levels were measured in HCT116 cells grown in CTR or STS condition, in presence or absence of vitamin C. Notably, my results indicate that STS as single intervention was able to increase ROS levels, as well as vitamin C. However, the combinatorial treatment strongly exacerbated cellular oxidative state by 3.6-fold (Figure 14).

HCT116

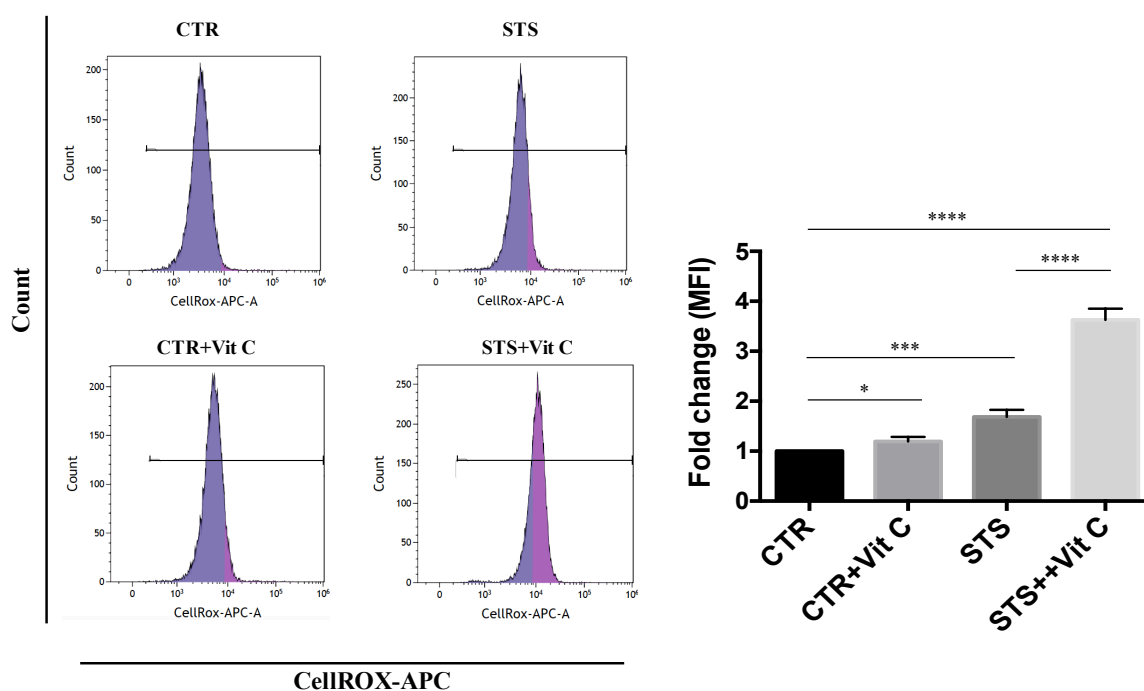


Figure 14. STS increases cellular ROS levels and enhances vitamin C pro-oxidant effect. HCT116 were grown in CTR or STS condition for 24 hours and then co-treated with vitamin C (Vit C; 1 mM) and the ROS-staining fluorescent probe CellROX Deep Red for 30 minutes. Median fluorescence intensity (MFI) was measured by flow cytometry ($n \geq 3$ biological replicates). Representative histogram (left panel) and quantification (right panel) were shown. Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed value (**p* value ≤ 0.05 , ****p* value ≤ 0.001 , *****p* value ≤ 0.0001).

To directly test whether high ROS level is a causative or a secondary event in STS-induced sensitization to vitamin C, KRAS mutant CRC cells (HCT116, DLD1, CT26.WT) grown in STS condition and exposed to vitamin C, were co-treated with glutathione (GSH), which is the major cellular antioxidant, as well as with the cell-permeable reducing agent and glutathione precursor N-acetyl cysteine (NAC). Confirming previous findings (Yun et al., 2015), both agents rescued vitamin C cytotoxic effect in CTR medium. Interestingly, GSH and NAC were also able to revert the massive cell death induction mediated by STS and vitamin C cotreatment, suggesting that STS may act by exacerbating the pro-oxidant action of vitamin C (Figure 15). Accordingly, GSH cotreatment fully reversed the increase in ROS mediated by STS and STS + vitamin C in HCT116 cell line (Figure 16). These

findings support the hypothesis that ROS production could represent the main route through which STS acts.

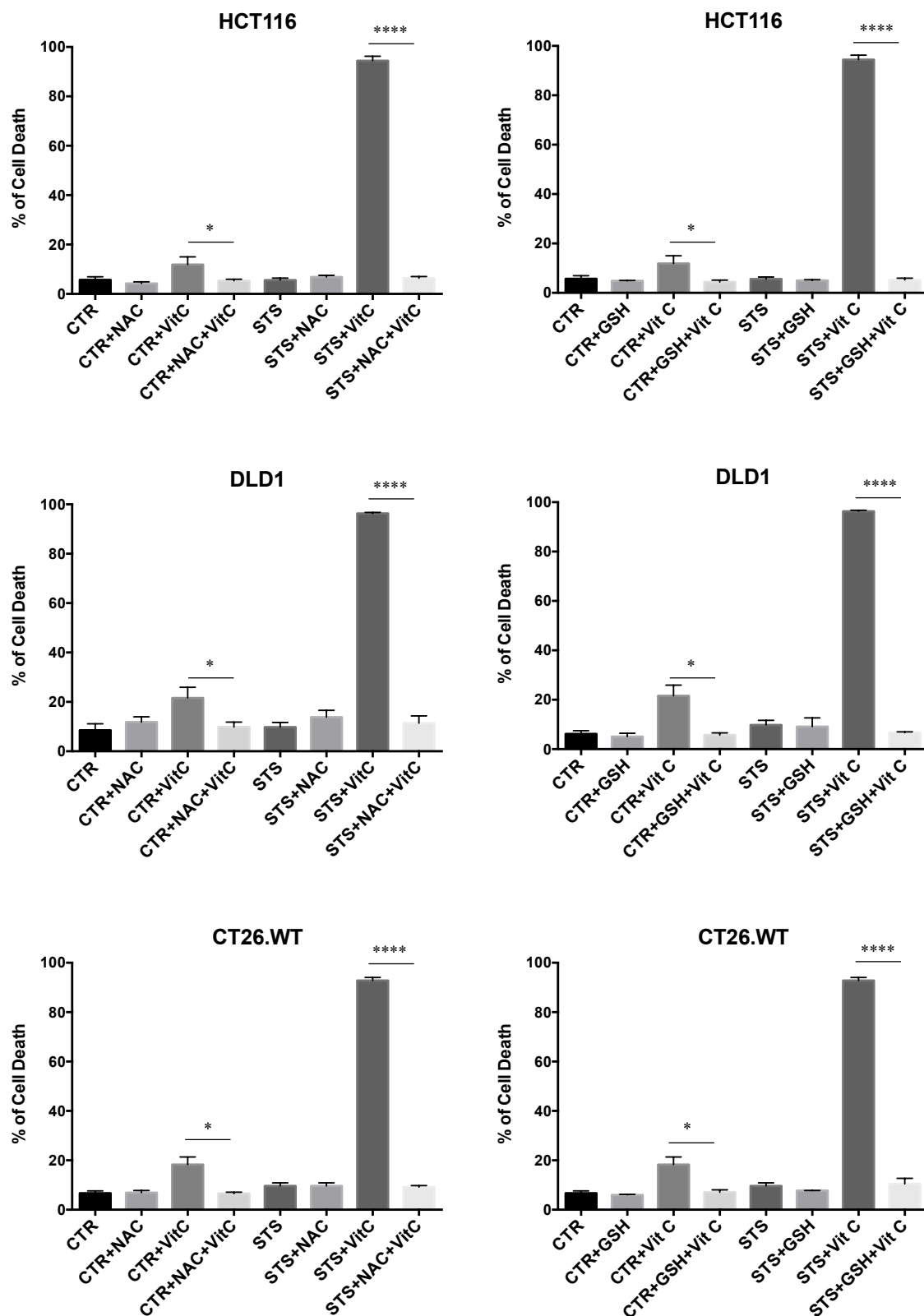


Figure 15. GSH and NAC reverse STS + vitamin C toxicity in KRAS mutant CRC cells.

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Figure 15. GSH and NAC reverse STS+vitamin C toxicity in KRAS mutant CRC cells. HCT116, DLD1 and CT26.WT, were grown in CTR or STS condition for 24 hours. Then, cells were co-treated with the antioxidants glutathione (GSH; 5 mM) and glutathione precursor N-acetyl cysteine (NAC; 5 mM) and vitamin C (Vit C; 350 μ M, for 24 hours). At 48 hours, viability was assessed by Muse Cell viability analyser ($n \geq 3$ biological replicates). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed value (**p* value ≤ 0.05 , *****p* value ≤ 0.0001).

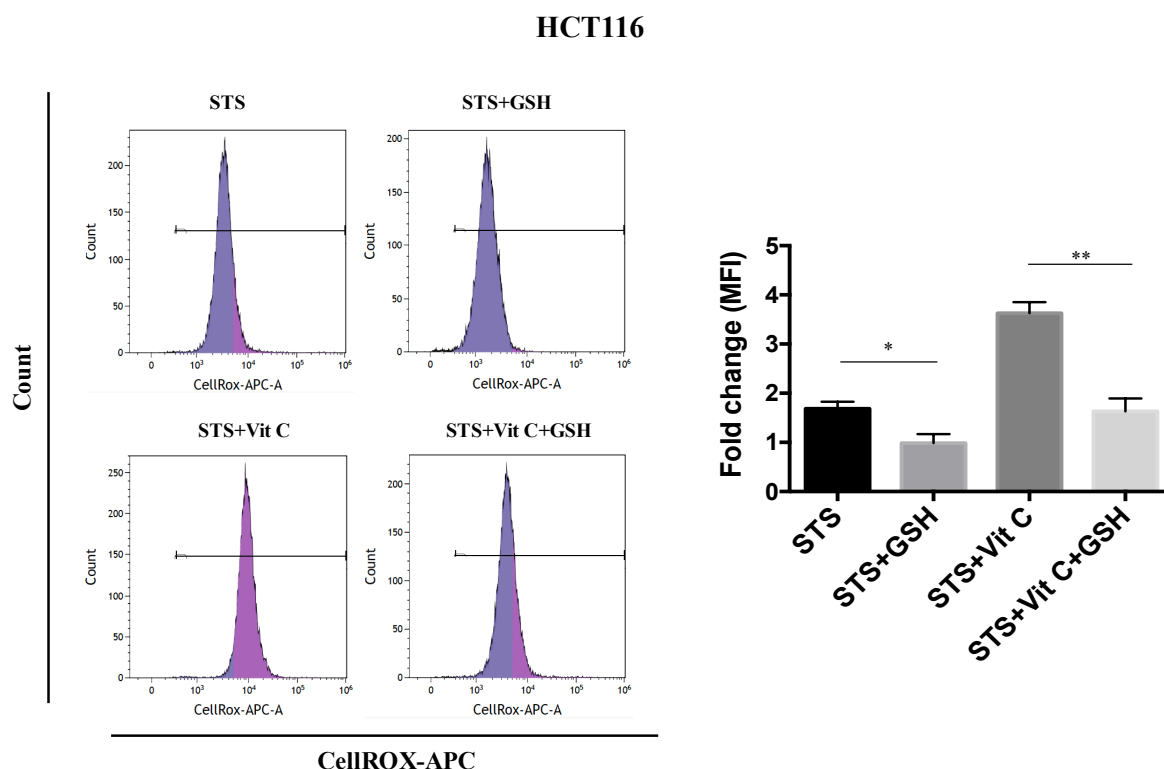


Figure 16. GSH reverses STS and STS + vitamin C-mediated increase in cellular oxidative stress in HCT116. HCT116 were grown in CTR or STS condition for 24 hours and then treated with GSH one hour prior to vitamin C treatment. Next, cells were cotreated with vitamin C (Vit C; 1 mM) and the ROS-staining fluorescent probe CellROX Deep Red for 30 minutes. Median fluorescence intensity (MFI) was measured by flow cytometry ($n \geq 3$ biological replicates). Representative histogram on the left, quantification on the right. Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed value (**p* value ≤ 0.05 , ****p* value ≤ 0.001).

Next, to further assess whether oxidative stress is the causative event in mediating STS + vitamin C cytotoxicity, I tested the effect of exogenous membrane-impermeable catalase, a well-known ROS scavenger able to specifically decompose hydrogen peroxide to water and molecular oxygen (Chelikani et al., 2004). My data show that exogenous catalase

exposure prior vitamin C treatment, was able to revert STS-mediated sensitization to vitamin C (Figure 17 A). In addition, the treatment with the membrane-permeable superoxide dismutase (SOD)/catalase mimetic MnTMPyP, which has been reported to scavenge both superoxide anions and also hydrogen peroxide, before vitamin C exposure, inhibited cell death upon combinatorial treatment (Figure 17 B).

Collectively my data strongly indicate that increased ROS levels are the causative factors responsible for STS-mediated sensitization to vitamin C anti-cancer effect.

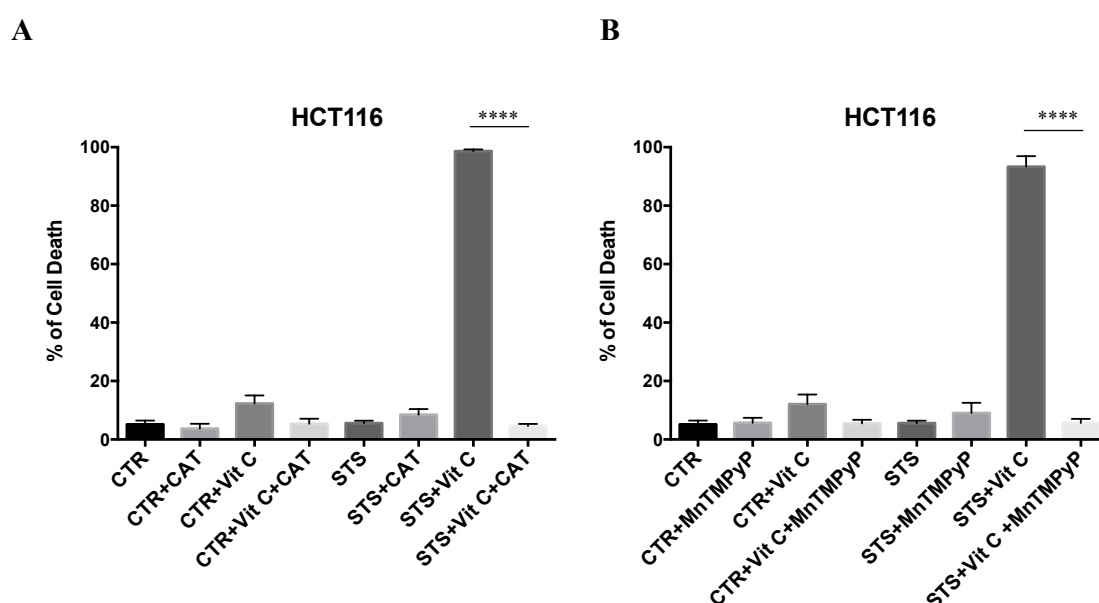


Figure 17. Catalase and MnTMPyP reverse STS-mediated sensitization to vitamin C toxicity in HCT116 cells. HCT116 were grown in CTR or STS condition for 24 hours and treated with catalase (CAT; 50 U/ml) (A) or the superoxide dismutase (SOD)/catalase mimetic MnTMPyP (MnTMPyP; 50 μ M) (B) prior to vitamin C (Vit C; 350 μ M for 24 hours). At 48 hours, viability was assessed by Muse Cell viability analyser ($n \geq 3$ biological replicates). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed value (*****p* value ≤ 0.0001).

Chapter 3

Alterations in intracellular iron levels induced by FMD are responsible for the enhancement of vitamin C efficacy

3.1 STS and vitamin C cotreatment increases intracellular free ferrous ions

A large body of studies shows that the mechanism of vitamin C anti-cancer effect is mediated by hydrogen peroxide production (Chen et al., 2008; Schoenfeld et al., 2017). Particularly, its toxicity is dependent on metal ion redox chemistry, which allows the generation of the hydroxyl radical, through the Fenton reaction (Chen et al., 2008; Schoenfeld et al., 2017).

Schoenfeld and colleagues recently reported that ROS-induced disruption of iron metabolism, significantly contributes to vitamin C toxicity in tumors, by increasing the redox-active labile iron pool involved in the Fenton reaction.

Given the role of FMD and vitamin C in increasing ROS levels, I started to investigate whether the oxidative stress enhancement could correlate with an increase in labile ferrous iron pool. Interestingly, I found that, in HCT116 cells, ferrous ion (Fe^{2+}) level was significantly higher upon STS and vitamin C combo treatment compared to all other conditions (Figure 18), thus suggesting a potential role of iron in mediating STS-sensitization effect to vitamin C treatment.

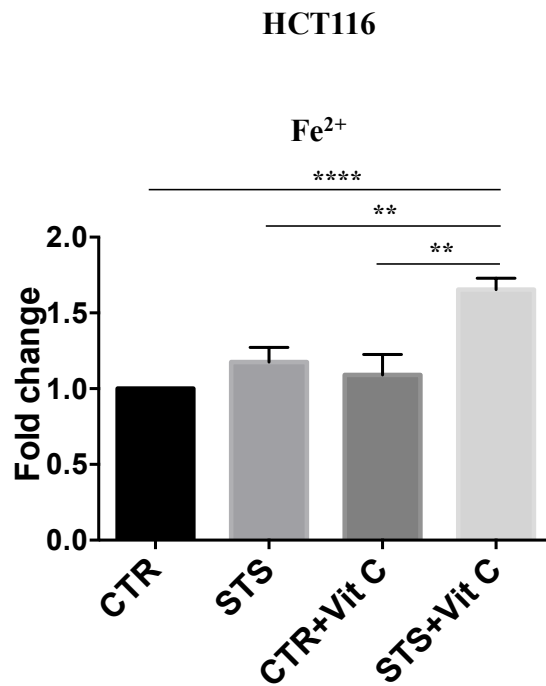


Figure 18. STS and vitamin C combination treatment increases free iron (Fe²⁺) level in HCT116 cells. HCT116 cells were grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M). Then, cells were lysed and free iron (Fe²⁺) was measured by iron assay kit (n = 5 biological replicates). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed (**p value $\leq$ 0.01, ****p value $\leq$ 0.0001).

3.2 FMD and vitamin C combo treatment down-regulates ferritin protein expression level

Ferritin is the main protein involved in iron binding and storage. Several studies have shown its central role in the intracellular modulation of the labile iron pool (LIP) (Kakhlon et al., 2002; Torti and Torti, 2013; Yang and Stockwell, 2008). Particularly, ferritin downregulation has been shown to be responsible for LIP increase in KRAS mutant cancer cells (Kakhlon et al., 2002; Yang and Stockwell, 2008).

Thus, I investigated whether ferritin could be in part involved in STS-mediated increase in ferrous ions observed upon combinatorial treatment. To this purpose, I evaluated the level of the heavy subunit of ferritin (FTH), which is responsible for the iron storage through its ferroxidase activity (Torti and Torti, 2002). Interestingly, I found that STS condition

down-regulated FTH protein expression in human HCT116 (Figure 19) and in murine CT26.WT KRAS mutant cells (Figure 20).

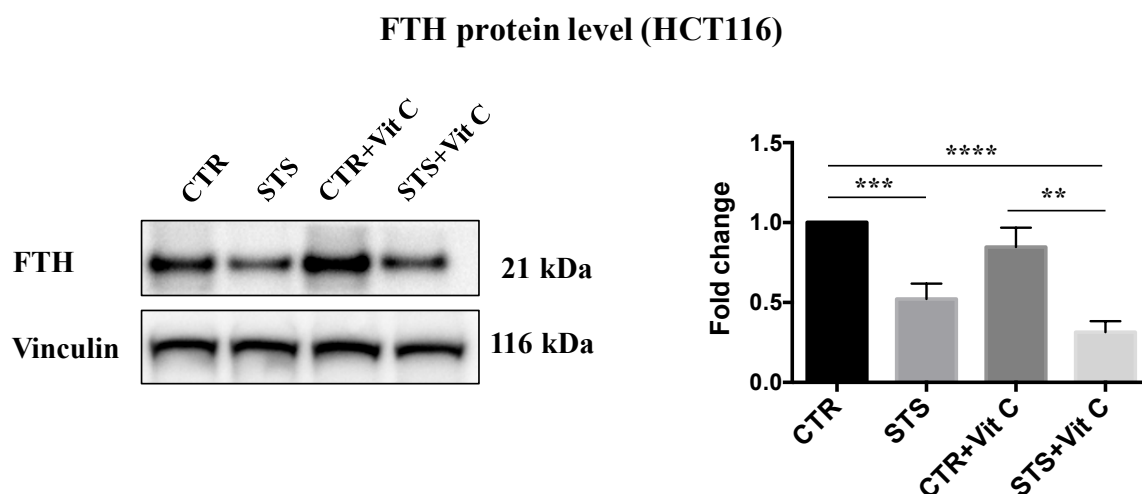


Figure 19. STS and vitamin C combination treatment reduces FTH protein expression level in HCT116 cells. FTH protein expression level in HCT116 grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M), were measured by western blotting ($n \geq 5$ biological replicates); Vinculin as loading control. Representative bands are shown (quantification on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed (***p* value ≤ 0.01 , ****p* value ≤ 0.001 , *****p* value ≤ 0.0001).

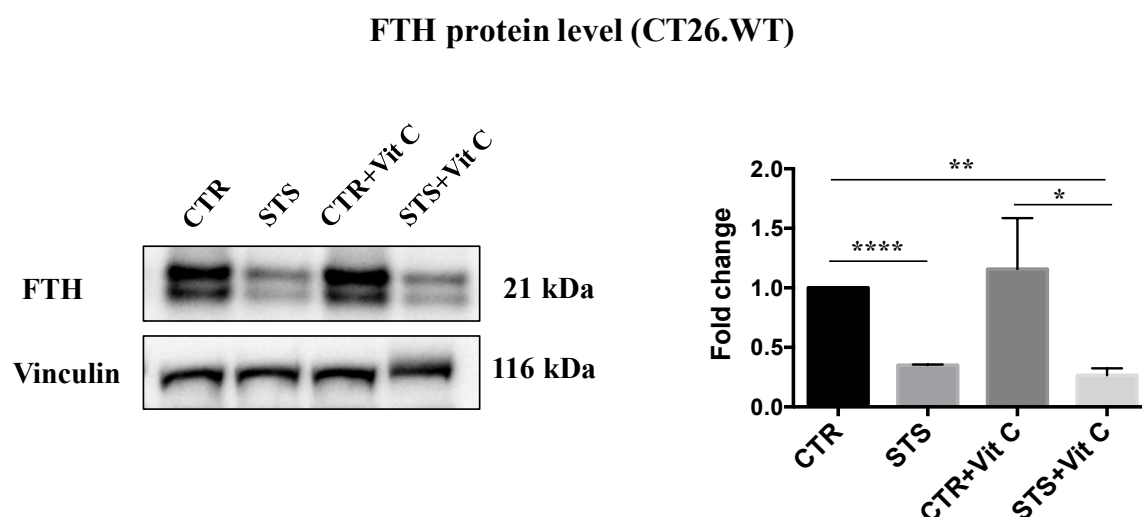
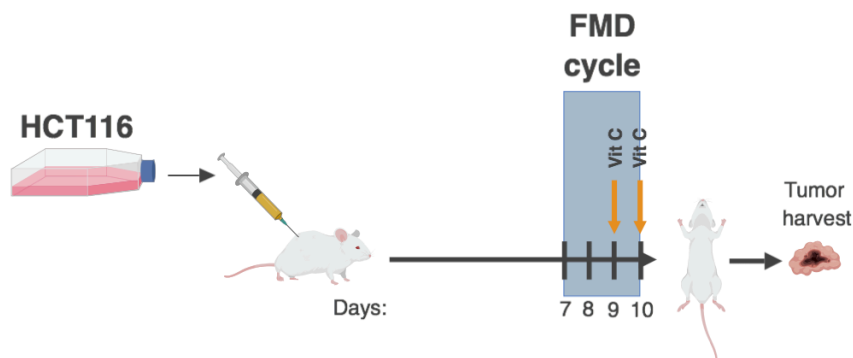


Figure 20. STS and vitamin C combination treatment down-regulates FTH protein expression level in CT26.WT. FTH protein expression level in CT26.WT grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M), were measured by western blotting ($n = 3$ biological replicates); Vinculin as loading control. Representative bands are shown (quantification on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed (**p* value ≤ 0.05 , ***p* value ≤ 0.01 , *****p* value ≤ 0.0001).

Importantly, the results obtained *in vitro* were also confirmed *in vivo*, where I found that FMD cycles down-regulated FTH protein expression level also in HCT116-derived grafted tumors (Figure 21).

A



B

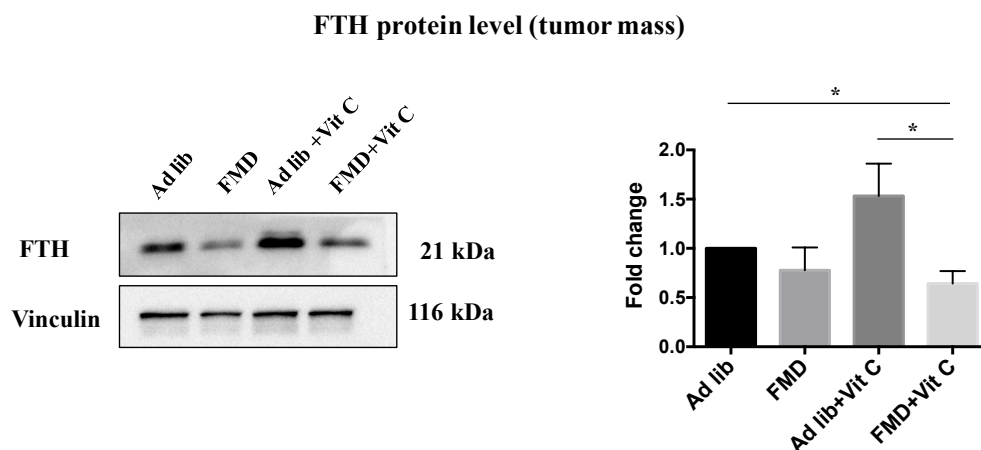


Figure 21. FMD and vitamin C combination treatment reduces FTH protein expression level *in vivo*. (A) Schematic representation of the *in vivo* experimental procedure. Mice were subcutaneously injected with HCT116 cells and when tumors were palpable (day 7) mice were subjected to FMD cycles, alone or in combination with high dose vitamin C (Vit C; 4 g/kg twice a day, i.p.). At the end of the cycle, mice were euthanized and tumors were excised and harvested for western blot analysis. (B) FTH protein expression level of grafted HCT116 tumors collected from mice fed *ad libitum* or undergoing FMD cycles ($n \geq 3$), were measured by western blotting; Vinculin as loading control. Representative bands are shown (quantification on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed (* p value ≤ 0.05).

3.3 Iron chelation reverses STS-mediated sensitization to vitamin C

Next, to assess whether the alteration in cellular iron content could be responsible for STS-mediated sensitization to vitamin C, HCT116 cells grown in STS condition were treated with the iron chelator desferrioxamine (DFO), before vitamin C exposure. To evaluate the intracellular effect of DFO in chelating iron and to avoid possible interactions with media components, cells were washed before vitamin C treatment. Supporting my hypothesis, DFO pre-treatment was able to rescue vitamin C-induced cell death (Figure 22), suggesting that the free iron increase mediated by FMD/STS could be, at least in part, responsible for the enhanced vitamin C oxidation and toxicity.

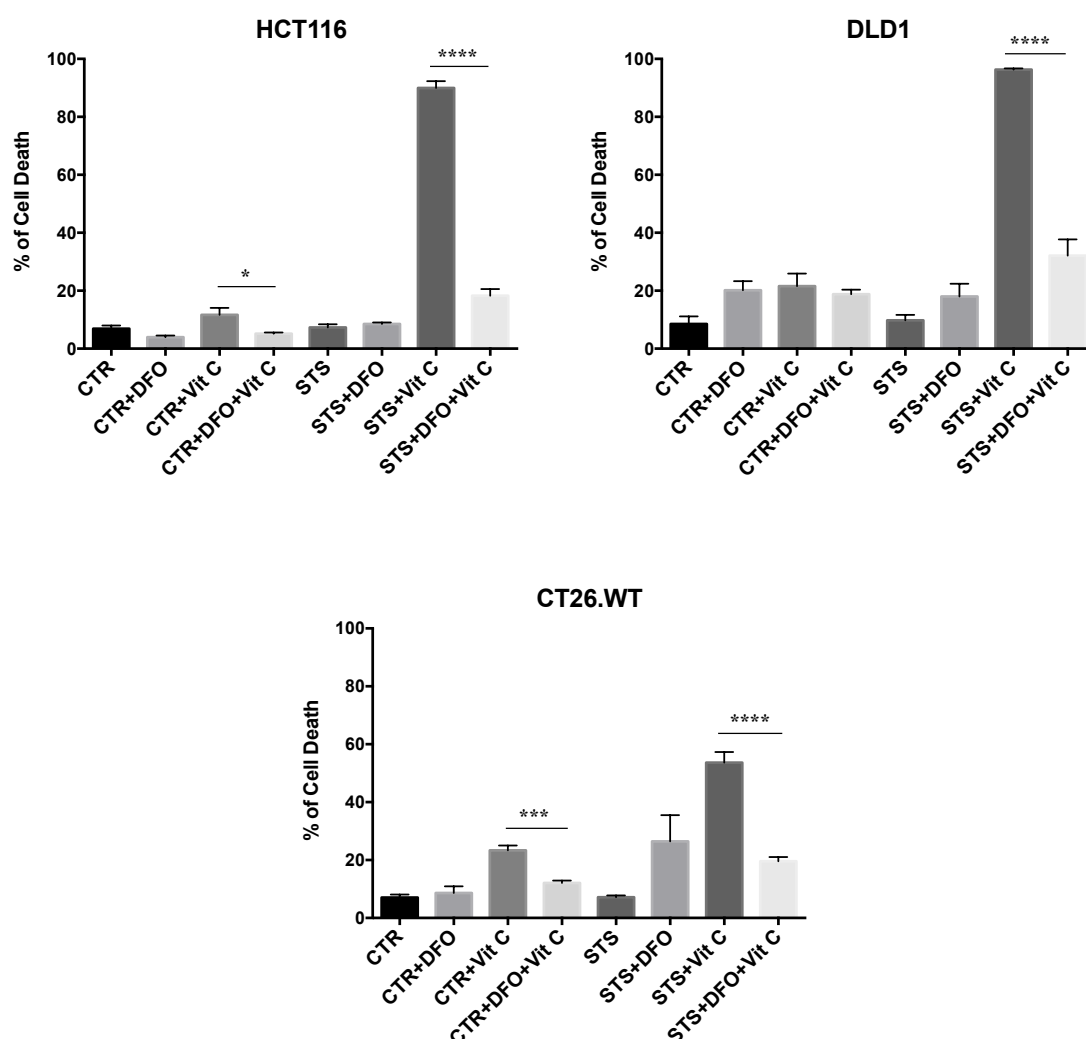


Figure 22. DFO rescues STS and vitamin C cytotoxic effect. Figure legend next page.

Figure 22. DFO rescues STS and vitamin C cytotoxic effect. HCT116, DLD1 and CT26.WT were grown in CTR or STS condition for 24 hours and treated with desferrioxamine (DFO; 500 μ M) for 6 hours (HCT116) or 12 hours (DLD1 and CT26.WT) before vitamin C treatment (Vit C; 350 μ M). At 48 hours, viability was assessed by Muse Cell viability analyser ($n \geq 3$ biological replicates). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed value (**p* value ≤ 0.05 , ***p* value ≤ 0.01 , *****p* value ≤ 0.0001).

Chapter 4

Heme-oxygenase-1 down-regulation is central in FMD-mediated sensitization to high dose vitamin C

4.1 Vitamin C up-regulates HO-1 expression level while FMD reverts this effect

Several studies have described the role of ferritin in protecting cells from oxidative damages by sequestering LIP (Torti and Torti, 2002).

Among the protective enzymes, which are involved in response to oxidative insults, the stress-inducible protein heme oxygenase-1 (HO-1) exerts its anti-apoptotic function in part by inducing ferritin expression (Ferris et al., 1999; Gonzales et al., 2002).

Supported by my data showing that FMD down-regulates FTH protein expression level, I investigated whether HO-1 could be involved in altering cellular iron content during FMD and vitamin C combo treatment. Notably, our previous works have already shown that FMD sensitizes breast cancer to chemotherapy in part by down-regulating HO-1, further supporting a possible role of this stress-inducible protein in mediating FMD beneficial effects (Di Biase et al., 2017).

To test my hypothesis, I evaluated the association between HO-1 expression level and FTH induction in my model system. To this purpose, HCT116 cells were treated with the HO-1-activator hemin, confirming that hemin increases both HO-1 and FTH protein expression level in CTR and STS growing conditions (Figure 23).

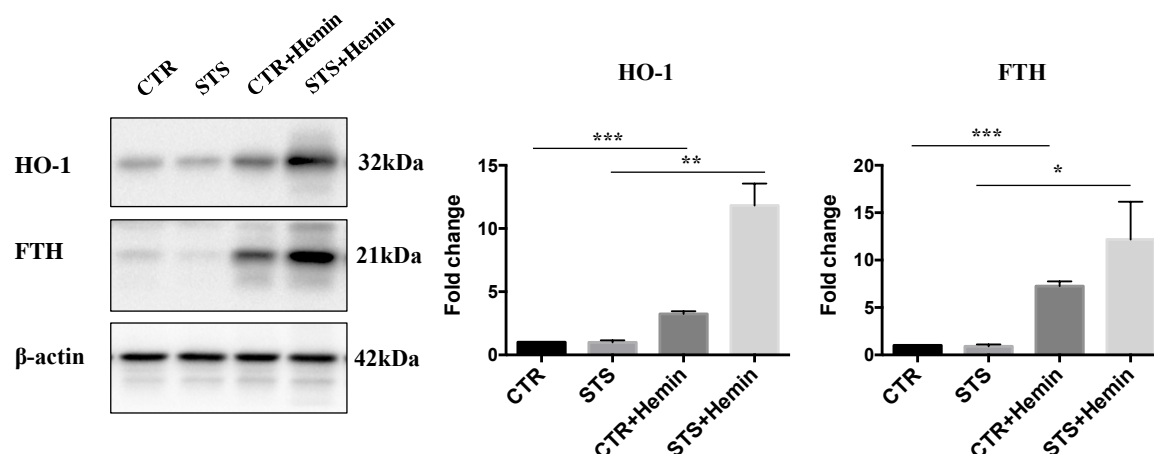


Figure 23. Hemin up-regulates HO-1 and FTH protein expression level. HCT116 were grown in CTR or STS condition for a total of 24 hours. At 12 hours, cells were treated with the HO-1 activator hemin (20 μ M) for the next 12 hours. HO-1 and FTH protein expression level were measured by western blotting; β -actin as loading control. Representative bands of three independent experiments are shown (quantifications on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed (**p* value $\leq$ 0.05, ***p* value $\leq$ 0.01, ****p* value $\leq$ 0.001).

Next, I evaluated HO-1 expression level in CTR and STS media and in presence or absence of vitamin C treatment. Interestingly, I found that vitamin C, as single intervention, significantly up-regulated HO-1 both at transcriptional and protein level in HCT116 cell line, while STS was able to revert this effect (Figure 24). Similar results were also obtained in the murine CT26.WT cell line (Figure 25)

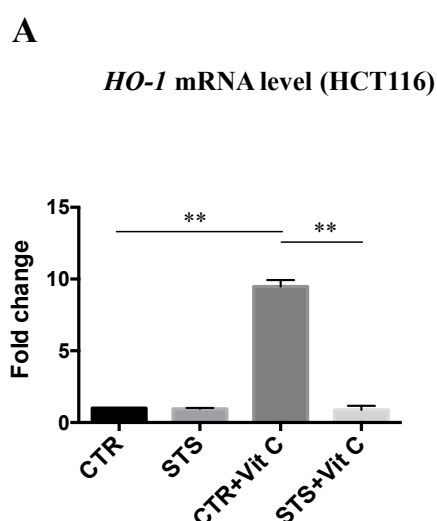


Figure 24. Vitamin C up-regulates HO-1 expression level and STS reverts this effect in HCT116 cells. Continued to next page.

B

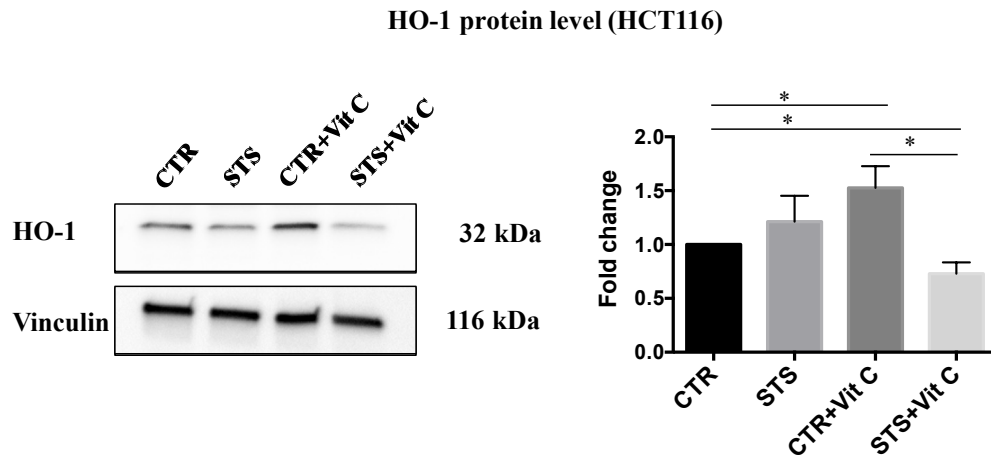


Figure 24. Vitamin C up-regulates HO-1 expression level and STS reverts this effect in HCT116 cells. (A) *HO-1* mRNA level in HCT116 grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M), were analysed by qPCR. (B) HO-1 protein expression level in HCT116 grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M), were measured by western blotting. Vinculin as loading control. Representative bands of at least three independent experiments are shown (quantification on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed (**p* value $\leq$ 0.05, ***p* value $\leq$ 0.01).

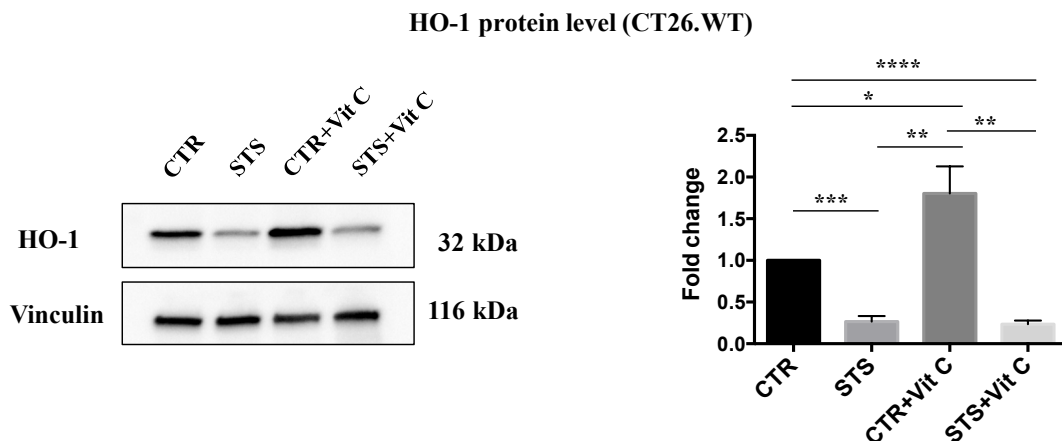


Figure 25. Vitamin C up-regulates HO-1 expression level and STS reverts this effect in CT26.WT. HO-1 protein expression level in CT26.WT grown in CTR or STS condition for 24 hours, followed by 3 hours of vitamin C treatment (Vit C; 350 μ M), were measured by western blotting (*n* = 4). Vinculin as loading control. Representative bands are shown (quantification on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed (**p* value $\leq$ 0.05, ***p* value $\leq$ 0.01, ****p* value $\leq$ 0.001, *****p* value $\leq$ 0.0001).

Importantly, also *in vivo* in grafted tumor masses derived from HCT116 cells, FMD cycles reversed the HO-1 up-regulation mediated by vitamin C treatment (Figure 26).

Collectively, these findings suggest a strong correlation between FTH and HO-1 expression in different *in vitro* and *in vivo* systems of KRAS mutant CRC, pointing out the importance of HO-1/FTH axis in FMD-sensitization of vitamin C cytotoxic effect.

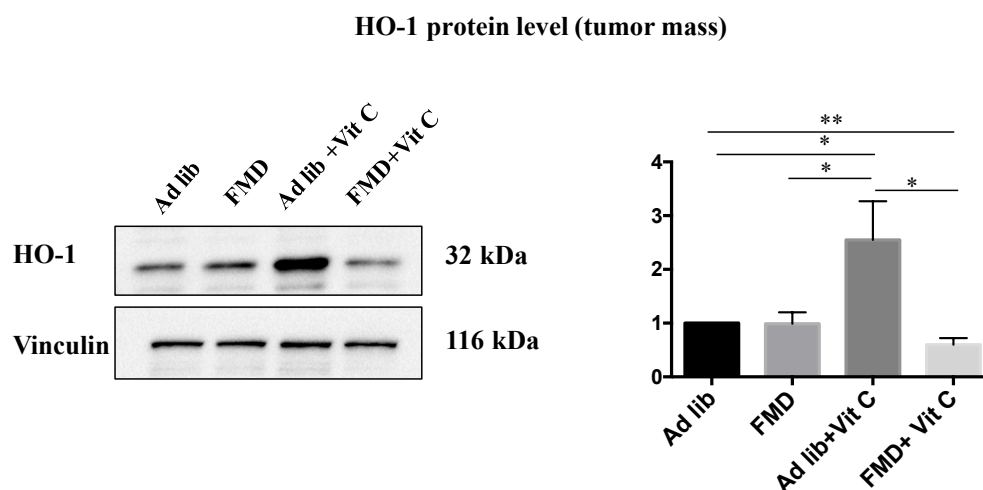


Figure 26. Vitamin C up-regulates HO-1 expression level and FMD reverts this effect in grafted tumors. HO-1 protein expression level of grafted HCT116 tumors collected from mice fed ad libitum or undergoing FMD cycles ($n \geq 5$), were measured by western blotting. Vinculin as loading control. Representative bands are shown (quantification on the right). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed (**p* value ≤ 0.05 , ***p* value ≤ 0.01).

4.2 Modulation of HO-1 expression level dictates cancer cell-sensitivity to vitamin C

The effect of FMD in reverting HO-1 up-regulation mediated by vitamin C, prompted me to investigate whether modulation in HO-1 expression level could alter cancer cell-sensitivity to pharmacological vitamin C treatment. To this purpose, human and murine KRAS mutant CRC cell lines (HCT116, DLD1, CT26.WT), grown in CTR or STS condition, were exposed to the HO-1 activator hemin. Notably, I found that, HO-1

activation was able to reverse cancer cell death induced by both vitamin C alone or in combination with STS (Figure 27).

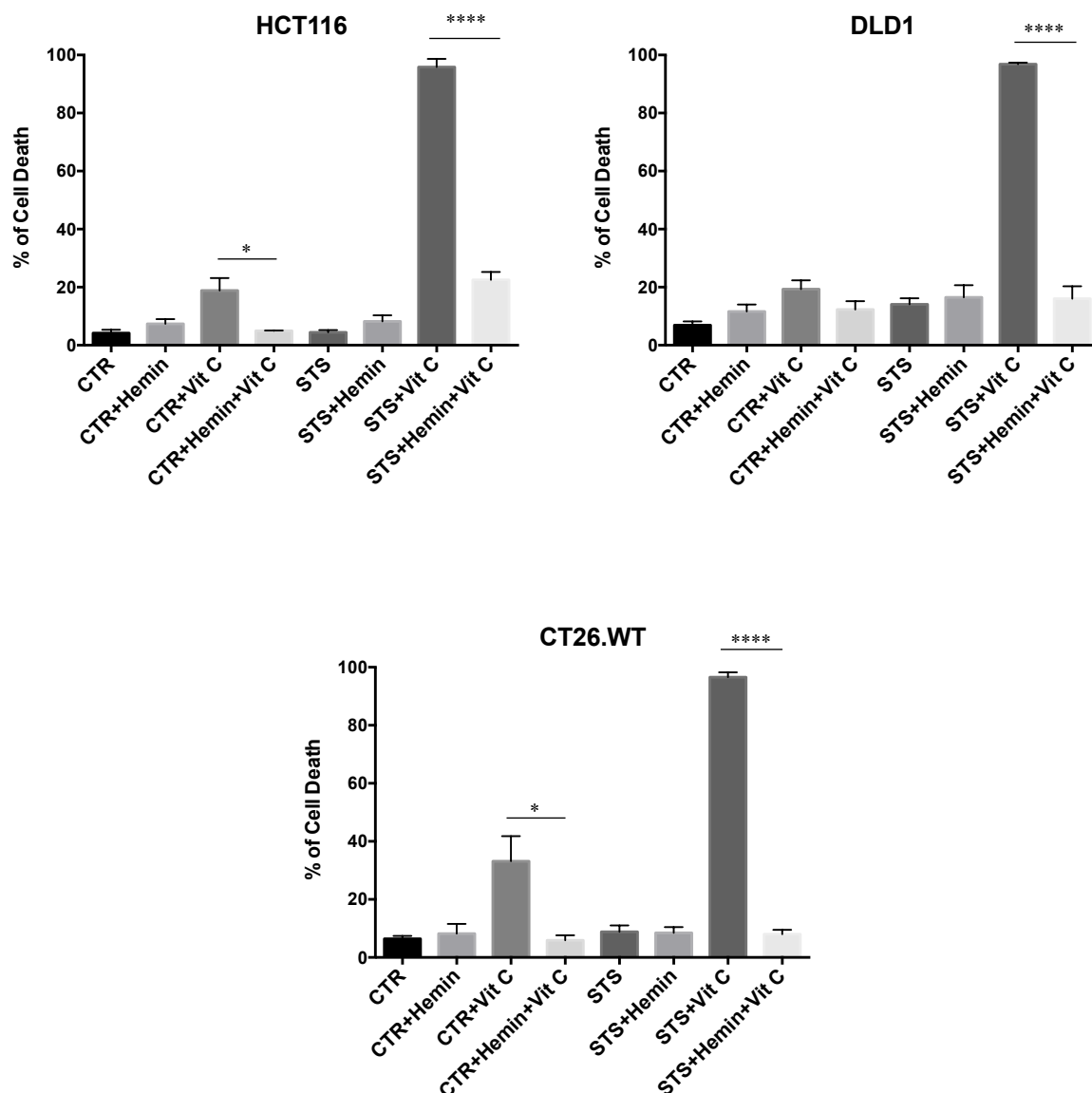


Figure 27. HO-1 activation reverses STS-mediated sensitization to vitamin C. HCT116, DLD1 and CT26.WT, were grown in CTR or STS condition for 24 hours. Cells were treated with hemin (20 μ M) for 3 hours, before vitamin C treatment (Vit C; 350 μ M, for 24 hours). At 48 hours, viability was assessed by Muse Cell viability analyser ($n \geq 3$ biological replicates). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired t -test was performed value (* p value ≤ 0.05 , **** p value ≤ 0.0001).

Next, I investigated whether HO-1 inhibition could sensitize cancer cells to the toxic effect of vitamin C in control condition. I found that the HO-1 inhibitor zinc protoporphyrin (ZnPP) was able to make KRAS mutant CRC cells more susceptible to vitamin C toxicity in control medium (Figure 28).

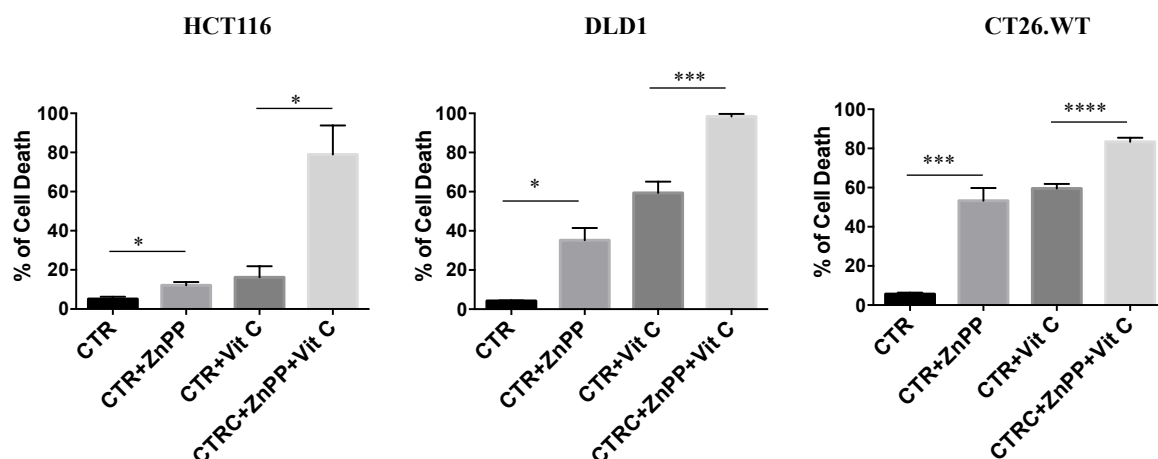


Figure 28. Pharmacological HO-1 inhibition sensitizes cancer cells to vitamin C toxicity. HCT116, DLD1 and CT26.WT cell lines, were grown in CTR or STS condition for 24 hours. Cells were treated with the HO-1 inhibitor zinc protoporphyrin (ZnPP; 20 μ M) for 3 hours, before vitamin C treatment (Vit C; 700 μ M, for 24 hours). At 48 hours, viability was assessed by Muse Cell viability analyser (n = 3 biological replicates). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed value (*p value $\leq$ 0.05, ***p value $\leq$ 0.001, ****p value $\leq$ 0.0001).

Accordingly, also HO-1 knockdown in HCT116 cells, increased cell death rate upon vitamin C treatment in nutrient-rich condition (Figure 29), supporting the key role of HO-1 in determining cancer cell sensitivity to vitamin C.

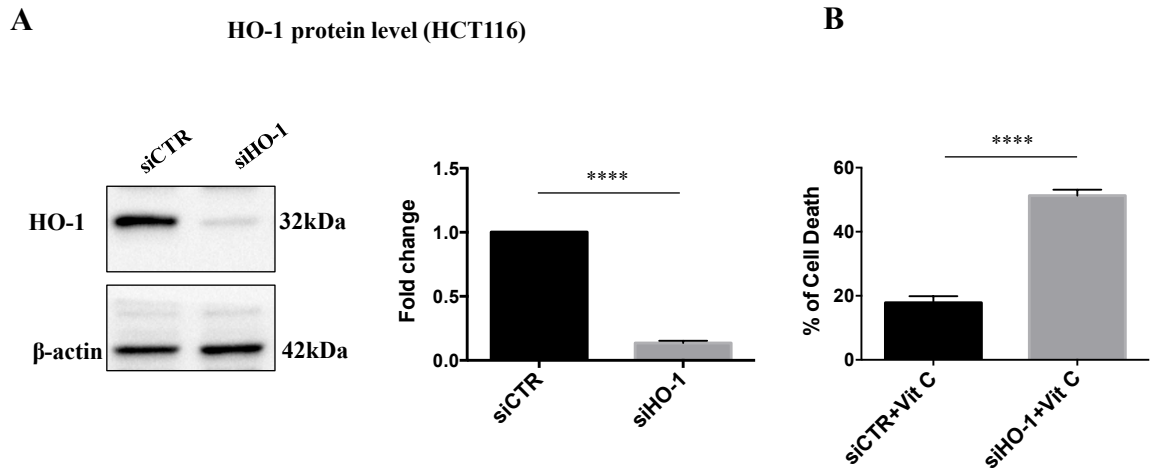


Figure 29. HO-1 knockdown sensitizes cancer cells to vitamin C toxicity. (A) HCT116 cells were transfected with siHO-1 or siCTR and HO-1 knockdown was assessed by western blotting. β -actin as loading control. Representative bands are shown (quantification on the right). (B) HCTT16, in CTR condition, were transfected with control siRNAs (siCTR) or a pool of siRNAs against HO-1 (siHO-1) and, after 24 hours, treated with vitamin C (Vit C; 700 μ M) for 24 hours. Viability was assessed by Muse Cell viability analyser (n = 5 biological replicates). Data are represented as mean $\pm$ SEM. Two-tailed, unpaired *t*-test was performed value (****p value $\leq$ 0.0001).

Chapter 5

FMD, vitamin C, oxaliplatin triple treatment is effective in reducing progression of KRAS mutant CRC

5.1 *In vitro* STS condition sensitizes KRAS mutant CRC cells to chemotherapy

We have recently shown that fasting and FMD are effective non-toxic interventions able to sensitize a wide range of cancer cells to the cytotoxic effect of chemotherapy, while protecting normal cells, through a mechanism which partially involve the lowering of IGF-1 level (Lee et al., 2012; Brandhorst et al., 2015, Di Biase et al., 2016).

To evaluate whether FMD potentiates chemotherapy efficacy in my *in vitro* model system, I used the chemotherapeutic agent oxaliplatin, which represents a first-line standard drug for oncological patient bearing KRAS mutant CRC (Brenner et al., 2014).

Supporting our previous published works (Lee et al., 2012; Brandhorst et al., 2015, Di Biase et al., 2016), my *in vitro* data on HCT116 cell line indicate that 24 hours-STS before and during oxaliplatin treatment is able to significantly decrease the percentage of metabolically active cells, as measured by MTT assay, and increase the percentage of cell death (Figure 30). Notably, the combination of a FMD-like condition and chemotherapy was also more effective compared to 48 hours-STS as single intervention or chemotherapy alone in decreasing viability of HCT116 cells, thus encouraging the use of FMD as an effective combination intervention in KRAS mutant cancers. (Figure 30).

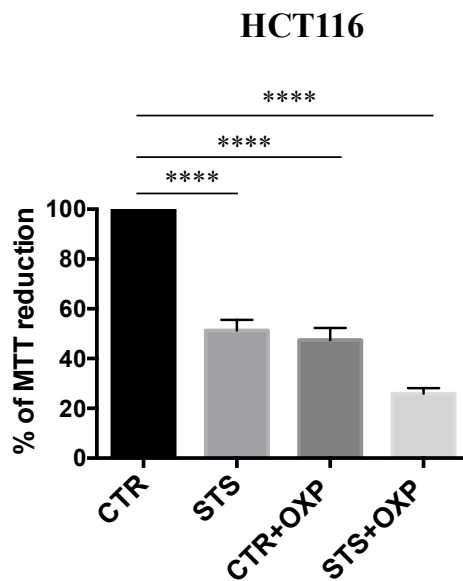
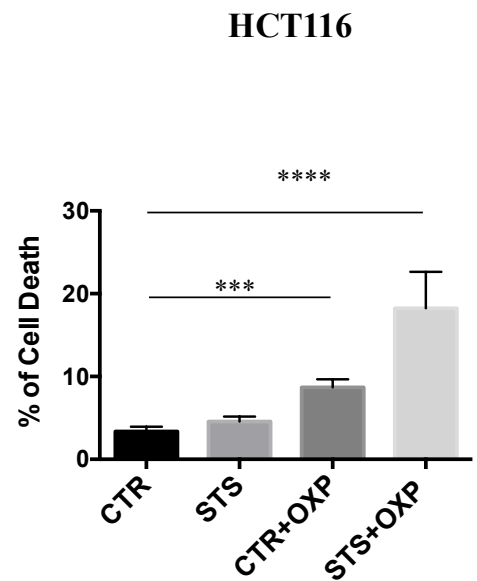
A**B**

Figure 30. STS sensitizes HCT116 to oxaliplatin toxicity. HCT116 were grown in CTR or STS conditions for a total of 48 hours. At 24 hours, cells were treated with oxaliplatin (OXP; 40 μ M) or vehicle for further 24 hours. At the end of the experiment, cell viability was assessed by MTT reduction (**A**), whereas percentage of cell death was assessed by erythrosine B exclusion assay (**B**) ($n \geq 3$ biological replicates). Data are represented as mean $\pm$ SEM (two-tailed unpaired t-test; ***p value ≤ 0.001 , ****p value ≤ 0.0001).

5.2 FMD and vitamin C combo treatment potentiates oxaliplatin efficacy in mouse models of KRAS mutant CRC

Several studies described the tolerability and potential efficacy of high dose vitamin C as adjuvant treatment during chemotherapy (Monti et al., 2012; Ma et al., 2014; Schoenfeld et al., 2017). In addition, our group has recently demonstrated the effectiveness of fasting or FMD cycles in combination with chemotherapy to reduce tumor growth in a wide range of cancer types compared to standard chemotherapy alone (Lee et al., 2012; Di Biase et al., 2016).

Supported by my data showing that FMD potentiates chemotherapy efficacy *in vitro* on KRAS mutant CRC cell line, I investigated whether FMD and vitamin C combo therapy, possibly by increasing cellular oxidative stress, could sensitize KRAS mutant cancer cells to the pro-oxidant action of chemotherapy *in vivo*. Mice bearing HCT116 xenografts were fed *ad libitum* with standard rodent diet or subjected to FMD cycles (3/2-days each) and treated with high dose vitamin C (4 g/kg i.p.) or saline, oxaliplatin (10 mg/kg) or vehicle, as single agents or in combination. Interestingly, I found that FMD and vitamin C combo treatment is as effective as oxaliplatin combined with FMD (FMD+vitamin C vs FMD+oxaliplatin) or vitamin C (FMD+vitamin C vs vitamin C+oxaliplatin), thus supporting the powerful action of these non-toxic combination in reducing tumor growth (Figure 31). Importantly, combination of FMD cycles, vitamin C and chemotherapy as triple treatment was the most effective therapeutic option in delaying tumor progression and tumor growth (Figure 31).

A

Human CRC (HCT116)

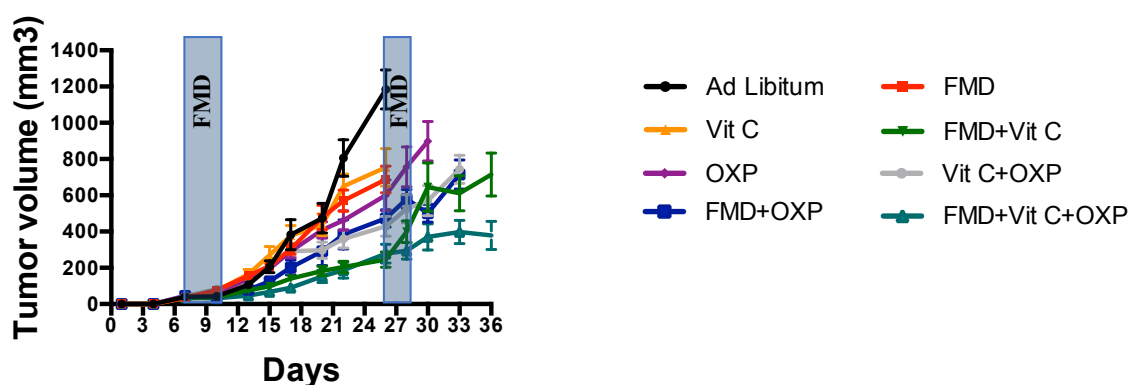


Figure 31. FMD, vitamin C, Oxaliplatin triple treatment is effective in delaying tumor progression in HCT116 xenograft mouse model. Continued on next page.

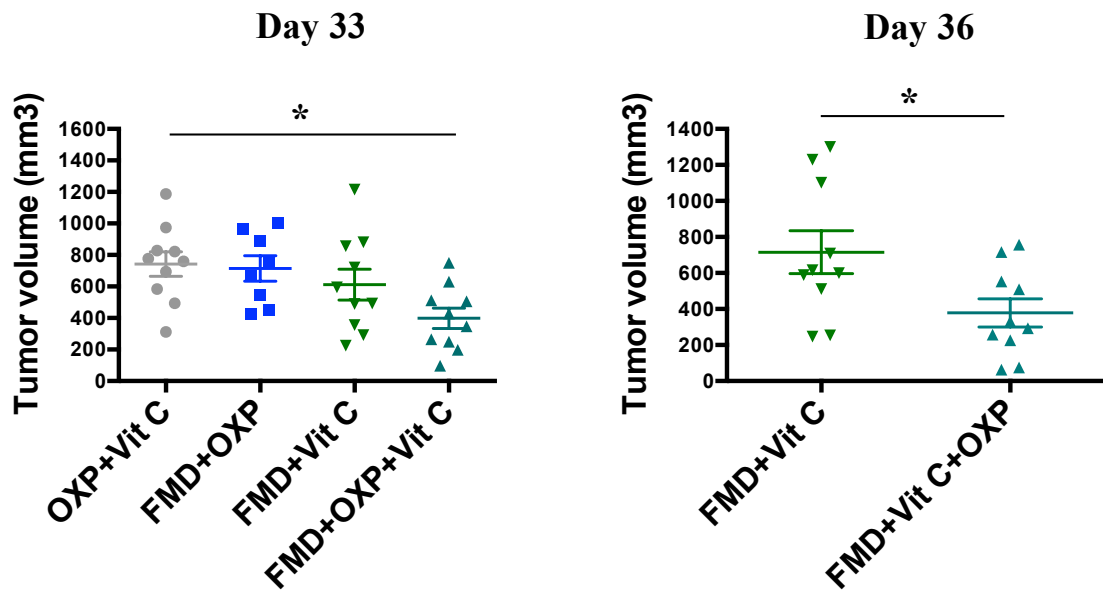
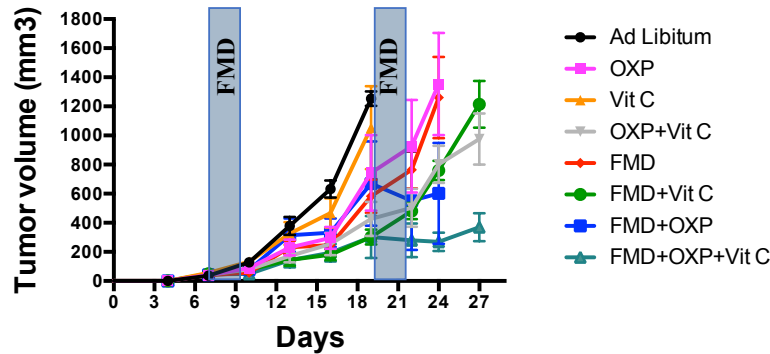
B

Figure 31. FMD, vitamin C, Oxaliplatin triple treatment is effective in delaying tumor progression in HCT116 xenograft mouse model. 12-weeks old female NOD scid gamma (NSG) mice were subcutaneously injected with HCT116 cells. Mice were fed *ad libitum* or subjected to FMD cycles, and treated with high dose vitamin C (Vit C; 4 g/kg twice a day, i.p.) or saline, Oxaliplatin (OXP; 10 mg/kg i.p. once every 15 days) or vehicle, as single agents or as combination. Tumor volumes at multiple time points (A) and before mice were euthanized (Days 33 and 36) (B) are reported (n = 8-10). Data are represented as mean $\pm$ SEM. One-way Anova (Tukey's post-analysis test) and two-tailed unpaired *t*-test (for day 36) were performed (*p value $\leq$ 0.05).

Next, the anti-cancer effect of the triple therapy was also evaluated in a syngeneic mouse model of KRAS mutant CRC (CT26.WT allograft). A first preliminary experiment showed that FMD and vitamin C combo treatment resulted to be as effective as oxaliplatin and vitamin C dual therapy in retarding tumor growth. Importantly the triple treatment (FMD + vitamin C + oxaliplatin) further delayed tumor progression, thus indicating its efficacy also in an immuno-competent model of KRAS mutant CRC (Figure 32).

A

Murine CRC (CT26.WT)



B

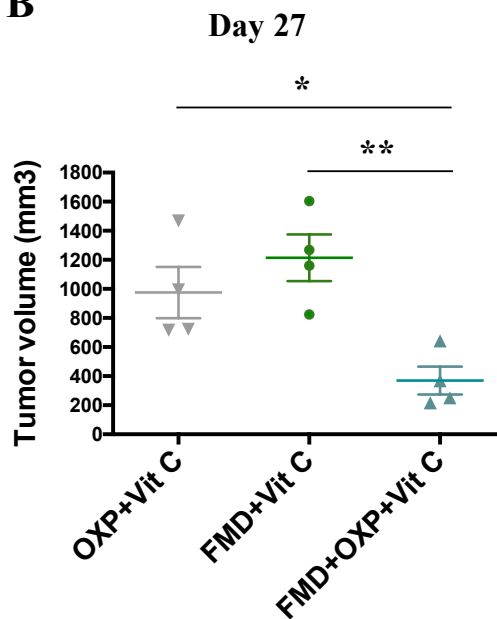


Figure 32. FMD, vitamin C, Oxaliplatin triple treatment is effective in delaying tumor progression in CT26.WT allograft mouse model. 8-weeks old female Balb c/OlaHsd mice were subcutaneously injected with CT26.WT cells. Mice were fed *ad libitum* or subjected to FMD cycles, and treated with high dose vitamin C (Vit C; 4 g/kg twice a day, i.p.) or saline, oxaliplatin (OXP; 10 mg/kg i.p. once every 10 days) or vehicle, as single agents or as combination. Tumor volumes at multiple time points (A) and before mice were euthanized (Day 27) (B) are reported (n= 3-4 per group). Data are represented as mean $\pm$ SEM. One-way Anova (Tukey's post-analysis test) was performed (*p value $\leq$ 0.05, **p value $\leq$ 0.01).

Finally, the combination of FMD cycles, vitamin C and oxaliplatin results to be well tolerated, as indicated by mouse bodyweight. FMD cycles causes a 20% weight loss, which is recovered upon refeeding, independently from vitamin C and oxaliplatin administration (Figure 33).

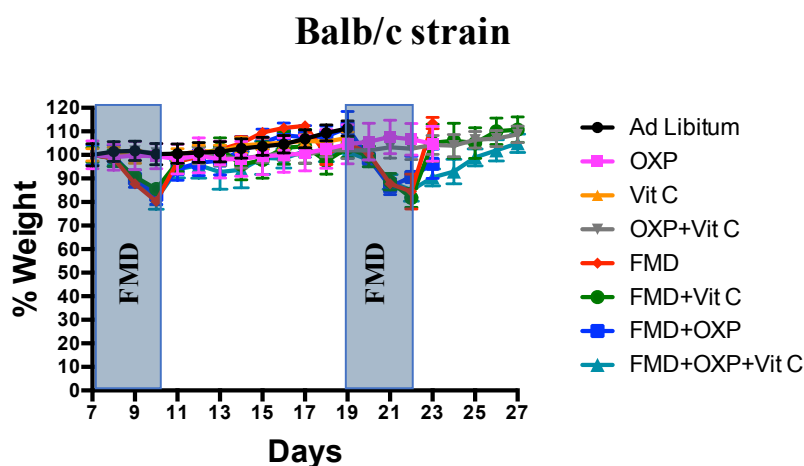


Figure 33. FMD, vitamin C, Oxaliplatin triple treatment is safe and well tolerated. Weight of Balb/cOlaHsd mice bearing CT26.WT-derived tumors underwent 3-days FMD alone or in combination with vitamin C (Vit C; 4 g/kg twice a day, i.p.) and Oxaliplatin (OXP; 10 mg/kg i.p. once every 10 days). Bodyweight was recorded daily and it is indicated as percentage of weight at day 7.

Chapter 6

FMD and chemotherapy cotreatment is safe and well tolerated in KRAS mutant CRC patients

In collaboration with Prof. Alessio Nencioni from San Martino hospital (Genoa, Italy) and Dr. Claudio Vernieri from Istituto Nazionale dei Tumori (Milan, Italy), we are evaluating the feasibility and efficacy of FMD cycles in combination with standard chemotherapy in KRAS mutant CRC patients. Here, I report three cases from ongoing feasibility studies.

6.1 Patient # 1

A 49 years-old woman was diagnosed with a KRAS mutant colon cancer with distant metastasis in lung and lymph nodes. The patient has been treated with FOLFOX, which is a standard chemotherapy regimen including oxaliplatin, 5-fluorouracil (5-FU) and folinic acid.

The patient underwent a first FMD cycle, while receiving chemotherapy. Then, the FMD regimen was skipped for one month due to the lowering of phase angle (Table 1). Phase angle (PhA) is defined as the relationship between resistance and reactance and it represents an indicator of lean mass. Healthy subjects show a phase angle higher than 5° . A PhA of 5° was considered as threshold below which FMD cycle was not feasible. After the first FMD cycle, the patient showed a slight lowering of PhA, from 5.3° to 4.8° . After one month, values return to be in the normal range (5.2°), thus starting a second FMD cycle. PhA values remained nearly constant even after the second and third FMD cycles as well as total body weight, indicating that even in patients with low PhA, multiple FMD cycles can be well tolerated (Table 1, Table 2).

PhA(°)	
Pre FMD	5.3
Post 1 st FMD cycle	4.8
Pre 2 nd FMD cycle	5.2
Post 2 nd FMD cycle	5.3
Post 3 rd FMD cycle	5.2

Table 1. FMD effect on phase angle (patient #1). Phase angle (PhA) of the patient before and 21 days after each FMD cycle was recorded. PhA pre 3rd FMD cycle was not collected.

BW (kg)	
Pre FMD	58.9
Post 1 st FMD cycle	61.7
Pre 2 nd FMD cycle	62.4
Post 2 nd FMD cycle	62.5
Post 3 rd FMD cycle	61.8

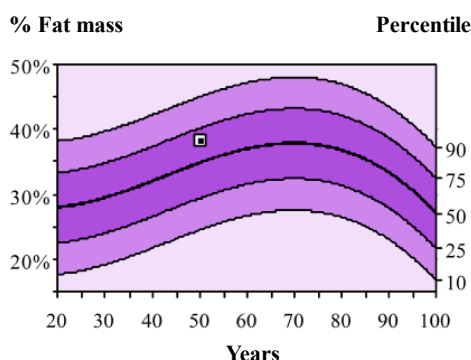
Table 2. FMD effect on bodyweight (patient #1). Bodyweight (BW) of the patient before and 21 days after each FMD cycle was recorded. Bodyweight pre 3rd FMD cycle was not collected.

After the third FMD cycle (22 days post refeeding), a dual-energy X-ray absorptiometry (DEXA) analysis was performed. These data indicate a normal body weight composition even after the administration of the diet. In particular, the body mass index (BMI) is classified as normal, with a value of 24,4 kg/m², according to the BMI classification of World Health Organization (WHO), with a lean body mass of 59.5 % and a fat mass of 38.5% (Figure 34).

A

BMI (kg/m ²)	24.4
Lean body mass (%)	59.5
Fat mass (%)	38.5

B



C

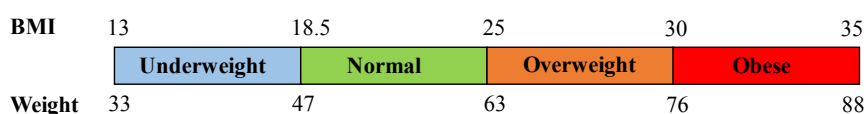


Figure 34. Body composition measurement (patient #1). (A) Bodyweight (kg/m²), lean mass (%) and fat mass (%) of the patient 22 days after the third FMD cycle, are reported. (B) The plot shows the percentage of patient's fat mass compared to the reference population. The curve in bold represents the average of the population and the square represents the result of the patient. (C) Representative scheme of BMI values classified as underweight, normal, overweight and obese according to the World Health Organization (WHO).

At the moment, the patient has completed the fourth FMD cycle in combination with 5-FU without evident side effects.

6.2 Patient # 2

A 51-year old man was diagnosed with a right-sided colorectal cancer, with lung, liver and abdominal lymph nodes metastasis. Next generation sequencing analysis and microsatellite evaluation revealed the presence of *KRAS G12* mutation, associated with *PI3KCA E542K* and *STK11 F354L* mutations, as well as stable microsatellite.

One month after diagnosis, the patient started FOLFOXIRI (5-FU, oxaliplatin and irinotecan) combined with the monoclonal antibody bevacizumab every two weeks and monthly FMD cycles. Treatments were well tolerated by the patient and grade 1 nausea was the only reported side effect, likely due to chemotherapy.

As shown in figure 35 the patient underwent reversible bodyweight loss during the FMD regimen (Figure 35). Blood and urine analysis revealed reversible reduction of plasma glucose, serum insulin and IGF-1 levels, and an increase of urinary ketones (Figure 36).

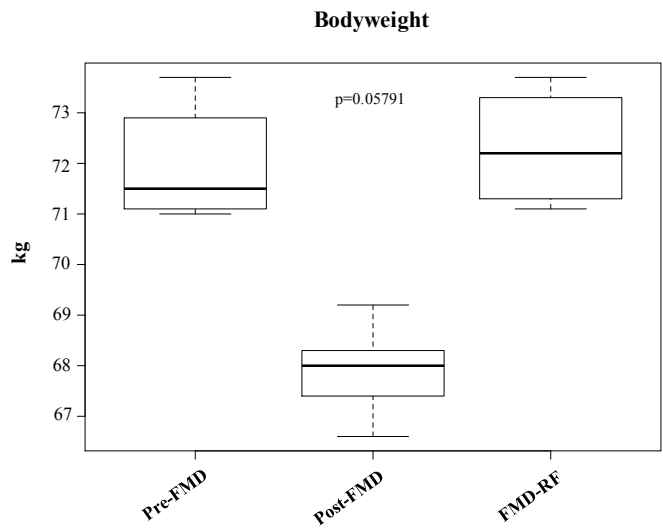


Figure 35. FMD effect on bodyweight (patient #2). Bodyweight was recorded before (pre-FMD), at the end (post-FMD) and 21-28 days of refeeding (FMD-RF) after each FMD cycle. Data are represented as median of 5 FMD cycles. P value was calculated with Wilcoxon test (non-parametric).

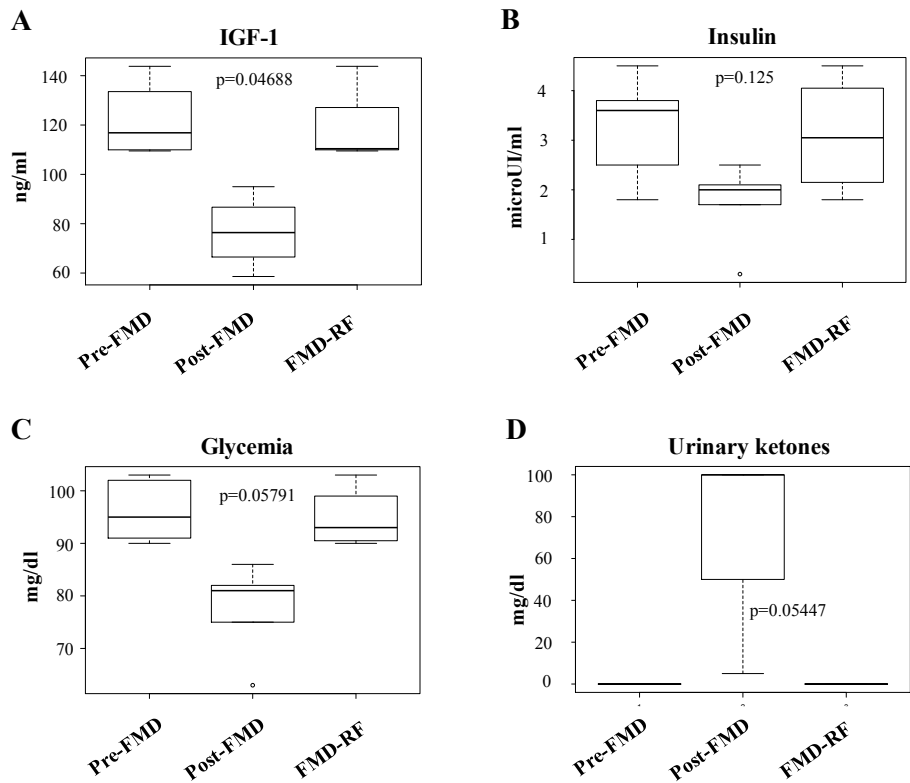


Figure 36. FMD effect on metabolic markers (patient #2). Figure legend next page.

Figure 36. FMD effect on metabolic markers (patient #2). IGF-1 (A), insulin (B), glucose (C) and urinary ketone (D) levels were measured before (pre-FMD), at the end (post-FMD) and 21-28 days of refeeding (FMD-RF) after each FMD cycle. Data are shown as median of 5 FMD cycles. P values were calculated with Wilcoxon test (non-parametric).

Computed tomography (CT) and positron emission tomography (PET) evaluation after 6 cycles of chemotherapy and 3 cycles of FMD revealed partial reduction and metabolic activity of liver metastases and complete disappearance of metabolic activity in the primary tumor (data not shown).

In summary, the patient has completed 12 cycles of FOLFOXIRI and bevacizumab and 5 FMD cycles. CT re-evaluation confirmed the response in the liver and in the primary tumor (data not shown), thus allowing the patient to undergo resection of liver metastases and peri-ilar lymph nodes. Pathological analysis revealed the presence of necrosis in nearly 50% of resected lesions (data not shown). After surgery, the patient started maintenance chemotherapy with 5-FU and bevacizumab. Nine months since the start of the treatment, CT-scan confirms the absence of radiological detectable disease (data not shown).

6.3 Patient # 3

After resection of transverse colon, histological examination revealed the presence of poorly differentiated grade 3 mucinous adenocarcinoma in a 49-year old man. Microsatellite analysis revealed microsatellite stability. After surgery, the patient underwent 8 cycles of adjuvant chemotherapy XELOX (capecitabine and oxaliplatin).

Eight months after diagnosis, CT scan revealed the presence of subcutaneous, hypogastric lesion. After surgical removal, histological examination revealed the presence of metastasis from mucinous colon carcinoma. Sequencing analysis revealed the presence of *KRAS* mutation. Two months after surgery, PET scan evaluation revealed diffuse abnormal abdominal uptake of 18F-fluoro-2-deoxy-D-glucose (FDG). The patient started first-line chemotherapy XELIRI (capecitabine and irinotecan) every three weeks combined with

FMD cycles (every three weeks). The patient underwent a total of 8 FMD cycles + chemotherapy.

The treatment was well tolerated by the patient. Grade 1 nausea and grade 2 vomiting were reported as side effects, likely due to chemotherapy and grade 2 fatigue which can be related either to FMD or chemotherapy.

FMD regimens caused bodyweight loss, which was recovered upon refeeding (Figure 37). Blood and urinary analysis revealed that the plasma glucose, serum insulin and IGF-1 levels showed reversible reduction and urinary ketones showed reversible increase (Figure 38).

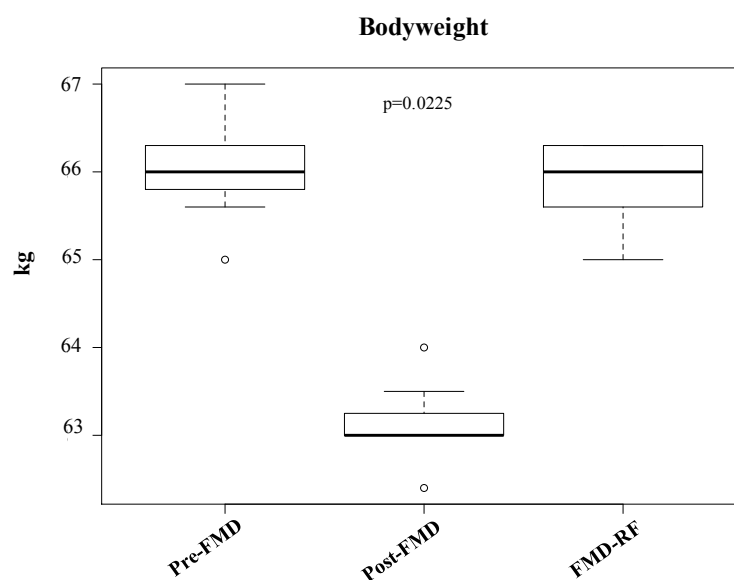


Figure 37. FMD effect on bodyweight (patient #3). Bodyweight was recorded before (pre-FMD), at the end (post-FMD) and 21-28 days of refeeding (FMD-RF) after each FMD cycle. Data are represented as median of 8 FMD cycles. P value was calculated with Wilcoxon test (no parametric).

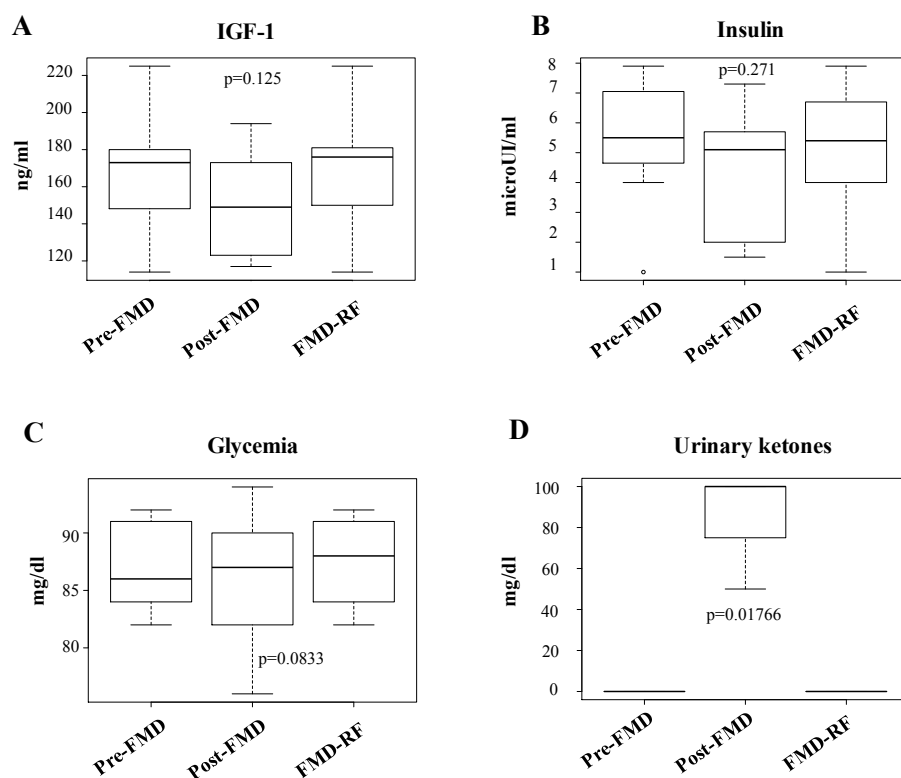


Figure 38. FMD effect on metabolic markers (patient #3). IGF-1 (A), insulin (B), glucose (C) and urinary ketone (D) levels were measured before (pre-FMD), at the end (post-FMD) and 21-28 days of refeeding (FMD-RF) after each FMD cycle. Data are shown as median of 8 FMD cycles. P values were calculated with Wilcoxon test (non-parametric).

PET scan evaluation after 4 cycles of chemotherapy and FMD revealed the disappearance of pathological ^{18}F FDG uptake in all disease sites (data not shown). This result was confirmed after 8 cycles.

One year since the start of the treatment, the patient is undergoing maintenance chemotherapy with oral capecitabine and he remains free of disease (data not shown).

Collectively these three cases indicate that FMD cycles in combination with chemotherapy represent a safe therapeutic option for cancer patients. Ongoing studies are evaluating the potential effectiveness for the treatment of KRAS mutant CRC.

Moreover, these results together with our molecular and pre-clinical studies, give the basis for future clinical investigation on the beneficial effect of FMD and pharmacological vitamin C in potentiating chemotherapy efficacy in patients harbouring KRAS mutant CRC.

DISCUSSION

DISCUSSION

Fasting or Short-Term Starvation (STS) reduces tumor progression and sensitizes different types of cancers to chemotherapy, while protecting normal cells (Lee et al., 2012; Bianchi et al., 2015). These phenomena are described as “Differential Stress Sensitization” (DSS) and “Differential Stress Resistance” (DSR), respectively (Raffaghello et al., 2008; Lee et al., 2010; Lee and Longo, 2011; Lee et al., 2012; Longo and Mattson, 2014). The differential protection of normal and cancer cells in response to fasting has been proposed to be mediated, at least in part, by the down-regulation of the insulin-like growth factor (IGF-1) pathway (Lee et al., 2010). By decreasing IGF-1 level, fasting allows normal cells to enter in a protection mode, reducing mitotic stimuli and inducing the expression of repair genes (Longo and Mattson, 2014; Raffaghello et al., 2008). Cancer cells, instead, harbouring oncogenic mutations, are unresponsive to growth signals, and thus fail to be protected (Lee et al., 2012; Lee et al., 2010). Studies showing that IGF-1 restoration reverts the fasting-mediated sensitization to chemotoxicity, sustain its fundamental role also in DSS (Lee et al., 2010; Lee et al., 2012). In addition, oxidative stress results to contribute to fasting effects, in fact, by increasing ROS generation, fasting sensitizes cancer cells to the oxidative damages exerted by chemotherapy (Lee et al., 2012).

However, fasting remains a challenging option for cancer patients, for this reason our group developed a more feasible and safer diet whose specific formulation mimics fasting effects. The fasting-mimicking diet (FMD) is a low calories, protein and sugar but high unsaturated fat diet with effects similar to fasting in delaying tumor progression and in sensitizing cancer cells to chemotherapy, as shown in breast cancer and melanoma models (Brandhorst et al., 2015; Di Biase et al., 2016).

In my thesis, I have focused my studies on the identification of very low toxicity combination therapy, with the potential to 1) replace chemotherapy and other high toxicity therapies, 2) lead to cancer free survival or high therapeutic index in combination with toxic therapies. For this purpose, I focused on the therapeutic effect of FMD on the highly aggressive KRAS mutant colorectal cancer, which is one of the most widespread and lethal cancer in the western world (Bardelli and Siena, 2010; Stephen et al., 2014; Van Emburgh et al., 2016).

KRAS mutant cancers are refractory to standard and targeted treatment, making the patient's prognosis very poor (Lievre et al., 2006). For this reason, there is an increasing and urgent need to identify effective therapeutic option able to delay or stop KRAS-driven tumor progression without causing the major side effects that eventually lead to death or force oncologist to discontinue treatment. However, several attempts have failed in efficacy and specificity for the treatment of this type of cancer so far (Cox et al., 2014; Verissimo et al., 2016). Indeed, patients bearing KRAS-mutant CRC result to be unresponsive to targeted-therapy, such as monoclonal antibody directed against EGFR (cetuximab and panitumumab), and still now, no effective therapeutic options are available. In fact, despite several improvements have been achieved, the survival rate at 5 years post diagnosis remains very poor (Bardelli and Siena, 2010; Brenner et al., 2014).

Recently, Yun and colleagues reported that high-dose vitamin C is effective in killing KRAS mutant CRC cells, arising the possibility that vitamin C could have a potential therapeutic use for the treatment of this aggressive tumor type (Yun et al., 2015).

The anti-cancer properties of high-dose vitamin C have been associated with controversial results. In 1976, vitamin C was proposed by Cameron and Pauling as an anti-tumoral agent, however two randomized clinical trials failed to demonstrate any beneficial effect of oral-administered vitamin C on cancer patient survival (Cameron and Pauling, 1976; Creagan et al., 1979; Moertel et al., 1985). These contradictory outcomes are explained, at least in part, by the different administration route. In fact, growing evidence sustains that

vitamin C requires to be delivered intravenously in order to bypass the gastric barrier and achieve plasma millimolar concentrations, which are toxic to cancer cells (Padayatty et al., 2010; Chen et al., 2008, Stephenson et al., 2013). For this reason, several studies are now focusing on vitamin C mechanism of action and on its effectiveness as anti-cancer drug, through several pre-clinical and clinical trials (Hoffer et al., 2008; Ma et al., 2014; Schoenfeld et al., 2017, NCT02344355; NCT03146962; NCT02420314; NCT01752491). The major aim of my thesis was to identify non-toxic but highly effective interventions with the potential to result in cancer free survival for the treatment of KRAS mutant cancers. Given the tolerability and efficacy of FMD and vitamin C as anti-cancer agents, I evaluated their combination as potential novel therapeutical option for colorectal cancer patients.

1. FMD/STS sensitizes KRAS-mutant CRC to vitamin C toxicity

My preliminary data support previous published findings showing that KRAS mutant cancer cells are more susceptible to vitamin C toxicity, compared to KRAS wild type cell lines. However, the anti-cancer effect is limited, only slightly increasing cell death *in vitro* and retarding tumor progression *in vivo*. For this reason, my goal was to evaluate whether vitamin C toxicity could be potentiated by FMD cycles.

To study the effect of FMD in a cell culture system, I used an *in vitro* growing condition previously established by our laboratories, referred as STS (short-term starvation) or FMD-like condition (Lee et al., 2012; Di Biase et al., 2016). STS medium is characterized by low level of glucose and low level of serum to mimic the physiological effect of fasting/FMD in reducing glucose and growth factor concentrations (Lee et al., 2012).

Importantly, I found that STS is able to act synergistically with vitamin C in causing toxicity to a wide range of KRAS mutant cell lines derived from colorectal, lung and pancreatic cancers, leaving unaffected KRAS wild type prostate and ovary cancer cells,

normal colon cells and normal fibroblasts. These findings suggest that KRAS oncogenic reprogramming is required for the FMD-mediated sensitization to vitamin C toxicity.

Notably, I confirmed the *in vitro* results also in *in vivo*, in two different mouse models of KRAS mutant CRC. These studies reveal that FMD cycles combined with pharmacological vitamin C treatment are more effective than vitamin C and FMD as single interventions in delaying tumor progression. Moreover, the tolerability of FMD and vitamin C combo therapy observed in both mouse strains, underlines its potential use in the clinic.

2. Vitamin C toxicity in cell culture systems

The *in vitro* cytotoxic action of vitamin C is object of debate. Several reports have shown that medium components contribute in determining vitamin C-dependent hydrogen peroxide generation (Clément et al., 2001; Mojic et al., 2014). Alpha-ketoglutarate and pyruvate, which directly interact with hydrogen peroxide, are able to quench its action (Nath et al., 1995). In addition, also different concentrations of iron, present in different commercially available growth media, could interfere with hydrogen peroxide production (Mojčić et al., 2014). Cell confluency is another important factor to be considered in evaluating vitamin C anti-cancer effect *in vitro* (Spitz et al., 1987). Cells are very often treated with high-dose vitamin C (> 1mM) at very low confluency, thus causing a massive and rapid cell death. Although *in vivo* vitamin C reaches millimolar concentrations, the *in vitro* cytotoxic effect could be overestimated.

Nevertheless, I found that my *in vitro* experimental system is quite representative of the results obtained *in vivo*. Cell treatment at 40-50% confluency with vitamin C at a high dose (defined as > 0.3 mM), but lower than 1mM, best mimic the *in vivo* effect on tumor progression reduction.

In my experimental system, CTR and STS media have identical composition, with the exception of glucose and serum concentration, which are responsible for the mimicking of the *ad libitum* feeding or fasting. Moreover, previous reports have shown that vitamin C toxicity is dependent on serum level, arising the possibility that this factor could be responsible for the enhancement of vitamin C toxicity mediated by STS condition. However, published data are controversial. In fact, it has been shown that serum levels can either enhance or inhibit vitamin C toxicity in different cell lines (Chen et al., 2005; Mojic et al., 2014).

Notably, in my model system, serum reduction and not glucose deprivation result to be the causative factor in mediating the enhanced toxicity of vitamin C *in vitro* (Appendix section, Figure 4). Importantly, my data support that the observed phenotype is not a medium artefact. In fact, the intracellular iron chelation, mediated by DFO, is able to rescue cell death induction, indicating that the observed effect is intracellular and does not depend on extracellular media components. Moreover, modulation of HO-1 expression by pharmacological and genetic intervention, indicates that STS sensitizes cells to vitamin C through a genetic program.

Finally, the *in vitro* results were confirmed by *in vivo* findings, supporting the consistency of my *in vitro* condition as a good model system to study the effect of STS.

3. STS/FMD-mediated sensitization to vitamin C is dependent on ROS generation

The molecular mechanism responsible for cancer cell-selective toxicity of vitamin C and the higher susceptibility of KRAS-mutant tumors, is still not completely elucidated. Recently, Yun and colleagues reported that the overexpression of glucose transporter GLUT1 in KRAS-mutant tumors contributes to the increased uptake of the oxidized form

of vitamin C (DHA) and thus its enhanced toxicity. In fact, it has been shown that under low glucose concentrations DHA uptake is increased and consequently cell death. However, GLUT1 overexpression is not sufficient to sensitize KRAS wild type cancer cells to vitamin C toxicity, suggesting that an oncogenic-reprogramming is required. Indeed, it has been found that intracellular DHA reduction to vitamin C depletes the glutathione reservoir causing an increase in ROS levels which in turn inhibits GAPDH. GAPDH inhibition results to be lethal in the highly glycolytic KRAS mutant cancer cells, leading to energetic crisis and cell death.

However, in my experimental condition, the lowering of glucose concentration, did not have a crucial role in STS-mediated sensitization to vitamin C. In fact, I found that the dramatic cell death induction caused by STS and vitamin C combo treatment is, as mentioned above, glucose level independent, but serum dependent (Appendix section, Figure 4).

Although I have not investigated the activity status of GAPDH in my model system, I found that STS and vitamin C combo treatment induces markers of metabolic stress, in particular causing the increase in AMPK activity (measured by its phosphorylation level), which indicates a drop of ATP levels. This energetic crisis could be compatible with glycolysis inhibition, however the effect of STS and vitamin C on glycolytic pathways requires additional studies.

Consistent with previous findings, ROS accumulation has a central role in STS-mediated effects. My data indicate that KRAS mutant CRC cells show an increase in ROS generation upon STS condition. However, despite the STS-induced effects, cancer cells do not show either DNA damage or death induction, possibly because of the up-regulation of a set of anti-oxidant enzymes, which can partially explain the limited efficacy of FMD in retarding tumor progression. The dramatic increase in ROS levels upon STS and vitamin C combo treatment, which induces both DNA damage and cell death, suggest that cancer cells are no longer able to counteract ROS generation. Indeed, the ability of anti-oxidants

(GSH, NAC), as well as hydrogen peroxide and anion superoxide scavengers (catalase and MnTMPyP) to reverse cell death induction, support the hypothesis that oxidative stress is the causative factor in STS-mediated sensitization to vitamin C.

My data, together with previously published work on the role of anti-oxidants in reverting vitamin C toxicity to cancer cells, have important implication for the clinic (Chen et al., 2011; Yun et al., 2015). In fact, pro-oxidant therapies, such as chemotherapy or pharmacological vitamin C, are very often co-administered with anti-oxidant agents, which could interfere with the treatment (Chen et al., 2011).

4. STS/FMD-mediated sensitization to vitamin C requires HO-1 down-regulation

Despite controversial results, a growing body of studies has consistently shown that high-dose vitamin C exerts its cytotoxicity to cancer cells by a hydrogen peroxide-dependent mechanism (Chen et al., 2005; Chen et al., 2007; Chen et al., 2008; Du et al., 2010; Ma et al., 2014).

However, normal cells are protected since they may both generate less hydrogen peroxide and maintain high antioxidant defenses. On the contrary, cancer cells, which are characterized by low expression level of anti-oxidant enzymes and increased ROS, are sensitive to vitamin C associated toxicity (Liu et al., 2004; Oberley, 2005; Du et al., 2012; Doskey et al., 2016). In fact, ROS-disruption of iron metabolism increases the level of labile iron, which in turn reacts with hydrogen peroxide to generate hydroxyl radical, through the Fenton reaction chemistry, consequently causing oxidative damages and cell death (Schoenfeld et al., 2017). Accordingly, it has been shown that the increase in ROS and LIP levels obtained through deletion of the mitochondrial *SOD* gene, makes tumor cells more sensitive to vitamin C toxicity (Schoenfeld et al., 2017). Interestingly, evidence

indicating that KRAS-driven cancers show higher level of ROS and LIP compared to KRAS wild type tumors, could partially explain their higher susceptibility to vitamin C toxicity (Kakhlon et al., 2002; Yang and Stockwell, 2008; Weinberg et al., 2010; Yun et al., 2015; Schoenfeld et al., 2017).

Here, I provide evidence showing that vitamin C treatment is able to up-regulate the expression level of the inducible-stress responsive protein heme-oxygenase-1 (HO-1). HO-1 catabolizes heme generating CO, biliverdin and free iron, which in turn induces ferritin expression. All these products show anti-oxidant and anti-inflammatory properties making HO-1 an anti-apoptotic and pro-survival enzyme (Was et al., 2010). It has been found that HO-1 is often overexpressed in tumors, in particular in response to chemotherapy, thus representing a mechanism of resistance and a poor prognostic factor for cancer patients (Was et al., 2010; Muliaditan et al., 2018).

My *in vitro* and *in vivo* data, together with those from previous published works, show that, despite vitamin C is safe to normal tissues, its anti-cancer effect in reducing tumor progression is limited (Hoffer et al., 2008; Padayatty et al., 2010; Stephenson et al., 2013; Welsh et al., 2013; Ma et al., 2014). The induction of HO-1 upon vitamin C administration indicates that it may represent a possible mechanism of oxidative stress defence that cancer cells put in place in order to limit vitamin C effectiveness. In accordance with the described mechanism of vitamin C action, HO-1 up-regulation, by inducing ferritin expression, may limit free iron pool, and consequently Fenton chemistry and oxidative damages.

My data show that FMD is able to revert vitamin C-mediated up-regulation of HO-1 both *in vitro* and *in vivo*. These findings raise the possibility that FMD potentiates vitamin C toxicity by impairing HO-1 pro-survival action. In support of this hypothesis, modulation of HO-1 expression dictates cancer cell susceptibility to vitamin C. In fact, HO-1 activation is able to revert STS-mediated cell death upon vitamin C co-treatment, whereas

HO-1 pharmacological inhibition, as well as genetic silencing, is able to sensitize cancer cells to its toxicity in nutrient-rich condition.

As a consequence of the decrease in HO-1 level, FMD and vitamin C co-treatment causes ferritin down-regulation, which is accompanied by an increase in free ferrous iron. Supporting the role of high ferrous iron level in enhancing pro-oxidant reactions, intracellular iron chelation is able to revert STS-mediated cell death upon vitamin C treatment.

These results support previous findings showing the connection between the level of free iron in cancer cells and their susceptibility to vitamin C toxic action. Thus FMD, increasing ferrous iron, possibly through the reduction of HO-1 and ferritin level, exacerbates Fenton chemistry, leading to an enhanced toxicity.

5. FMD and vitamin C combo treatment potentiates standard chemotherapy efficacy in KRAS mutant CRC mouse models

A large body of studies supports the potential role of vitamin C in enhancing standard therapy efficacy in pre-clinical and clinical studies (Ma et al., 2014; Du et al., 2015b; Schoenfeld et al., 2017). Vitamin C in combination with chemotherapy and/or radiotherapy reduces tumor progression in mouse model of ovary and pancreatic cancer and extends survival in mouse model of glioblastoma and lung cancer (Ma et al., 2014; Du et al., 2015b; Schoenfeld et al., 2017).

Moreover, clinical studies show that vitamin C combined with chemotherapy and/or radiotherapy results to be well tolerated and effective by extending patient's survival and in certain cases causing disease regression (Monti et al., 2012; Welsh et al., 2013; Schoenfeld et al., 2017). In addition, vitamin C is also able to even reduce chemo-associated side effects (Ma et al., 2014; Du et al., 2015b). In fact, in normal tissues, which

show efficient system of hydrogen peroxide removal, vitamin C acts as an excellent anti-oxidant agent, participating to protection from chemo-associated oxidative damages (Ma et al., 2014; Schoenfeld et al., 2017).

Given the promising results on the safety and effectiveness of pharmacological vitamin C integration in standard therapy, I evaluated whether a more potent intervention as FMD and vitamin C combo treatment, could further potentiate chemotherapy efficacy.

The standard care for KRAS mutant CRC patients often includes oxaliplatin, a DNA synthesis inhibitor, with limited efficacy. Importantly, I found that FMD-like condition is able to sensitize KRAS-mutant CRC cells to oxaliplatin toxicity. Notably, FMD and vitamin C combinatorial treatment, further potentiates chemotherapy effectiveness in delaying tumor progression in two different mouse models.

Our preliminary results on three patients bearing KRAS mutant colorectal cancer, show the tolerability, feasibility and possibly efficacy of FMD cycles in combination with standard chemotherapy, prompting us to translate our discoveries into the clinic.

Collectively, these data indicate that FMD and vitamin C co-treatment could represent a promising therapeutic option to enhance the efficacy of standard therapy, that still now show a very limited impact on survival of patients bearing KRAS-mutant cancer.

6. Conclusions

Collectively, my findings show that FMD is a potent non-toxic intervention able to enhance the efficacy of pharmacological vitamin C in KRAS mutant CRC cells.

The proposed mechanism of action responsible for FMD-mediated sensitization to vitamin C involves the down-regulation of the inducible stress-responsive protein HO-1 (Figure 1). FMD, by reverting vitamin C-dependent HO-1 induction, causes an increase of free ferrous ions. The boost of free iron levels, together with the FMD-mediated ROS increase, possibly enhance Fenton chemistry, finally leading to oxidative damage and cell death.

The expansion of ferrous ions seems to occur through ferritin down-regulation, however it can't be excluded that the FMD-induced ROS could also be partially involved in iron metabolism disruption. Thus, future analyses are required to elucidate all these possibilities.

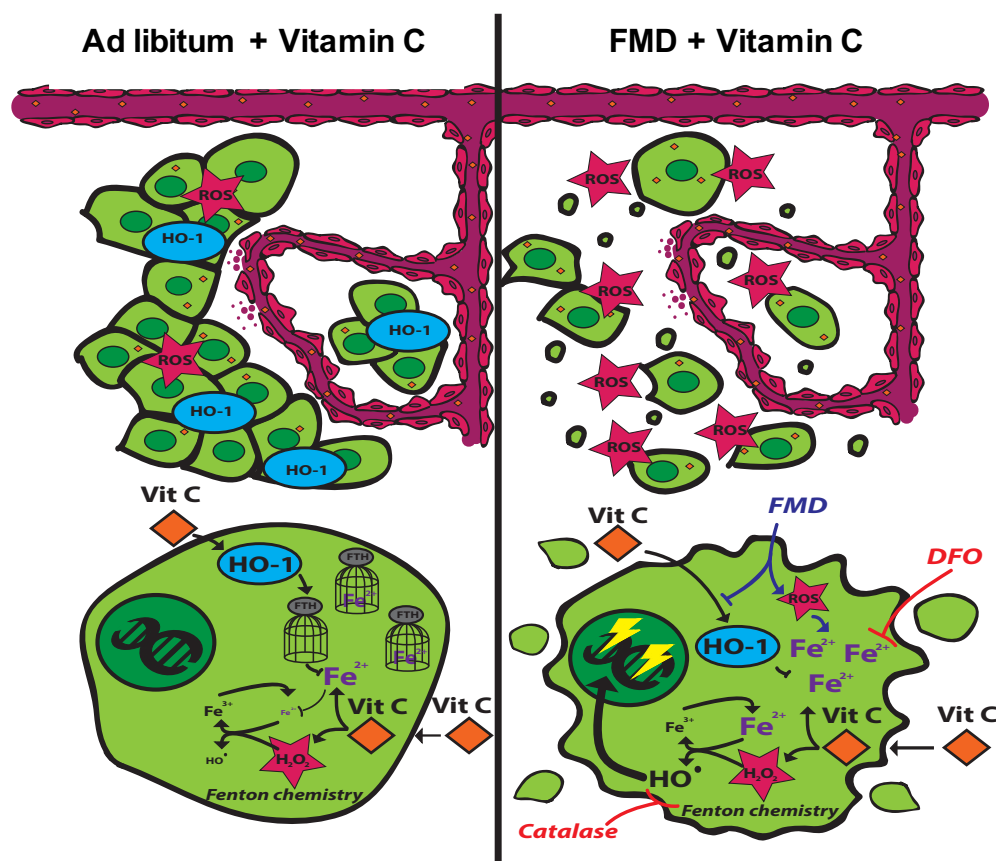


Figure 1. Working model of FMD-mediated sensitization to vitamin C. Vitamin C oxidation generates H_2O_2 and, by reacting with Fe^{2+} (Fenton chemistry), produces $\text{HO}^\bullet$. Vitamin C-mediated up-regulation of HO-1 induces FTH, thus limiting LIP and consequently the pro-oxidant chemistry responsible for vitamin C toxicity (left side). FMD is able to revert the HO-1 up-regulation induced by vitamin C. By doing this, FMD expands Fe^{2+} pool possibly through FTH down-regulation. The increase in Fe^{2+} , together with the FMD-induced boost in ROS levels possibly exacerbate Fenton chemistry leading to DNA damage (yellow bolts) and cell death. Catalase, by scavenging H_2O_2 , and DFO, by chelating iron, inhibit Fenton reaction and prevent cell death (right side).

In conclusion, FMD and vitamin C combination treatment represents a safe therapeutic option which can be easily integrated with standard therapy, to ameliorate the prognosis

for patients bearing KRAS-driven cancers. Furthermore, my pre-clinical results support the testing of the combination of FMD, vitamin C and oxaliplatin triple treatment in a future clinical trial on KRAS mutant CRC patients.

APPENDIX

APPENDIX

Chapter 1. Short-Term-Starvation effect on KRAS mutant CRC sensitivity to high-dose vitamin C toxicity

RESULTS - Chapter 1

1. Stable isotope labelling with amino acids (SILAC) analysis of proteome alteration of KRAS mutant CRC cells upon Short Term-Starvation

In collaboration with Dr. Paolo Soffientini (IFOM, Milan), we performed a SILAC (stable isotope labeling with amino acids in cell culture) coupled to LC-MS/MS (Liquid chromatography - mass spectrometry/ mass spectrometry) analysis on HCT116 cells, grown in CTR or STS condition.

STS condition, triggers considerable alterations in the protein expression profile of HCT116 KRAS mutant CRC cell line, compared to CTR. We identified 308 ($p < 0.05$) proteins that are statistically significantly ($p < 0.05$) altered in their expression. In particular, 167 proteins were up-regulated (fold change >1), whereas 141 were down-regulated (fold change < -1) upon STS.

Statistically significant proteins (red dots) were presented as scatter plot for graphical visualization (Figure 1).

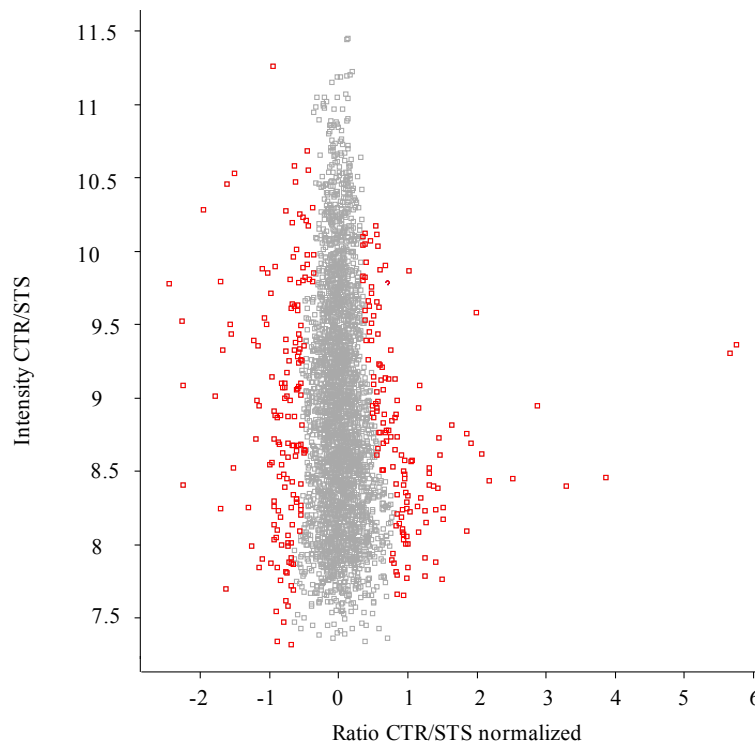


Figure 1. SILAC analysis of proteome alteration of KRAS mutant CRC cells upon STS.

HCT116 were grown in SILAC DMEM media supplemented with 1 g/l glucose, 10% dialysed serum (CTR) and "heavy" amino acids arginine and lysine, whereas cells that have incorporated "light" amino acids, were then grown in SILAC DMEM media supplemented with 0.5 g/l glucose and 1% dialysed serum (STS). Statistically significant proteins that differ in intensity in CTR versus STS condition are indicate by red boxes. Unaffected proteins are in grey boxes. Significance B outlier test was applied ($p < 0.05$).

Next, lists of up-regulated and down-regulated proteins in STS condition were analysed by using Enrichr tool, a comprehensive gene set enrichment analysis web server (<http://amp.pharm.mssm.edu/Enrichr>) (Kuleshov et al., 2016).

Notably, enrichment analysis of gene ontology (GO) Molecular Function gene set library of up-regulated proteins upon 48 hours of STS, reveals a significative enrichment in molecular processes involved in anti-oxidant response (oxidoreductase activity, acting on CH-CH group of donors [GO:0016628], $p = 0,001496$; oxidoreductase activity, acting on CH-OH group of donors [GO:0016616], $p = 0,005658$; oxidoreductase activity, acting on the aldehyde or ketone (oxo) group of donors [GO:0016620], $p = 0,03018$) (Figure 2). The

list of proteins involved in the anti-oxidant response [GO:0016209] is also reported (Table 1).

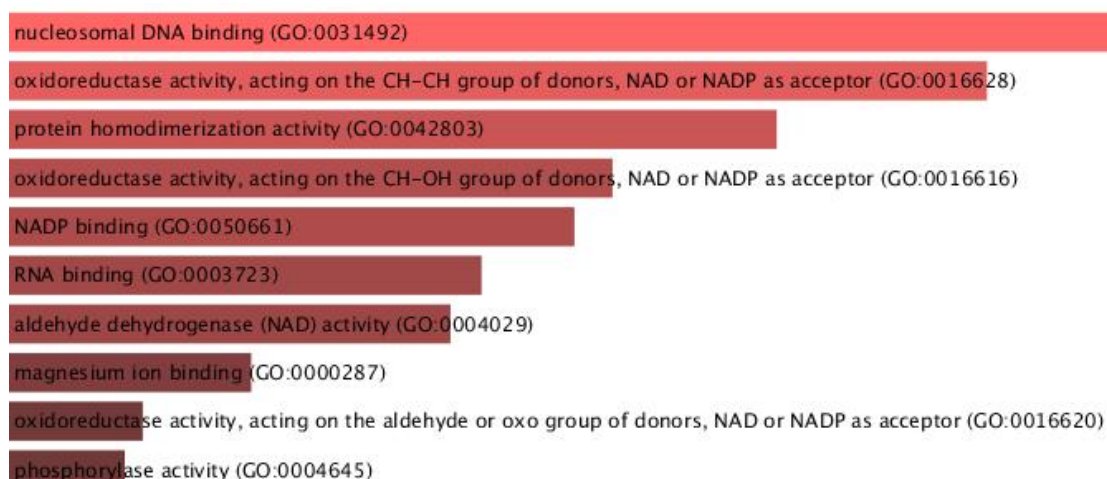


Figure 2. Gene Ontology (GO) enrichment analysis of proteins up-regulated upon STS. Gene Ontology (GO) enrichment analysis with Enrichr of the 167 proteins up-regulated in STS, compared to CTR condition, in KRAS mutant HCT116 cells. Bar graph show the top 10 enriched Molecular function GO gene-set library sorted by p-value ranking. The length of bars indicates the relative significance (Fisher Exact test; adjusted p-value: from 0,001045 [GO:0031492] to 0,03018 [GO:0004645]).

Enrichment analysis of GO Molecular Function gene set library of down-regulated proteins upon 48 hours of STS is listed in figure 3 (Figure 3).

Gene Symbol	Protein name
GSTO1	Glutathione S-transferase omega-1
PRDX2	Peroxiredoxin-2
APOM	Apolipoprotein M
MGST1	Microsomal glutathione S-transferase 1
PRDX5	Peroxiredoxin-5, mitochondrial
NQO1	NAD(P)H dehydrogenase [quinone] 1
SOD1	Superoxide dismutase [Cu-Zn]

Table 1. Proteins with anti-oxidant activity up-regulated upon STS. List of proteins with anti-oxidant properties [GO:0016209] up-regulated in KRAS mutant HCT116 grown for 48 hours in STS condition.

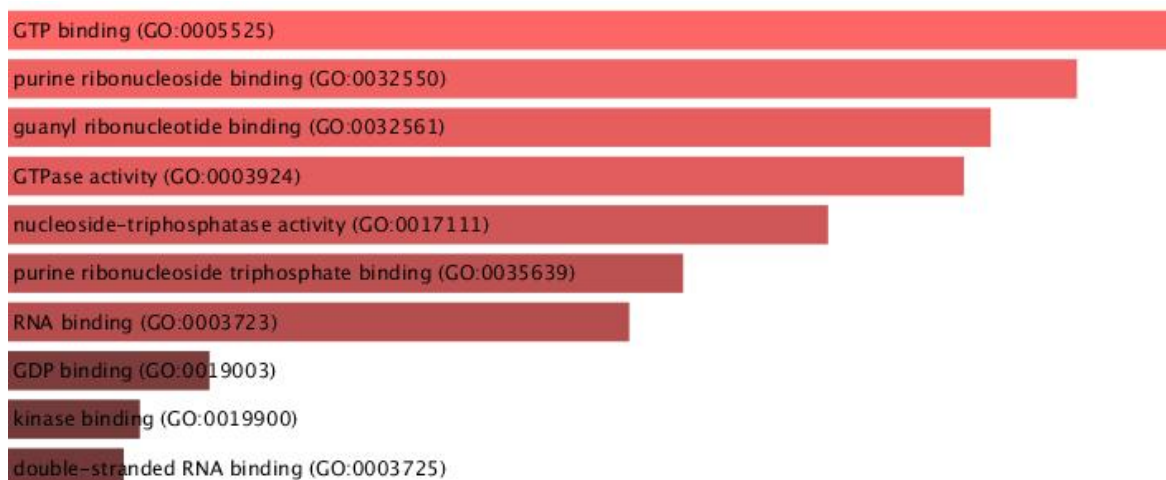


Figure 3. Gene Ontology (GO) enrichment analysis of proteins down-regulated upon STS.

Gene Ontology (GO) enrichment analysis with Enrichr of the 141 proteins down-regulated in STS, compared to CTR condition, in KRAS mutant HCT116 cells. Bar graph show the top 10 enriched Molecular function GO gene-set library sorted by p-value ranking. The length of bars indicates the relative significance (Fisher Exact test; adjusted p-value: from 0,01487 [GO:0005525] to 0,02052 [GO:0003725]).

2. Medium dissection to identify factors responsible for STS-mediated sensitization to vitamin C toxicity

2.1 STS-mediated sensitization to vitamin C is glucose-independent but serum-dependent

Given the dramatic cell death induction mediated by STS and vitamin C combinatorial treatment, I aimed at identifying medium component(s) which could be responsible for the observed effect.

The rationale of STS medium is to mimic the physiological effect of *in vivo* FMD cycles by lowering glucose and growth factors levels (reducing serum) (Raffaghello et al., 2008; Lee et al., 2012).

Firstly, I evaluated whether cell death induction depends on glucose or serum reduction or if the effect is mediated by reduction of both. To this purpose, HCT116 cells were grown

in media containing either low glucose and normal level of serum (fetal bovine serum, FBS), or normal glucose and low serum concentration, to investigate which condition best mimics the effect of STS in enhancing cell death upon vitamin C treatment. Interestingly I found that STS-mediated sensitization to vitamin C is fully dependent on serum reduction (Figure 4). Notably, even at higher concentration (4,5 g/L), glucose was not able to rescue the low serum-mediated effect (Figure 4).

Collectively, these data indicate that the cytotoxic action of STS and vitamin C combinatorial treatment results to be glucose-independent and serum-dependent.

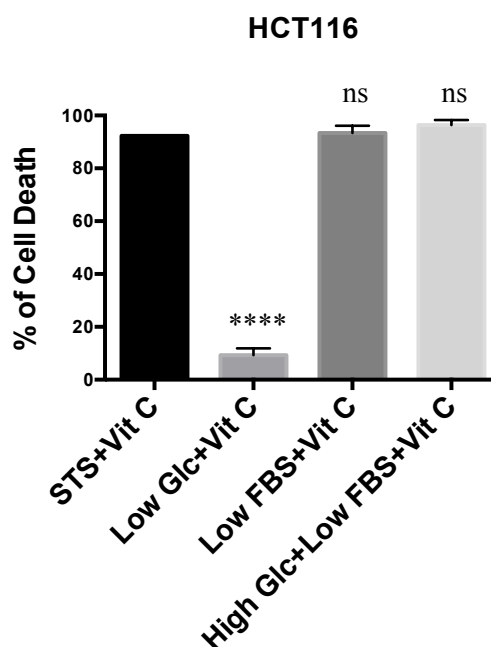


Figure 4. STS and vitamin C toxicity is glucose-independent but serum-dependent. HCT116 cells were grown in different conditions: STS (0,5 g/l glucose (Glc), 10% FBS); low glucose-normal serum medium (0.5 g/l Glc, 10% FBS), normal glucose-low serum medium (1g/L Glc, 10% FBS) and high glucose-low serum medium (4.5 g/l Glc, 1%FBS), for a total of 48 hours. At 24 hours, cells were treated with vitamin C (Vit C; 350 μ M) for further 24 hours. At 48 hours, viability was assessed by Muse Cell viability analyser. Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test; *****p* value $\leq$ 0.0001, ns not significant).

2.2 STS-mediated sensitization to vitamin C is IGF-1-, insulin- and EGF-independent

Next, I aimed at investigating which is/are the serum component(s) responsible for STS sensitizing effect.

Our previous works have already shown that STS/FMD-mediated sensitization to chemotherapy involves, at least in part, the reduction of IGF-1 level (Lee et al., 2010; Lee et al., 2012). For this reason, I tested whether IGF-1 supplementation during STS exposure, could, at least in part, reverse the cell death induction. Notably, I found that IGF-1 was not able to revert the STS-mediated sensitization to vitamin C (Figure 5A).

Given this result, I investigated whether other factors contained in the serum could be involved. Firstly, I tested insulin and epidermal growth factor (EGF) and I found that both of them were not involved STS-mediated effect (Figure 5B-C). The unresponsiveness of HCT116 to these three growth-factors could be explained by their mutational status in KRAS, which make the growth factor-dependent pathways constitutively active.

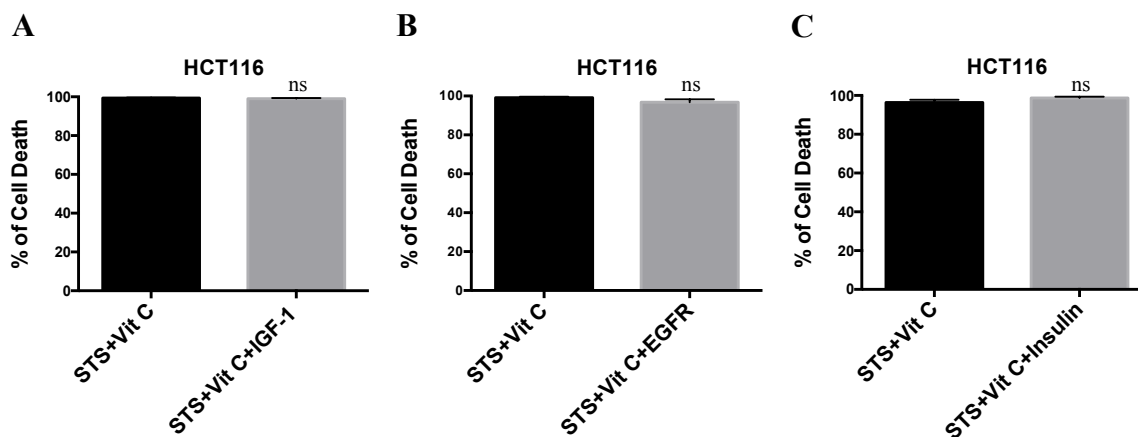


Figure 5. IGF-1, insulin and EGFR do not revert STS-mediated sensitization to vitamin C toxicity. HCT116 were grown in STS medium supplemented with (A) IGF-1 (250 ng/ml), or (B) insulin (100 ng/ml) or (C) epidermal growth factor (EGF; 200 ng/ml) for a total of 48 hours. At 24 hours, cells were treated with vitamin C (Vit C; 350 μ M) or vehicle for further 24 hours. At 48 hours, viability was assessed by Muse Cell viability analyser. Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test; ns not significant).

2.3 Glutamine and serine are not responsible for STS effects

Furthermore, I investigated whether a reduction in amino acids level could be the causative factor in mediating the serum-dependent effect. From a previous screening, I found that HCT116 are highly addicted to glutamine and, among the non-essential amino acid, to serine (Ser) (Figure 6). Given these results, I tested whether serine and glutamine supplementation could be responsible for STS-induced cell death upon vitamin C treatment.

Despite the addiction of HCT116 to both amino acids, I found that neither glutamine nor serine, were able to reverse the STS-sensitization to vitamin C (Figure 7).

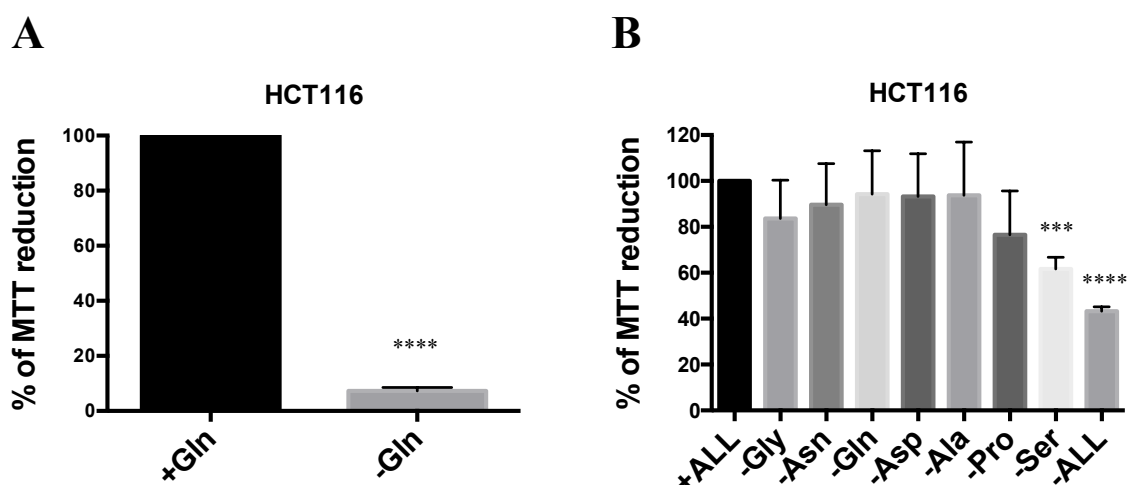


Figure 6. HCT116 cells are addicted to glutamine and serine. HCT116 were grown in a medium containing 1g/L glucose, 10% dialyzed serum in presence or absence of glutamine (A), or in presence or absence of non-essential amino acids (all, or excluded one by one) (B). At 48 hours, viability was assessed by MTT reduction. Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test; ****p* value $\leq$ 0.001, *****p* value $\leq$ 0.0001).

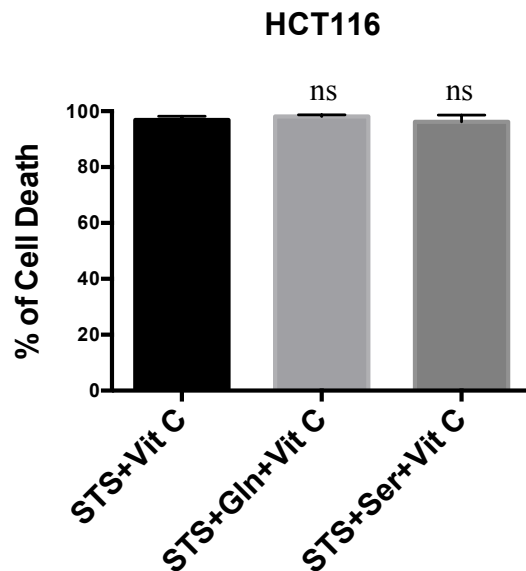


Figure 7. Glutamine and serine do not revert STS-mediated sensitization to vitamin C toxicity. HCT116 were grown in STS medium supplemented with glutamine (Gln; 2 mM) or serine (Ser; 5 mM) for a total of 48 hours. At 24 hours, cells were treated with vitamin C (Vit C; 350 μ M) for further 24 hours. At 48 hours, viability was assessed by Muse Cell viability analyser. Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test; ns not significant).

Next, I investigated whether essential amino acids (EAA) could be involved. To this purpose, cells were grown in STS medium supplemented with essential amino acids in double concentrations respect to standard medium. Also in this case, I found that essential amino acids are not able to reverse cell death induction (Figure 8). Collectively, these data support that amino acids are not the causative factors for the low serum-mediated effect.

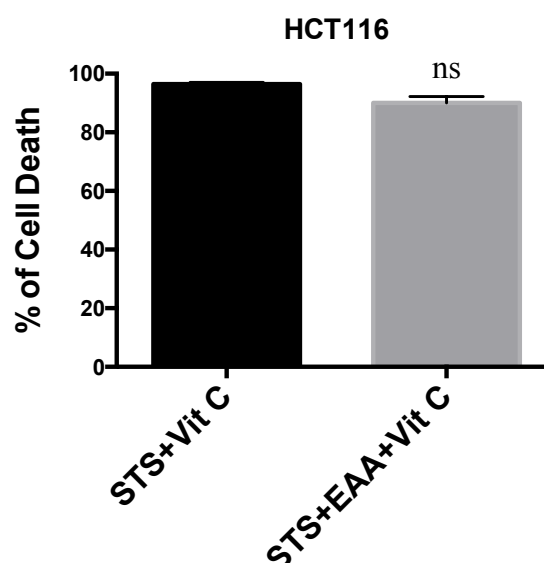


Figure 8. Essential amino acids are not involved in STS-mediated sensitization to vitamin C toxicity. HCT116 were grown in STS medium supplemented with a solution of all essential amino acids (EAA) for a total of 48 hours. At 24 hours, cells were treated with vitamin C (Vit C; 350 μ M) for further 24 hours. At 48 hours, viability was assessed by Muse Cell viability analyser. Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test; ns not significant).

2.4 Holo-transferrin, but not Apo-transferrin, is able to reverse STS and vitamin C cytotoxic effect

Iron bound-transferrin (holo-transferrin) and iron-free transferrin (apo-transferrin) are fundamental components present in the serum, which sustain cell growth in culture condition (Trowbridge and Shackelford, 1986)

For this reason, I tested whether transferrin supplementation could reverse cell death induction mediated by STS and vitamin C cotreatment. Interestingly, I found that only holo-transferrin, but not apo-transferrin, is able to rescue the observed cytotoxicity (Figure 9).

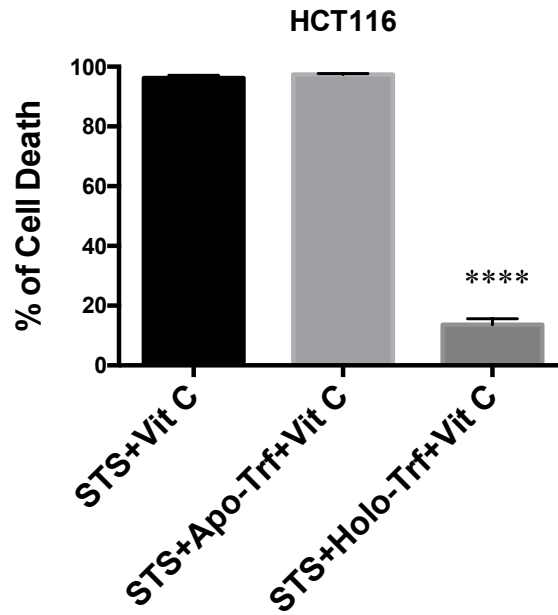


Figure 9. Holo-transferrin, but not apo-transferrin, reverses STS-mediated sensitization to vitamin C toxicity. HCT116 were grown in STS medium supplemented with apo-transferrin (Apo-Trf; 25 ng/ml) or holo-transferrin (Holo-Trf; 25 ng/ml) before vitamin C (Vit C; 350 μ M) exposure. At 48 hours, viability was assessed by Muse Cell viability analyser. Data are represented as mean $\pm$ SEM (two-tailed unpaired *t*-test; *****p* value $\leq$ 0.0001).

MATERIALS AND METHODS - Chapter 1

Medium dissection experiments

For medium dissection experiments, cells were seeded in 12-well plate (34'000 cells per well) in CTR medium (1g/l glucose, 10% FBS). The day after, cells were rinsed twice in 1X PBS, and grown in the following experimental conditions:

- CTR medium: DMEM no glucose (Life Technologies, #11966025) supplemented with 1 g/l glucose (Sigma-Aldrich, #G8769) and 10% FBS (Biowest, Cat. #: S1810)
- STS medium: DMEM no glucose (Life Technologies, #11966025) supplemented with 0.5 g/l glucose (Sigma-Aldrich, #G8769) and 1% FBS (Biowest, Cat. #: S1810)
- Low glucose and normal serum medium: DMEM no glucose (Life Technologies, #11966025) supplemented with 0.5 g/l glucose (Sigma-Aldrich, #G8769) and 10% FBS (Biowest, Cat. #: S1810)
- Normal glucose and low serum medium: DMEM no glucose (Life Technologies, #11966025) supplemented with 1 g/l glucose (Sigma-Aldrich, #G8769) and 1% FBS (biowest, Cat. #: S1810)
- High glucose and low serum medium: DMEM no glucose (Life Technologies, Cat. #: 11966025) supplemented with 4.5 g/l glucose (Sigma-Aldrich, Cat. #: G8769) and 1% FBS (Biowest, Cat. #: S1810)

For serum dissection experiments, STS media was supplemented with IGF-1 (PeproTech, Cat. #: 100-11) (final concentration of 250 ng/ml), EGF (Biomol, Cat. #: BPS-90201-3) (final concentration of 200 ng/ml) and insulin (Sigma-Aldrich, Cat. #: 11376497001) (final concentration of 100 ng/ml). Essential amino acids (Life Technologies, Cat. #: 11130) were supplemented as 2X concentration compared to standard media, L-serine (Sigma Aldrich, Cat. #: S4311) was supplemented to a final concentration of 5 mM, L-glutamine (Biowest, Cat. #: X0550) was supplemented to a final concentration of 2 mM. Holo-

transferrin (Sigma Aldrich, Cat. #: T0665) and apo-transferrin (Sigma Aldrich, Cat. #: T2252) were supplemented to a final concentration of 25 mg/ml.

After 24 hours, media were refreshed to ensure that glucose and serum levels were not completely exhausted, and after medium pH stabilization, cells were treated with 350 μ M vitamin C or vehicle (deionized water) (sodium ascorbate, Sigma-Aldrich, Cat. #: A4034) for the next 24 hours. Cell death was measured as indicated.

Non-essential amino acid and glutamine deprivation experiments

For measurement of the contribution of non-essential amino acids and glutamine in sustaining HCT116 growth, cells were seeded in minimal essential medium (MEM, no phenol red, 1g/L glucose) (Life Technologies, Cat. #: 51200), supplemented with 1% NEAA, 1% glutamine, 1% penicillin/streptomycin and 10% dialyzed serum (Life Technologies, Cat. #:26400044). The use of dialyzed serum is required, since it is designed to reduce the concentrations of small molecules, among which amino acids. In this way, it is possible to control the exact concentration of amino acids in the media, without variability due to the serum amino acidic content. Each single non-essential amino acid was purchased from Sigma-Aldrich. The day after, cells were rinsed twice in 1X PBS and grown in the followed experimental conditions for 48 hours:

- MEM supplemented with 10% dialyzed FBS, all NEAA and glutamine
- MEM supplemented with 10% dialyzed FBS, all NEAA, without glutamine
- MEM supplemented with 10% dialyzed FBS, glutamine, all NEAA except L-serine
- MEM supplemented with 10% dialyzed FBS, glutamine, all NEAA except L-proline
- MEM supplemented with 10% dialyzed FBS, glutamine, all NEAA except L-glutamic acid
- MEM supplemented with 10% dialyzed FBS, glutamine, all NEAA except L-aspartic acid
- MEM supplemented with 10% dialyzed FBS, glutamine, all NEAA except L-asparagine

- MEM supplemented with 10% dialyzed FBS, glutamine, all NEAA except L-alanine
- MEM supplemented with 10% dialyzed FBS, glutamine, all NEAA except L-glycine
- MEM supplemented with 10% dialyzed FBS, glutamine, without all NEAA

At the end of the experiment, cell viability was evaluated by MTT assay.

SILAC and analysis of proteins by LC-MS-MS

HCT116 cells were grown in medium for SILAC (DMEM without lysine and arginine, Life Technologies, Cat. #: A2493901) supplemented with 10% dialyzed serum, 1% penicillin/streptomycin, 2mM glutamine, 1% non-essential amino acids and isotopically labelled forms of arginine and lysine (heavy) or normal arginine and lysine (light). Cells were grown in “heavy” or “light” media for 6 passages to allow amino acids incorporation. Heavy lysine and arginine incorporation was checked by LS-MS-MS and results to be more than 95%.

Cells that have incorporated heavy amino acids arginine and lysine were then grown in SILAC DMEM media supplemented with 1 g/l glucose and 10% dialysed serum (CTR), whereas cells that have incorporated “light” amino acids, were then grown in SILAC DMEM media supplemented with 0.5 g/l glucose and 1% dialysed serum (STS), SILAC heavy and light labelled cells were lysed with in Urea buffer (8 M Urea, 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA), and proteins quantified by Bradford. CTR (heavy) and STS (light) samples were mixed proportionally to 1:1 based on protein extract amount. About 30 µg of mixed proteins for each sample were reduced by TCEP, alkylated by chloroacetamide, digested by Lys-C and Trypsin (Kulak et al., 2014) and peptides desalted on StageTip C18 (Rappsilber et al., 2003). 1 µl of each sample was analyzed as technical replicate on a LC (liquid-chromatography)–ESI–MS-MS quadrupole Orbitrap QExactive-HF mass spectrometer (Thermo Fisher Scientific). Peptides separation was achieved on a linear gradient from 93% solvent A (2% ACN, 0.1% formic acid) to 60% solvent B (80% acetonitrile, 0.1% formic acid) over 110 minutes and from 60 to 100% solvent B in 8 min

at a constant flow rate of 0.25 μ l/min on UHPLC Easy-nLC 1000 (Thermo Scientific) connected to a 23-cm fused-silica emitter of 75 μ m inner diameter (New Objective, Inc. Woburn, MA, USA), packed in-house with ReproSil-Pur C18-AQ 1.9 μ m beads (Dr Maisch GmbH, Ammerbuch, Germany) using a high-pressure bomb loader (Proxeon, Odense, Denmark). MS data were acquired using a data-dependent top 20 method for HCD fragmentation. Survey full scan MS spectra (300–1650 Th) were acquired in the Orbitrap with 60000 resolutions, AGC target 3^{e6} , IT 20 ms. For HCD spectra, resolution was set to 15000 at m/z 200, AGC target 1^{e5} , IT 80 ms; NCE 28% and isolation width 2.0 m/z .

For quantitative proteomic, raw data were processed with MaxQuant (ver. 1.5.2.8) searching against the database uniprot_cp_human_2015_03 setting labelling Arg10 and Lys8, trypsin specificity and up to two missed cleavages. Cysteine carbamidomethyl was used as fixed modification, methionine oxidation and protein *N*-terminal acetylation as variable modifications. Mass deviation for MS-MS peaks was set at 20 ppm. The peptides and protein false discovery rates (FDR) were set to 0.01; the minimal length required for a peptide was six amino acids; a minimum of two peptides and at least one unique peptide were required for high-confidence protein identification. The lists of identified proteins were filtered to eliminate reverse hits and known contaminants.

Statistical analyses were done using the Perseus program (Version 1.5.1.6) in the MaxQuant environment. Ratios H/L and proteins intensity normalized were transformed in log2 and log10 respectively. Significance B outlier test was applied and statistical significance based on magnitude fold-change was established at $p\text{-value} < 0.05$.

Chapter 2

Effects of dietary macronutrient composition on longevity and health-span

INTRODUCTION - Chapter 2

Aging and age-related diseases represent one of the greatest socio-economic challenges that developed countries will face in the near and distant future. Over the last 100 years, life expectancy has dramatically increased, however this was not accompanied by an improvement in the quality of life, defined as “health-span” (Hung et al., 2011). In fact, more than 70% of people over 65, experience at least two chronic conditions such as: cardiovascular diseases, chronic respiratory diseases, neurodegenerative disorders and cancer (Hung et al., 2011). For this reason, there is an urgent need to identify novel therapeutic interventions able to slow aging, thus extending lifespan and improving health-span.

A large body of studies shows that diet can exert a powerful effect on life expectancy and disease incidence (Longo and Finch, 2003; Fontana et al., 2010). We have previously shown that fasting and fasting-mimicking diet (FMD), which is a low calorie, protein and sugar diet, is able to improve health-span, cognitive function and multi system regeneration in rodents and to reduce risk factors for age-related disorders in humans (Brandhorst et al., 2015; Wei et al., 2017). In particular, mouse studies reveal that FMD impairs the progression of a wide range of tumor types, by sensitizing cancer cells to chemotherapy effects, while preserving normal tissues, promoting immune system

rejuvenation and tumor immune-surveillance (Lee et al., 2012; Cheng et al., 2014; Di Biase et al., 2016). The molecular mechanism responsible for the protective role of FMD against age-related pathologies, involves, at least in part, the down-regulation of glucose and insulin growth factor (IGF-1) levels, and the enhancement of protective pathways including stress resistance genes and DNA repair machinery (Lee et al., 2012; Cheng et al., 2014; Di Biase et al., 2016).

Studies on centenarians from different geographical areas such as Japan (Okinawa), Costa Rica (Nicoya), California (Loma Linda), Greece (Ikaria) and Southern Italy, have shown that these individuals follow a common type of diet, which is mainly based on high level of vegetables, unsaturated fats, omega-3 fats, low protein intake derived mainly from legumes and moderate fish consumption (Fraser, 1999; Davinelli et al., 2012; Grosso et al., 2014; Willcox et al., 2014). This common dietary regimen is associated with a reduced incidence of chronic diseases, such as diabetes, inflammation, cardiovascular defects and different types of cancers (colon, prostate and breast malignancies) (Willcox et al., 2014). Whereas, in developed countries the incidence of obesity and age-related pathologies has risen to very high levels, mainly due to a shift toward diets rich in refined oils, cereals, sugars and processed-meats (Stoll, 1998; Cordain et al., 2005; Kanoski and Davidson, 2011). These data, together with rodent and molecular studies, strongly support the role of a “longevity diet” in protecting individuals from aging processes and age-related diseases.

Our project has the goal to identify specific dietary interventions able to slow aging and decrease the incidence of age-related pathologies. Our main goal is to act on the entire aging process, thus preventing not only each single pathology but a wide range of disorders.

To this purpose, we will evaluate the effects of different macronutrients on lifespan and health-span in mouse models. In particular, we want to test typical western diet, characterized by high level of sugars, animal-derived proteins, saturated fats; and a longevity-centenarian’s based diet, which is composed by high complex carbohydrates,

high unsaturated fats, low proteins level and modest fish consumption. In line with this, we will assess how the different macronutrient compositions could impact on risk factors and incidence of cancer, inflammatory, neurological, cardiovascular disorders, motor and cognitive function.

Therefore, the major aim of this project is to determine potential dietary interventions able to slow aging processes and protect individuals from pathologies of the elderly, that nowadays result to be more diffused and also anticipated at young age.

PRELIMINARY RESULTS – Chapter 2

Here, I present the preliminary data obtained in the first 150 Days (5 months) of administration of different types of diets, including bodyweight and caloric intake. Blood was collected at 16 months of age (before diet administration) and it will be collected after 8 months of diet administration for analysis of additional markers for age-related pathologies (cholesterol; triglycerides; ketone bodies; alanine transaminase; creatinine; urea; IGF-1; IGF-1BP1 and insulin).

1. Effect of standard diet, western diet and longevity diet on mouse bodyweight

Our goal is to evaluate the effect of macronutrient composition on longevity and health-span. To this purpose, C57/J male mice were fed with different types of diet starting at 16 months of age.

Three types of diets were tested:

- standard diet: a nutritional balanced and complete regimen utilized for maintenance of mice in laboratories;
- western diet: a diet containing high levels of glucose and fats, which is typical of industrialized countries;
- longevity diet: a diet which has been formulated on the basis of the centenarian dietary regimen, containing low levels of proteins and refined sugars, high levels of complex carbohydrates and unsaturated fats.

The composition of each diet is listed in table 2 (Table 2).

Western Diet	% kcal from
Protein	15 (<i>Caseine</i>)
Fats	43 (<i>Milk fats</i>)
Carbohydrates	42 (<i>Sucrose, starch</i>)

Longevity Diet	% kcal from
Protein	9 (<i>Chickpeas</i>)
Fats	25 (<i>Olive oil, fish oil</i>)
Carbohydrates	66 (<i>Vegetable soup</i>)

Standard Diet	% kcal from
Protein	22 (<i>Soya meal</i>)
Fats	13 (<i>Soya oil</i>)
Carbohydrates	65 (<i>Wheatfeed</i>)

Table 2. Macronutrient composition of western, longevity and standard diets. Proteins, fats and carbohydrates of each diet are indicated as percentage of gross energy.

Moreover, we aimed also at evaluating the potential beneficial effect of human-like fasting-mimicking diet (HFMD) (5 days regimen) in mice undergoing standard diet or western diet.

Mouse bodyweight and caloric intake have been recorded weekly since the start of the experiment. As shown in figure 10, mice undergoing western diet have shown a dramatic increase (more than 30%) of their initial bodyweight, over the first 5 months of the new dietary regimen. Whereas, mice fed with longevity diet, have lost more than 10 % of their initial bodyweight. Mice under a standard diet regimen have maintained a constant weight.

Mice undergoing monthly FMD have not lost more than 20% of weight during each cycle that has been immediately recovered upon refeeding. Particularly, mice refed with standard diet have maintained a nearly constant bodyweight, whereas mice refed with western diet have gained nearly 20 % of their initial bodyweight (Figure 10).

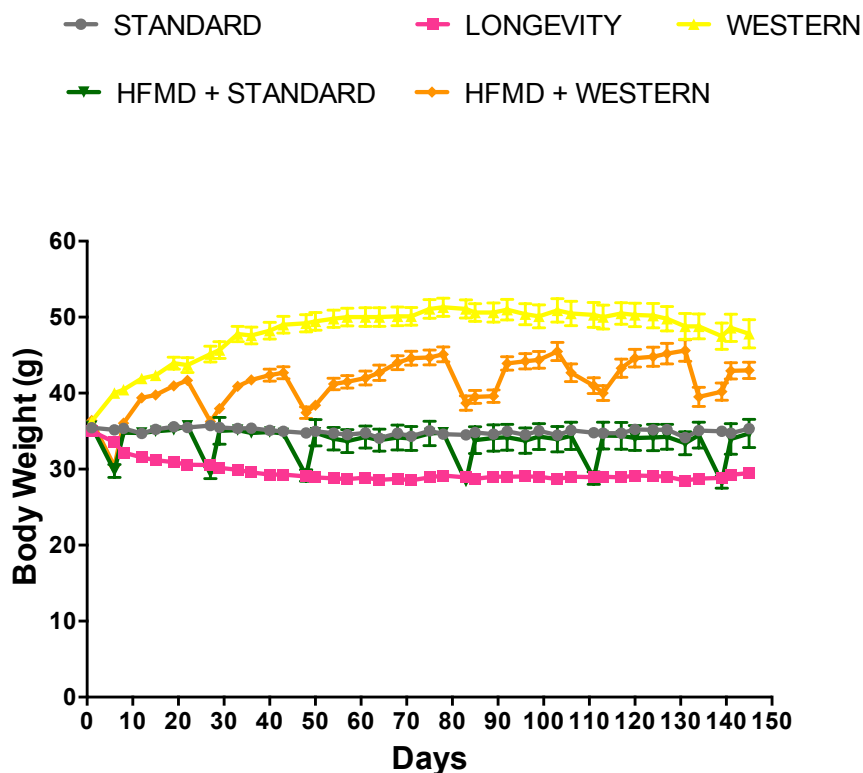


Figure 10. Mouse bodyweight. Bodyweight (g) of mice fed with standard, longevity and western diets was recorded twice a week. Mice undergoing cycles of human-like fasting-mimicking diet (HFMD) are also reported. Data are represented as average $\pm$ SEM (n=24-31 per group).

In addition, weekly caloric intake measurement indicates that despite bodyweight, mice fed with longevity diet has the highest caloric intake (Figure 11), supporting the fundamental role of macronutrient ratio and not caloric intake in determining bodyweight and possibly health status (Solon-biet et al., 2014).

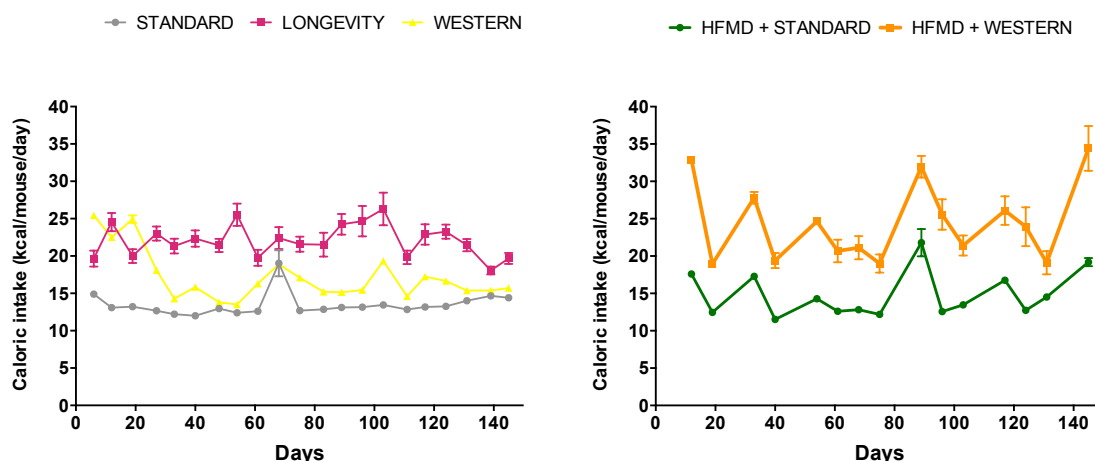


Figure 11. Mouse caloric intake. Caloric intake of mice fed with standard, longevity and western diet is shown in left panel. Caloric intake of mice undergoing human-like fasting-mimicking diet (HFMD) cycles followed by standard or western diet is shown in right panel (HFMD days are excluded from the graph and only caloric intake in refeeding period is shown). Caloric intake is recorded every week. Data are represented as average $\pm$ SEM (n=24-31 per group).

2. Effect of longevity diet supplemented with fish proteins on mouse bodyweight

Given the excessive weight loss observed in mice fed with longevity diet regimen, we decided to add a supplementation of fish as additional protein sources increasing the final percentage of kcal from protein to 11%. The composition of the supplemented longevity diet is listed in table 2 (Table 3).

Longevity Diet-supplemented	% kcal from
Protein	11 (<i>Chickpeas and fish</i>)
Fats	27 (<i>Olive oil, fish oil</i>)
Carbohydrates	62 (<i>Vegetable soup</i>)

Table 3. Macronutrient composition of longevity diet supplemented with fish proteins. Proteins, fats and carbohydrates of longevity diet are indicated as percentage of gross energy.

Despite the addition of fish, we noticed that longevity diet contains low level of methionine (0.12%) compared to standard diet (0.29% from natural sources + 0.14% supplement). Thus, a supplementation of 0.14% methionine was added in longevity diet formulation.

From the moment (Day 150) that the diet was switched, mice have partially recovered their bodyweight as shown in figure 12 (Figure 12). These results support that legumes couldn't be a sufficient source of proteins, thus promoting the healthy effect of a fish-based diet compared to a vegan diet.

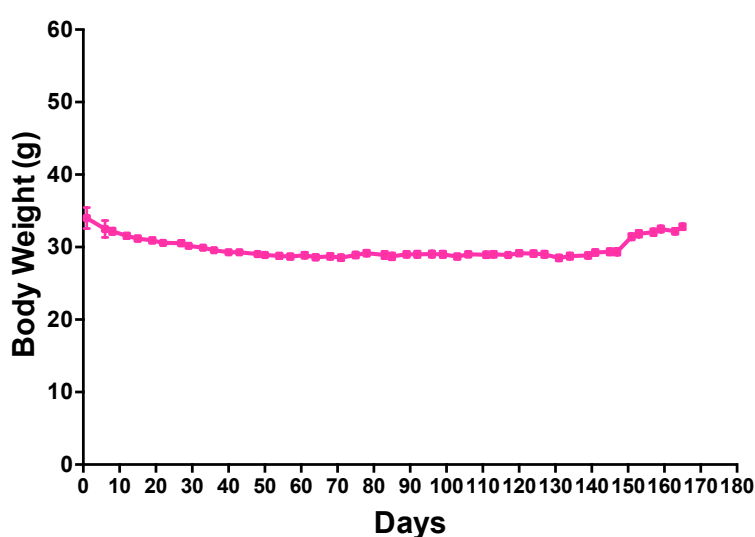


Figure 12. Mouse bodyweight post switching to modified-longevity diet. Bodyweight of mice fed with longevity diet supplemented with fish proteins and methionine was recorded every week. Diet switch occurred at day 150. Data are represented as average $\pm$ SEM (n=26).

MATERIALS AND METHODS - Chapter 2

Mice

Male C57/J mice were purchased from Charles River at 4 months of age and housed under specific pathogen-free conditions. Mice showing signs of pain and stress were sacrificed. Mice were analysed by gross necropsy and organs were fixed for future immunohistochemical analysis.

All experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC) and were approved by the Italian Ministry of Health.

Rodent diets

Longevity diet, western diet and FMD cycles started at 16 months of age. Mice were provided *ad libitum* access to the diets.

Longevity diet was formulated by our laboratory and produced by Mucedola (Milan). The first version of longevity diet is a low protein, high complex carbohydrates and high unsaturated fats dietary regimen (gross energy: 4.49 kcal/g). In the second version, longevity diet was supplemented with fish proteins and methionine supplementation, as described in the result section (gross energy: 4.41 kcal/g). Western diet was purchased by Envigo (Cat #: TD88137), which is characterized by high level of saturated fats and sugar (gross energy: 4.5 kcal/g). Standard diet (VRFI (P)) was purchased by Special Diet Services (SDS) (Cat #: 801900) (gross energy: 3.88 kcal/g).

Human version of fasting-mimicking diet (HFMD) is a plant-based diet (containing a mixture of vegetable soup, olives, energy bars) causing fasting-like effects, while providing high nourishment. HFMD is a 5 day-regimen; during day 1 mice undergo a caloric reduction of 50% of their normal caloric intake, whereas from day 2 to day 5 a 70%

caloric intake reduction. Mice undergoing FMD and western diet were refed with standard diet for three days before switching to western diet to avoid overfeeding.

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