UNIVERSITÀ DEGLI STUDI DI GENOVA

SCUOLA DI SCIENZE MEDICHE E FARMACEUTICHE

CORSO DI DOTTORATO DI RICERCA in EMATO ONCOLOGIA E MEDICINA INTERNA CLINICO-TRASLAZIONALE - CURRICULUM GERONTOLOGIA, FISIOPATOLOGIA DELLE MALATTIE GERIATRICHE E MEDICINA ANTI-AGING (XXXIV CICLO)

TESI DI SPECIALIZZAZIONE

PERIODIC FASTING AS A TOOL FOR DRUG REPURPOSING: ENHANCEMENT OF CHOLESTEROL BIOSYNTHESIS INHIBITORS ANTITUMOR EFFECTS VIA DIETARY RESTRICTION

Relatore:

Prof. Alessio Nencioni

Candidato:

Amr Khalifa

ANNO ACCADEMICO 2021-2022

INDEX

1. ABSTRACT

Drug repurposing through fasting could pinpoint new cancer cell liabilities and define new treatment options. By screening over 800 approved drugs in PK9 pancreatic ductal adenocarcinoma (PDAC) cells, we identified azoles, inhibitors of cholesterol biosynthesis, as agents whose cytotoxic activity against cancer cells was synergistically enhanced by starvation conditions. We hypothesized that starvation and azoles would cooperate by blunting cholesterol production in PDAC cells. Interestingly, we found other cholesterol inhibitors had their antitumor effects strongly enhanced by starvation. In addition, combined starvation and cholesterol inhibitors reduced tumor growth in gastrointestinal mouse models and intracellular cholesterol levels both *in vitro* and *in vivo*. Furthermore, methyl-beta-cyclodextrin, which depletes intracellular cholesterol, and starvation showed a synergistic cytotoxic effect against Capan-1 and MIA PaCa-2 cells (another PDAC cell line). Adding back cholesterol or LDL in gastrointestinal tumor cells/xenografts prevented the synergistic interaction between starvation and cholesterol inhibitors. Combined therapy inhibited pAKT (partially restored with LDL add-back) whereas, its antitumor activity was abolished by the simultaneous supplementation with circulating growth-promoting factors (IGF1, insulin, and leptin). In addition, combined therapy reduced mitochondrial oxidative phosphorylation (OXPHOS) and energy status in gastrointestinal tumor xenografts, whereas cholesterol restoration abolishes this effect. Taken together, these findings support the rationale for conducting clinical studies to assess the safety, feasibility, and activity of combining periodic cycles of fasting with inhibitors of cholesterol biosynthesis in cancer patients.

1. INTRODUCTION

One key issue that cancer treatment currently faces consists in its extremely substantial costs, high attrition rates, and deliberate pace of traditional drug discovery which are all becoming hard to sustain by health care systems. Identifying oncological applications for drugs that are already approved for other medical indications, drug repurposing, is considered to be a possible solution for such an issue (1). Indeed, while it is estimated that the costs of designing, testing, and bringing to the market a new drug typically exceed 10 billion dollars, bringing a repurposed drug to market was calculated to cost 300 million US dollars on average (2, 3). An additional advantage of repositioning agents is that the time needed for translating discovery into a possible therapy is much shorter since old drugs have normally already passed clinical trials and FDA/EMA-approval, leading to a speed-up of the entire process, but also to higher predicted safety compared to a newer agent (1, 3). There is strong hope that metabolic/dietary interventions will help with drug repurposing in oncology (4) and indeed here our recent findings indicate that fasting-based dietary regimens may well succeed in unleashing antiproliferative/anticancer effects of already approved drugs.

Dietary and lifestyle-related factors are key determinants of the risk of developing cancer, with certain cancers being more dependent on dietary habits than others (5-9). Consistent with this notion, obesity was independently associated with subsequent cancer mortality in seven prospective cohort studies from the U.S., Australia, and Finland (10), leading to guidelines on nutrition and physical activity for reducing the risk of developing cancer (8). In addition, given the emerging propensity of cancer cells, but not of normal tissues, to disobey anti-growth signals (11) and their inability to properly adapt to fasting conditions (12, 13), there is growing interest in the possibility that certain calorie-limited diets could also become an integral part of cancer prevention

and, perhaps, of cancer treatment as a means to increase efficacy and tolerability of anticancer agents (12-14). To date, the main treatment options for cancer include surgery when possible and chemotherapy and/or radiotherapy, plus target-specific drugs such as tyrosine kinase inhibitors, immunotherapy, hormone therapy, and others. Treatment planning is usually guided by tumor type and stage and available resources. Although these novel target-specific drugs may largely replace chemotherapy and radiotherapy, traditional treatments are unlikely to be phased out for decades. In addition, the high cost of many novel cancer therapies such as immunotherapy will limit their availability to a large portion of the world population, making chemotherapy a viable treatment option for many years to come. Most chemotherapeutic drugs target rapidly dividing cancer cells but can also damage normal cells (e.g., bone marrow, gastrointestinal tract, heart, hair follicle) generating various side effects including myelosuppression, fatigue, vomiting, diarrhea, and even, in some cases, death. This greatly limits the use of chemotherapy and treatment remains suboptimal (15). Thus, some dietary approaches (Table 1) have the potential to both promote the protection of normal cells against chemotherapy, radiotherapy, and other treatments and enhance their efficacy by generating a hostile environment for cancer cells.

1.1 DIETARY APPROACHES IN CANCER

1.1.1 Fasting

The dietary approaches based on periodic fasting [or short-term starvation (STS)] that have been investigated more extensively in oncology, both preclinically and clinically, include water-only fasting (abstinence from all food and drinks except for water) and modified fasting, i.e. vegan, very low-calorie, low-sugar, low-protein diets that typically last two-to-five days. Modified fasting regimens include medical food kits, such as the “fasting-mimicking diet” (FMD) that was developed by the University of Southern California spin-off company, L-Nutra (12, 13, 16, 17).

Preclinical data indicate that fasting should protect normal cells that, when nutrients are absent, can reallocate energy from reproduction and growth processes toward maintenance pathways. According to the model proposed by the Longo lab, this switch to a protected mode is not possible for cancer cells because oncogenes stimulate continued growth, preventing the activation of stress resistance. Preliminary clinical data indicate that a fast of at least 48 hours may be required to achieve clinically meaningful effects in oncology, such as preventing chemotherapy-induced DNA damage to healthy tissues and helping to maintain patient quality of life during chemotherapy (18-20).

In a clinical study of 3 monthly cycles of a 5-day FMD in generally healthy subjects, the diet was well tolerated and reduced trunk and total body fat, blood pressure, and insulin-like growth factor-1 (IGF1) levels (21). In previous oncological clinical trials, fasting or modified fasting have typically been administered every 3–4 weeks, for example, in combination with chemotherapy regimens, and their duration has ranged between 1 and 5 days (18-20, 22-24). Importantly, no serious adverse events (level G3 or above, according to Common Terminology Criteria for Adverse Events) were reported in these studies.

1.1.2 Ketogenic diets

Ketogenic diets (KDs) consists of high fat (55% - 60%), moderate protein (30% - 35%), and low carbohydrates (5% - 10%) (25-27). In humans, a KD decreases IGF1, glucose, and insulin levels, though these effects are affected by the types and levels of protein and carbohydrates in the diet (28). Notably, KDs may be effective for preventing the increase in glucose and insulin that typically occurs in response to phosphatidylinositol 3-kinase (PI3K) inhibitors, which were proposed to limit their efficacy (29). KDs, as single agents, can induce anticancer effects, (26, 29-34). Additionally, KDs were reported to be an effective adjuvant to chemotherapy, antiangiogenic

treatments, radiotherapy, and PI3K inhibitors for the treatment of various types of malignancies (29, 30, 35, 36). It was previously reported that KDs produce neuroprotective effects in the central nervous system (37, 38). However, it is not fully known whether KDs also have pro-regenerative effects similar to fasting and whether KDs might protect against chemotherapy-induced toxicity in living mammals. Interestingly, the regenerative effects of starvation appear to be maximized by switching from the fasting-response mode, which involves the death of many cells and breakdown of cellular components, and the re-feeding time, in which tissues and cells undergo reconstruction (39). Since in KDs there is no entry into a starvation mode, cannot promote a major breakdown of intracellular components and tissues, and do not include a refeeding period, they are unlikely to cause the type of coordinated regeneration observed during the fasting refeeding.

1.1.3 Caloric restriction

Caloric restriction (CR) involves a decrease of overall caloric intake by 30–40% typically through carbohydrate limitation that would allow an individual to maintain a normal weight (40, 41). While chronic CR and diets deficient in specific amino acids are very different from periodic fasting, they share with fasting and modified fasting a more or less selective restriction in nutrients, and they have anticancer effects (33, 34, 42-50). CR is proved to be a potent intervention in increasing the median end maximum lifespan in mammals (51) and protecting against age-related diseases (52). In addition, it is very effective in robustly inhibiting the growth of diverse tumor types, including breast, lung, prostate, brain, pancreatic, and colorectal cancers (53-55). However, CR can cause side effects, such as changes in physical appearance, increased cold sensitivity, reduced strength, menstrual irregularities, infertility, loss of libido, osteoporosis, slower wound healing, food obsession, irritability, and depression. Furthermore, there are substantial concerns that it may exacerbate malnutrition and that it will unavoidably cause excessive loss of lean body mass (40,

41, 53, 56, 57). CR reduces fasting blood glucose levels, though they remain within the normal range (41). In humans, chronic CR does not affect IGF1 levels unless a moderate protein restriction is also implemented (58). Studies show that by reducing mTORC1 signaling in Paneth cells, CR augments their stem cell function and that it also protects reserve intestinal stem cells from DNA damage (59, 60), but it is unknown whether pro-regenerative effects in other organs are also elicited by CR. Thus, the available data suggest that fasting or modified fasting creates a metabolic, regenerative, and protective profile that is distinct and probably more potent than that elicited by a KD or CR.

1.1.4 Effects of fasting on hormone and metabolite levels

Metabolic and circulating hormonal changes (reduced levels of glucose, IGF1, insulin, and leptin and increased levels of adiponectin) that are typically observed in response to fasting can exert anticancer effects (61-63) and/or afford protection of healthy tissues from side effects (reduced levels of IGF1 and glucose). Because ketone bodies can inhibit histone deacetylases (HDACs), the fasting-induced increase of ketone bodies may help slow tumor growth and promote differentiation through epigenetic mechanisms (64). However, the ketone body acetoacetate has been shown to accelerate, instead of reducing, the growth of certain tumors, such as melanomas with mutated v-raf murine sarcoma viral oncogene homolog B (BRAF) (65). Those changes for which there is the strongest evidence for a role in the beneficial effects of fasting against cancer are the reductions in the levels of IGF1 and glucose. At the molecular level, fasting reduces intracellular signaling cascades including IGF1R–AKT–mTOR–S6K and cAMP–PKA signaling, increases autophagy, helps normal cells withstand stress, and promotes anticancer immunity (17, 66-68).

Table 1 | Dietary approaches used in oncology (4, 69, 70).

Diet type	Composition & restriction in calories	Length	IGF1 & glucose reduction (humans)	Ketone bodies increase (humans)	Regenerative & protective effects	Anticancer effect
Fasting	- Water only fasting - >50%	Fast for 16 hours, with an 8-hour eating window for 2–5 consecutive days per month	Yes	Yes	Potent	All have an anticancer effect which is augmented when used as a complement for standard therapeutics
Modified fasting	- Vegan and low-protein and low-sugar, high-plant-based fat composition, with micronutrient supplementation - >50%	2–5 consecutive days per month	Yes	Yes	Potent	
CR	- Reduction in all diet constituents except for vitamins and minerals - 20-40%	Chronic	IGF1; decrease only in the presence of protein restriction (58) Glucose; No	No	Yes, but not as potent as fasting or modified fasting	
Ketogenic diet	- High-fat, low carbohydrate composition, with adequate protein content - None	Chronic	IGF1; Yes Glucose; No	Yes	NA	

NA, not available.

1.1.5 Differential stress sensitization: increasing the death of cancer cells

Most dietary interventions when used alone have limited effects on cancer treatment. According to the differential stress sensitization (DSS) hypothesis, combining these dietary interventions such as fasting with another treatment is much more promising (12, 13). This hypothesis predicts that tumor cells can adapt to limited concentrations of nutrients and oxygen, however, many types of tumor cells are not able to prevent changes accompanied by the combination of fasting and standard therapeutics such as nutrient-poor and toxic environment, which allow cancer cell survival. In response to fasting, melanoma, glioma, and breast cancer cells had a significant increase in ribosome biogenesis/assembly genes such as elongation factor 1γ (Eef1g) and components of the 60S and 40S ribosomal proteins and the expression of proliferation-associated

genes (12, 13). Such changes were associated with unexpected AKT and S6K activation, these changes lead to an increase in oxidative stress and may contribute to DNA damage and a sensitization to DNA- damaging drugs through DSS (12). Such response of tumor cells to the altered conditions including the reduction in IGF1 and glucose levels caused by fasting is considered a key regulatory mechanism underlying the antitumor properties of these dietary interventions and their potential usefulness for distinguishing the effects of anticancer drugs on normal versus cancer cells (12, 13) (Fig. 1). In line with the DSS hypothesis, periodic cycles of fasting are sufficient to slow the growth of many types of cancer cells, including solid cancer cell lines and lymphoid leukemia cells, *in vivo* and, most importantly, to sensitize cancer cells to tyrosine kinase inhibitors (TKIs), radiotherapy, and chemotherapy (12, 16, 17, 39, 67, 71-74).

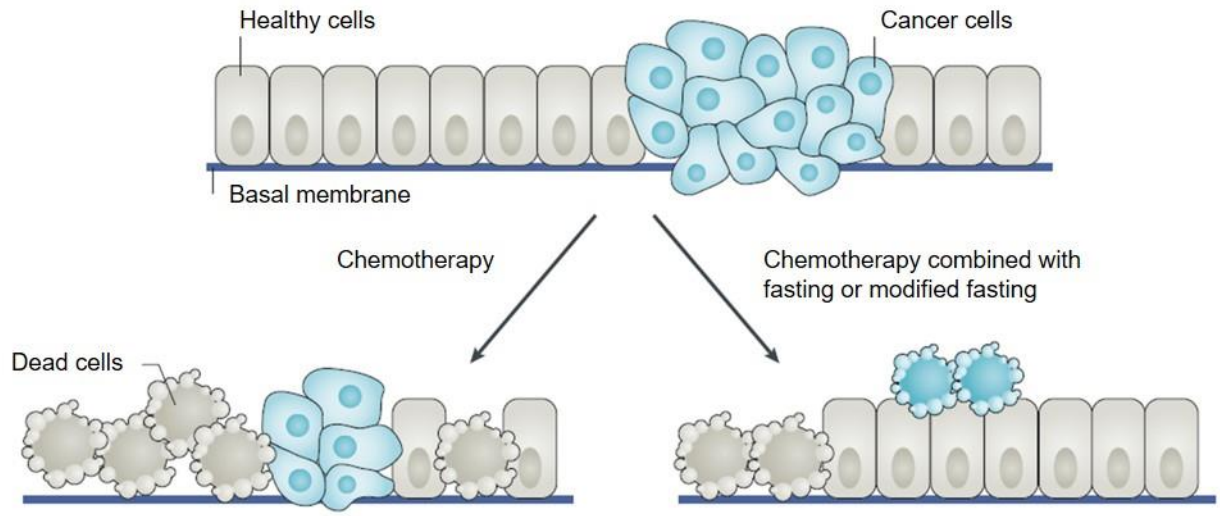

Fig. 1 | Differential stress resistance versus differential stress sensitization (69). Chemotherapy acts on both cancer cells and normal cells, inducing tumor shrinkage but almost inevitably also causing side effects that can be severe or even life-threatening because of the damage to many epithelial and non- epithelial tissues. Based on the available preclinical data, fasting or modified fasting could prove useful to separate the effects of chemotherapy, and possibly of newer cancer drugs, on normal versus cancer cells. Owing to the presence of oncogenic mutations that constitutively activate growth-promoting signaling cascades, cancer cells fail to properly adapt to starvation conditions. As a result, many types of cancer cells, but not normal cells, experience functional imbalances, becoming sensitized to toxic agents, including chemotherapy (differential stress sensitization). Conversely, fasting or modified fasting initiates an evolutionarily conserved molecular response that makes normal cells but not cancer cells more resistant to stressors, including chemotherapy (differential stress resistance). The predicted clinical translation of these differential effects of fasting or modified fasting on normal versus cancer cells is a reduction in the side effects of cancer treatments, on the one hand, and improved tumor responses, patient progression-free survival, and overall survival, on the other. Adapted from Nencioni et al. Nat Rev Cancer 2018.

By reducing glucose availability and increasing fatty acid β- oxidation, fasting can promote the switch from aerobic glycolysis (Warburg effect) to mitochondrial oxidative phosphorylation (OXPHOS), in cancer cells (71) (Fig. 2). This switch leads to an increased mitochondrial respiratory activity consequently increasing reactive oxygen species (ROS) production (12) and may also involve a reduction in glutaminolysis, lactate generation, and ATP synthesis (71). The combined effect of ROS production and reduced antioxidant protection elevates oxidative stress in cancer cells and boosts the activity of chemotherapies (71).

Notably, because a high glycolytic activity demonstrated by high- lactate production is predictive of aggressiveness and metastatic propensity in several types of cancer (75), the anti-Warburg effects of fasting or modified fasting have the potential to be particularly effective against aggressive and metastatic cancers. Apart from the metabolic change, fasting or modified fasting induce other changes that can promote DSS in pancreatic ductal adenocarcinoma (PDAC) cells. Starvation increases the expression levels of equilibrative nucleoside transporter 1 (ENT1), a plasma membrane transporter of gemcitabine, leading to enhanced activity of this chemotherapy (74). In breast cancer cells and mouse B16 melanoma cells, fasting causes small ubiquitin-like modifier 2 (SUMO2)-mediated and/or SUMO3-mediated post-translational modification of REV1, a specialized DNA polymerase involved in DNA repair and a p53-binding protein (76). This modification reduces the inhibitory effects of REV1 on p53, leading to increased p53-mediated transcription of pro- apoptotic genes and p53-dependent cell death (Fig. 2). Fasting also increases the ability of commonly administered TKIs to prevent cancer cell growth by inhibiting MAPK signaling and, thereby, blocking E2 transcription factor-dependent gene expression and reducing glucose uptake (16, 72). FMD downregulated the expression of haem oxygenase 1 (HO1), a protein that provides protection against apoptosis and oxidative damage, in tumor cells in mice but increased its expression in normal cells (68, 77). HO1 reduction in tumor cells mediates FMD- induced chemosensitization by increasing CD8+ tumor-infiltrating lymphocyte-dependent cytotoxicity, consequently making cancer cells more susceptible to CD8+ cytotoxic T cells, possibly by countering the immunosuppressive effect of regulatory T (Treg) cells (68) (Fig. 2). In another study, 2 weeks of alternate-day fasting in a mouse colon cancer model downregulated CD73 expression and consequently decreased the production of immunosuppressive adenosine by tumor cells, via activating autophagy (78). Ultimately, CD73 downregulation via fasting was

shown to prevent macrophage shift to an M2 immunosuppressive phenotype (Fig. 2). Based on these data, it was recently reported that fasting synergizes with anti-PD-1 immunotherapy to inhibit lung cancer progression and metastasis in mice. This antitumor activity in mice was linked to a reduction in circulating IGF-1 and downregulation of IGF-1 receptor (IGF-1R) signaling in cancer cells. This antitumor effect increased the intratumoral CD8/Treg ratio and led to the development of tumor-specific immunity. Thus, a major goal for the near future will be to identify the types of cancer that are most susceptible to these dietary regimens by means of biomarkers.

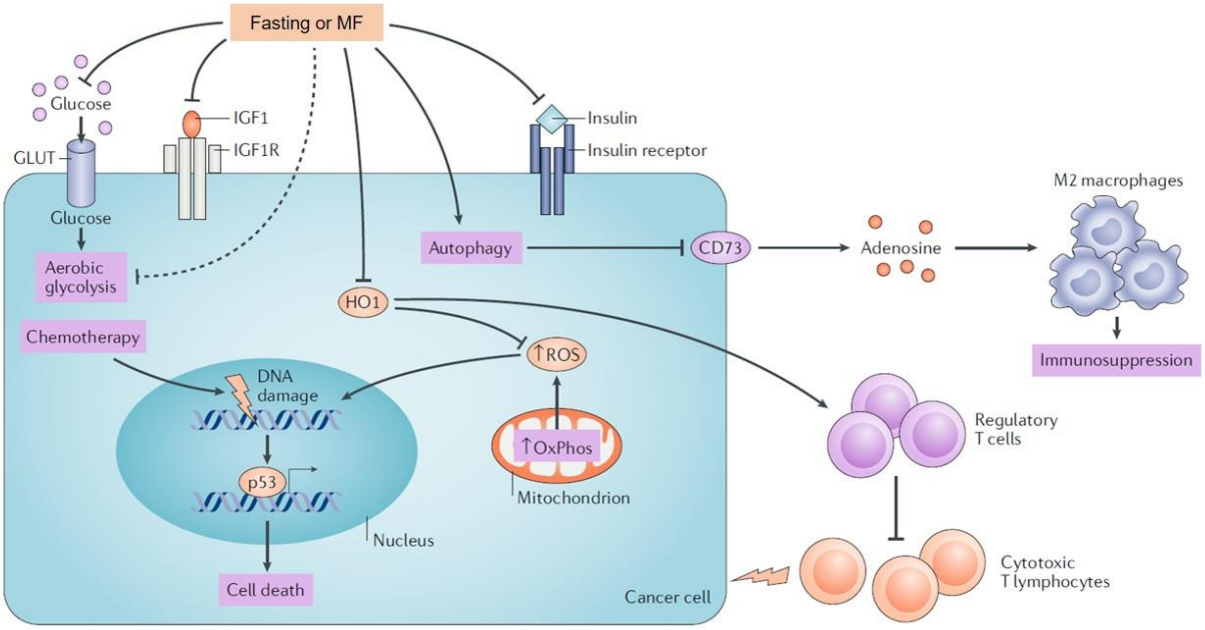

Fig. 2 | Mechanisms of fasting or fasting-dependent killing of cancer cells in solid tumors (69). Preclinical and initial clinical data indicate that fasting or modified fasting (MF) reduce the levels of tumor growth-promoting nutrients and factors, including glucose, IGF1, and insulin. Fasting can cause an anti-Warburg effect by reducing glucose uptake via glucose transporters (GLUTs) and aerobic glycolysis and forcing cancer cells to increase oxidative phosphorylation (OXPHOS); this increases the production of reactive oxygen species (ROS) in cancer cells and, resultantly, oxidative DNA damage, p53 activation, DNA damage, and cell death, particularly in response to chemotherapy. By activating autophagy, fasting can reduce CD73 levels in some cancer cells, thereby blunting adenosine production in the extracellular environment and preventing the shift of macrophages towards an immunosuppressive M2 phenotype. Finally, fasting or or modified fasting can downregulate haem oxygenase 1 (HO1) expression in breast cancer cells, which makes them more susceptible to CD8+ cytotoxic T cells, possibly by countering the immunosuppressive effect of regulatory T (Treg) cells. Notably, fasting or an or modified fasting can have very different and even opposite effects in different cancer cell types or even within the same cancer cell type. Adapted from Nencioni et al. Nat Rev Cancer 2018.

1.2 GASTROINTESTINAL (GI) CANCER

Gastrointestinal (GI) cancer is a leading cause of cancer-related mortality and remains a major challenge for cancer treatment (79, 80). Despite the combined administration of surgical resection and adjuvant chemotherapy or radiotherapy, there were 4.8 million new cases of GI cancers and 3.4 million related deaths, worldwide, in 2018 (80), due to rapid disease progression, metastasis, toxicity and resistance of adjuvant treatments (81-83). Thus determining effective treatment strategies for GI cancers are urgently needed. The most common and major types of GI cancers are esophageal cancer, gastric (stomach) cancer, liver cancer, pancreatic ductal adenocarcinoma (PDAC) and colorectal cancer (CRC) (80). In this study we focused on the latter (pancreatic and colorectal cancers) due to the increased incidence and mortality rates of these two types of cancers worldwide (80, 81, 84, 85). In addition, several studies demonstrated that upregulation of the cholesterol synthesis pathway, that was experimentally proved to be affected by the combination treatment used in the current study, plays a crucial role in maintaining growth and metastasis of PDAC and CRC (86-89).

1.3 PANCREATIC DUCTAL ADENOCARCINOMA (PDAC)

Global analyses predict that pancreatic ductal adenocarcinoma (PDAC) will be the second most common cause of cancer-associated mortality within the next decade as a result of the lack of early diagnosis, a poor therapeutic arsenal, and increased burden of this tumor regardless of gender and age (84, 90-92). Though the overall survival of patients with advanced PDAC has doubled over the past 15 years (median survival has increased from 6 to 12 months), the 5-year survival rate has just increased from 3% in the 1970s to 9% in 2020 (79, 93, 94). This slow improvement in survival

rate is much less than that seen in many other cancer types, thus PDAC is considered a formidable medical challenge (95).

1.3.1 PDAC risk factors

Due to lifestyle changes and modifiable risk factors (smoking, obesity, high-fat diets, inactivity, and increases in alcohol intake) (96), the worldwide incidence of PDAC is expected to increase more shortly (91). Prognosis remains poor despite diagnostic progress. Eighty-five percent of patients are diagnosed with locally advanced tumors and/or metastases since they are asymptomatic and lack the early markers of this highly aggressive disease (97).

1.3.2 PDAC therapy

PDAC treatment efficacy largely depends on the disease stage at the time of diagnosis. Survival can be prolonged by adjuvant therapy after surgery, but only a few PDAC patients (10–20%) have resectable tumors (98). Treatments comprise mainly chemotherapeutic regimens such as (gemcitabine, gemcitabine plus nab-paclitaxel or FOLFIRINOX (folinic acid, 5-fluorouracil, irinotecan, oxaliplatin)) (93, 99, 100). The different treatment strategies commonly used for PDAC are summarized in Table 2.

Table 2 | Current treatment options for pancreatic ductal adenocarcinoma (93, 97, 98).

Tumor type	**1st line treatment**	**2nd line treatment**	**Median survival**
Resectable tumor	Pancreatic resection	Adjuvant chemotherapy: gemcitabine–capecitabine or FOLFIRINOX	26 months
Nonresectable borderline and/or nonresectable locally advanced tumor (ECOG score 0/1)	Neoadjuvant radiotherapy or chemotherapy (borderline); chemotherapy (e.g. FOLFIRINOX)	Stable or response: surgical exploration or continued chemotherapy; progression: gemcitabine or FOLFOX or nanoliposomal irinotecan + 5FU	6–18 months
Nonresectable borderline and/or nonresectable locally advanced tumor (ECOG score 2)	Gemcitabine; BSC if ECOG score >2	FOLFOX or nab- paclitaxel + gemcitabine or nanoliposomal irinotecan + 5FU or BSC	6–8 months
Metastatic tumor	ECOG score 0/1: FOLFIRINOX or nab- paclitaxel + gemcitabine; ECOG score 2: gemcitabine; ECOG score >2: BSC	FOLFOX or nab- paclitaxel + gemcitabine or nanoliposomal irinotecan + 5FU or BSC	6–11 months

Treatments are indicated according to phase III randomized trials and international recommendations (93, 99, 100). 5-FU, fluorouracil; BSC, best supportive care; ECOG, Eastern Cooperative Oncology Group; FOLFIRINOX, a combination of folinic acid and 5-FU, irinotecan and oxaliplatin; FOLFOX, a combination of folinic acid, 5-FU, and oxaliplatin; nab, nanoparticle albumin-bound.

1.3.3 Role of diet in PDAC

Given the decline of cigarette smoking, which represents the main PDAC risk factor, a possible explanation for the registered augmented incidence and mortality of PDAC may reside in the type of diet that the general population is eating, especially in adulthood and childhood. In preclinical studies on mice, experimental pancreatitis induced by caerulein, amino acid oligopeptide that stimulates smooth muscle and increases digestive secretions, or high-fat diets activated the oncogenic Kirsten rat sarcoma viral oncogene homolog (KRAS) to induce the occurrence and development of PDAC (101-103). In another study, metformin completely abrogated PDAC incidence induced by a diet high in fats and calories in transgenic mice that express oncogenic KRAS (104). Consistent with this notion, Yuan et al (105) showed that pre-diagnostic high BMI is associated with both increased risk of developing PDAC and mortality. Interestingly, they demonstrated a statistically significant positive correlation between the stages of PDAC and pre-diagnostic BMI, showing that non-obese patients present less frequently at the diagnosis with metastatic disease when compared with obese patients (105).

1.4 COLORECTAL CANCER (CRC)

Colorectal cancer (CRC) is the third most commonly diagnosed cancer among men and the second most common cancer among women worldwide (81). An estimated 18.1 million new cancer cases and 9.6 million cancer deaths occurred worldwide in 2018. Among them, colorectal cancer (CRC) ranked third for incidence (10.2%, with 1.8 million new cases) and second for mortality (9.2%, with 881,000 deaths) (81, 106). In addition, the number of new cases may increase to nearly 2.5 million in 2035 (85). According to statistics in the USA, the death rate declined by ~50% in 2016 (13.7 per 10,000 patients) compared with that in 1970 (29.2 per 10,000 patients) because of the rapid development of screening methods and improved treatment methods. However, this trend seems to be observed only in highly developed countries (85). Meanwhile, the 5- year survival rate for CRC is ~64% but drops to 12% for metastatic CRC, and further investigation is still required to develop effective approaches for medical intervention (107). Despite reductions in the overall CRC burden, the incidence of CRC among individuals younger than age 50 (early-onset CRC; EOCRC) has increased over the past two decades worldwide. Long-term projections suggest that by 2030, incidence rates for colon and rectal cancers may increase by 90.0% and 124.2%, respectively, for EOCRC patients (108). In addition, an alarming increase of CRC in the population under the age of 55 has also recently been detected (82).

1.4.1 CRC risk factors

Progression of CRC is influenced by obesity, geography, human development index, age, genetic, environmental, and lifestyle factors (82, 109). Hereditary colorectal cancer syndromes include Lynch Syndrome (Hereditary nonpolyposis colorectal cancer), Familial adenomatous polyposis (FAP), MUTYH-associated polyposis (MAP). Lynch syndrome and Familial adenomatous polyposis contribute to a vast majority of hereditary CRC syndrome, which accounts for only about

5% of entire CRC incidence (110). The presence of a family history of colon cancer in first-degree relatives, even in the absence of the above hereditary colon cancer syndromes, increases the risk of the development of CRC in about 20% of cases. The risk increases twofold when compared to the general population, with a history of CRC in first-degree relatives. Other well-known associations with colorectal cancer include African American ethnicity, male sex, inflammatory bowel disease (Ulcerative colitis, and Crohn's disease), obesity, sedentary lifestyle, red meat and processed meat, tobacco use, alcohol use, history of abdominal radiation, acromegaly, renal transplant with use of immunosuppressive medications, diabetes mellitus and insulin resistance, androgen deprivation therapy, cholecystectomy, and coronary artery disease (110).

1.4.2 CRC stages

Clinical manifestations of CRC are categorized into five stages (O, I, II, III, and IV). These stages determine treatment and prognosis and are based on histopathological features, the degree of bowel wall invasion, lymph node spreading, and the appearance of distant metastases (111). Early stages are often asymptomatic or concomitant with non-specific symptoms (i.e., loss of appetite or weight loss, anemia, abdominal pain, or changes in bowel habits) (112). Later stages are concomitant with the dissemination of cancer cells to the lymph system or other organs in the body. Colorectal cancer diagnosed in adults aged 85 and older is often associated with a more advanced stage, with a 10% less likely to be diagnosed at a local stage when compared with patients diagnosed at the age of 65 to 84 (113). The most relevant mechanisms of CRC carcinogenesis identified to date include genetic chromosomal instability, microsatellite instability, serrated neoplasia, specific gene signatures, and specific gene mutations, such as Adenomatous Polyposis Coli (APC), SMAD4, BRAF, or KRAS (114-116).

1.4.3 CRC molecular subtypes

To correlate cancer cell phenotype with clinical behavior and guide rational treatment with specific targeted therapies, the CRC Subtyping Consortium unified six different molecular classification systems, based on gene expression analysis, into a single consensus system with four distinct types, known as the consensus molecular subgroups (CMS) (117) summarized in (Table 3).

Table 3 | Biological characterization and clinical prognosis of the consensus molecular subtypes (115, 118, 119).

	CMS1	**CMS2**	**CMS3**	**CMS4**
Alternate Name	MSI Immune	Canonical	Metabolic	Mesenchymal
Frequency	14% of CRCs	37% of CRCs	13% of CRCs	23% of CRCs
Predominance	Proximal colon	Distal colon & rectum	Mixed	Distal colon & rectum
Main Hallmarks	- Hypermethylation of the promoter regions of the DNA MMR genes causes MSI. - High BRAF mutation 42% - KRAS mutation 25% - Hypermethylation of CpG islands (CIMP) - Low SCNA counts -Activation of JAK/STAT & caspases	- High degrees of CIN, CIMP - Activation of WNT-β catenin, MYC & EGFR signaling pathways - Low BRAF mutation <1% - KRAS mutation 25% - Mutations in APC, p53, & PIK3CA	- Low, intermediate MSI & CIMP hypermethylation - Low SCNA counts - Low BRAF mutation <10% - Prevalent KRAS mutation (68%) - Mutations in APC, p53, & PIK3CA	- Low levels of hypermutation & MSI - High SCNA counts - Low BRAF mutation <10% - KRAS mutation 40% - overexpression of stromal invasion & neo-angiogenesis genes, activation of TGF-β pathway, & EMT. - Mutations in APC, p53, & PIK3CA - More chemoresistant

DNA mismatch repair (MMR); Microsatellite Instability (MSI); Chromosomal instability (CIN); SCNA, somatic copy number alterations; tissue growth factor (TGF-β); epithelial-mesenchymal transition (EMT); Adenomatous Polyposis Coli (APC).

1.4.4 CRC therapy

Given the advances in primary and adjuvant treatments, the survival time in CRC has been improving. Typically, the ideal CRC treatment is to achieve complete removal of the tumor and metastases, which mostly requires surgical intervention (120). However, despite the emergence of numerous screening programs to reduce CRC incidence, nearly a quarter of CRCs are diagnosed at an advanced stage with metastases, and 20% of the remaining cases may develop metachronous metastases (metastasis developed after completion of the initial curative treatment), which result in difficulties in curative surgical control and subsequent tumor-related deaths (121-124). For those patients with unresectable lesions or who are intolerant to surgery, the goal is a maximum shrinkage of the tumor and suppression of further tumor spread and growth, and radiotherapy and chemotherapy are the leading strategies for controlling disease in such patients (116). Metastatic

CRC is termed resectable when the primary tumor and all metastases are amenable to complete surgical removal. Resection of metastatic CRC achieves a long-term cure for less than 20% of metastatic CRC patients (114). Systemic chemotherapy regimens are the primary treatment for metastatic CRC and are summarized in Table 4. Although the cure for metastatic CRC is rare, recent large clinical trials that included patients healthy enough to receive chemotherapy have shown that intensive treatment with multiple systemic therapies can help patients survive for 2 to 3 years (114, 116).

Table 4 | Chemotherapy regimens most commonly used for the treatment of metastatic CRC (114, 116).

Regimen name and component drugs[a]	First-line use	Toxicities/adverse effects	Additional comments	Mechanism of action and FDA approval date
FOLFOX (fluorouracil, leucovorin, & oxaliplatin)	Yes	Pancytopenia, neuropathy, hypersensitivity	The most commonly used adjuvant regimen	**Fluorouracil** Pyrimidine analog; interrupts DNA synthesis. FDA approved in 1962. **Leucovorin** Folic acid analog; interrupts DNA synthesis. FDA approved in 1952. **Oxaliplatin** Alkylating agent; causes DNA breaks. FDA approved in 2002. **Capecitabine** Pyrimidine analog; interrupts DNA synthesis. FDA approved in 1998. **Irinotecan** Topoisomerase I inhibitor; interrupts the breaking and rejoining of DNA strands during replication. FDA approved in 1996. **Cetuximab** Recombinant chimeric monoclonal antibody to EGFR; interrupts or stops cell growth. FDA approved in 2004. **Panitumumab** Humanized monoclonal antibody to EGFR; interrupts or slows down cell growth. FDA approved in 2006. **Bevacizumab** Humanized monoclonal antibody to VEGF; interrupts the growth of blood vessels. FDA approved in 2004. **Pembrolizumab** Humanized monoclonal antibody against PD-1 receptor; activates T-cell‑mediated immune Response. FDA approved in 2017.
CAPOX (capecitabine & oxaliplatin)c	Yes	Pancytopenia, diarrhea, hand/foot -syndrome, neuropathy, hypersensitivity	A common adjuvant regimen; substitutes oral capecitabine for IV fluorouracil	
FOLFOXIRI (fluorouracil, leucovorin, oxaliplatin, & irinotecan)	Yes	Pancytopenia, diarrhea, neuropathy, hypersensitivity	Intensive regimen used for patients who are possible candidates for surgical resection of limited metastatic disease	
FOLFOX plus cetuximab or panitumumab	Yes	Pancytopenia, diarrhea, hand/foot-syndrome, hypomagnesemia, hypersensitivity reactions. neuropathy, skin toxicity	For tumors that are KRAS/NRAS/BRAF wild type; ineffective for tumors with sequence variations in these genes	
IROX (irinotecan & oxaliplatin)	Rare	Diarrhea, neuropathy	Nonstandard regimen but useful for patients intolerant of fluorouracil due to severe dihydropyrimidine dehydrogenase deficiency or coronary vasospasm	
FOLFIRI (fluorouracil, leucovorin, & irinotecan)	Yes	Pancytopenia, diarrhea;	Not used in adjuvant regimens	
CAPIRI (capecitabine orally & irinotecan)	Yes	Pancytopenia, diarrhea, hand/foot-syndrome	Substitutes oral capecitabine for intravenous fluorouracil	
FOLFIRI plus cetuximab or panitumumab	Yes	Pancytopenia, diarrhea, Hypomagnesemia, infusion reaction, skin toxicity	KRAS/NRAS/BRAF wild type	
Fluorouracil & leucovorin (**Bolus and continuous infusion regimen**)	Yes	Pancytopenia, mucositis	Single-agent regimen; often optimal for frail patients with major comorbidities	
Bevacizumab plus FOLFOX, CAPOX, FOLFIRI, CAPIRI, FOLFOXIRI, fluourouracil & leucovorin, or capecitabine (**VEGF-containing regimen**)	Yes	Hypertension, bowel perforation, poor wound healing, proteinuria, thrombosis	All molecular subtypes	
Pembrolizumab (**Immunotherapy regimen**)	Yes	Fatigue, colitis, dermatitis, hepatitis, pneumonitis, thyroiditis	Used in MSI-H and/or MMR-D: CRC patients only	

Abbreviations: KRAS, Kirsten rat sarcoma vial oncogene homolog; NRAS, neuroblastoma RAS viral oncogene homolog; *BRAF*, v-raf murine sarcoma viral oncogene homolog B; CRC, colorectal cancer; *EGFR*, epithelial growth factor receptor; FDA, Food and Drug Administration; MMR-D, mismatch repair-deficient; MSI-H, microsatellite instability-high; PD-1, programmed cell death-1; VEGF, vascular endothelial growth factor. a Drugs are administered intravenously except where noted.

1.4.5 Role of diet in CRC

The relationship between dietary factors and different cancers is now well recognized. Several review studies have discussed the evidence for the associations between dietary factors (eg, foods and food groups, beverages, alcohol, macronutrients, and micronutrients) and the incidence of CRC (111, 125). Although fasting exerts extensive antitumor effects in numerous contexts as previously discussed, the impact of fasting on metabolic changes in CRC remains poorly studied. In a recently published study, fasting negatively regulated glucose metabolism and proliferation via the FDFT1/AKT-mTOR-HIF1α axis in CRC. Besides, it upregulated a cholesterol biosynthesis enzyme Farnesyl-diphosphate farnesyltransferase 1 (FDFT1) in CRC either *in vitro* or *in vivo*. In addition, FDFT1 overexpression played an important tumor-suppressive role when combined with fasting an effect that was reversed in case of its knockdown (126). Another study evaluated how glucose concentration influences CRC cells' response to metformin, using two CRC cell lines with different growth rates (fast-growing more glycolytic cell line SW948 and slow-growing cell line SW111 which is reliant on mitochondrial respiration in terms of metabolism). The authors found that both cell lines show inhibitory growth after metformin treatment under physiological glucose conditions, but not in high glucose conditions. In addition, metformin-treated SW1116 shifted toward a more glycolytic profile resembling that of the SW948 cell line. This metabolic shift of SW116 to be more like SW948 could then also result in increased vulnerability to chemotherapy. This demonstrates that cells having different metabolic phenotypes show a clear differential response to metformin treatment based on glucose concentration (127).

1.5 CHOLESTEROL

The discovery of cholesterol dates back to the second half of the eighteenth century when Poulletier de la Salle isolated for the first time this molecule from human gallstone and bile. Cholesterol is a ubiquitous sterol found in vertebrate organisms with a plethora of biological functions that are essential for proper cellular growth and activity (128, 129). It is a precursor for steroid hormones, and an essential component of plasma membranes (130). Due to its alicyclic nature, cholesterol is highly hydrophobic and resides predominantly within the phospholipidic bilayer of cell membranes, where it preserves the barrier function by modulating permeability, fluidity, and rigidity (131, 132). In this setting, cholesterol preferentially interacts with the saturated fatty acids of adjacent sphingolipids and glycophosphatidylinositol-anchored proteins of the outer leaflet, forming small ordered and tightly packed microdomain assemblies, physically separated from the shorter and unsaturated phospholipids of the bilayer (132-134). Cholesterol is enriched in these assemblies, usually called lipid rafts, and plays a key role in several biological processes, including signal transduction pathways related to cell proliferation such as IGF1/PI3K/AKT signaling (130, 135-138).

1.5.1 Cholesterol homeostasis and metabolism

Cholesterol is an essential lipid for maintaining cellular homeostasis (130). Due to the crucial role played by this sterol in several physiological settings, disruption of cholesterol homeostasis and metabolic reprogramming may be responsible for the development of cardiovascular disorders and is implicated in the pathogenesis of diabetes, Alzheimer disease, and many types of cancer (139-143). Cholesterol is either acquired from the diet (exogenous) or endogenously synthesized in our body (~70% of total body cholesterol, endogenous). Beyond *de novo* cholesterol biosynthesis, most cells acquire cholesterol from low-density lipoprotein (LDL) taken up from the

circulation via LDL receptor (LDLR)-mediated endocytosis (131). The liver, 50% of total synthesis in humans occurs in the liver (144), delivers both endogenously synthesized and exogenously acquired cholesterol to the bloodstream as very- low-density lipoproteins (VLDLs). After processing in the bloodstream, the VLDLs generate circulating LDLs, which can be taken up by peripheral cells via receptor-mediated endocytosis (131). LDL is taken into cells by clathrin-mediated endocytosis and transported to the lysosomes through the endocytic pathway, where is then hydrolyzed to free cholesterol molecules, which are shuttled to the cell membrane and other cell membrane-bound organelles (130, 145). Cholesterol derived from dietary sources can be absorbed from enterocytes in the intestine and bile in the biliary ducts by hepatocytes in the liver by Niemann–Pick type C1-like 1 (NPC1L1) protein present on the apical surface of these cells (146). The cholesterol biosynthesis *de novo* synthesis cascade occurs in every mammalian cell. In brief, cholesterol synthesis starts from acetyl- CoA and is orchestrated by a series of ~30 enzymes, which are distributed between cytosol and endoplasmic reticulum (ER) membrane as summarized in fig. 3 (147). Within the cell, cholesterol is dynamically transported between various organelles by vesicular and nonvesicular mechanisms to fulfill its multifaceted functions (148). Although all mammalian cells can produce cholesterol, most (except for hepatocytes, adrenal cells, and gonadal cells) are unable to catabolize the molecule (147). Therefore, excess cholesterol is exported outside of the cell either to the blood by ATP- binding cassette (ABC) subfamily A member 1 (ABCA1), or the homodimer of ATP-binding cassette subfamily G member 1 (ABCG1), or to the intestinal lumen and bile ducts by the ABCG5 and ABCG8 heterodimer (147, 149). Lipid-free apoA- I is the primary acceptor for cholesterol efflux by ABCA1 (150). This generates nascent high-density lipoprotein (HDL) particles that, under the action of lecithin:cholesterol

acyltransferase (LCAT), become mature and competent for acquiring cholesterol from ABCG1 (151, 152).

Surplus intracellular cholesterol can also be esterified by acyl-coenzyme A (CoA):cholesterol acyltransferase (ACAT) to produce cholesteryl esters (CEs) (153). Indeed, produced cholesteryl esters is stored as a cholesterol reservoir in cytosolic lipid droplets (LDs) preventing free-cholesterol lipotoxicity (154) or released as a major constituent of plasma lipoproteins such as HDL, VLDL, LDL, and chylomicrons (large triglyceride-rich lipoproteins produced in enterocytes from dietary lipids) (155). HDLs are finally transported from peripheral tissues back to the liver and intestine, where cholesterol is eliminated or recycled, and also to steroidogenic organs to generate steroid hormones, thus maintaining cholesterol homeostasis (147).

1.5.2 Cholesterol role in cancer

Highly proliferative cancer cells are strongly dependent on cholesterol to satisfy their increasing demand for substrates for membrane biogenesis and other functional needs (156). Cancer cell satisfies their needs by either increasing the uptake of exogenous (or dietary) cholesterol or over-activating their endogenous synthesis (*de novo* biosynthesis) (Fig. 3). For example, the cholesterol-derived oncometabolite 6-oxo-cholestan-3β,5α-diol, which is enriched in patients with breast cancer, binds glucocorticoid receptors and subsequently promotes tumor growth (157). Accordingly, cholesterol metabolism is generally beneficial for cancer growth and progression, as it promotes oncogenic signaling and evasion of apoptosis, and cell proliferation, migration, and invasion (158-164). As a consequence, cholesterol depletion or trafficking blockade hinders tumor growth and invasion in a variety of cancers (165-168).

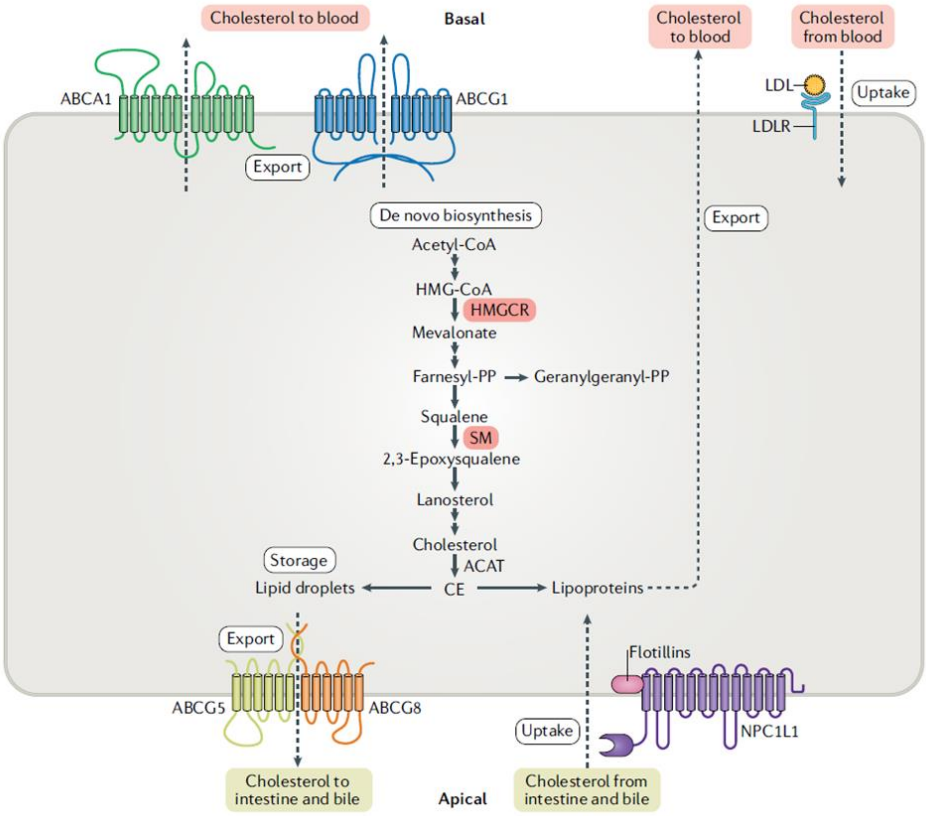

Fig. 3 | Major pathways of cholesterol metabolism in a polarized cell (147). Cholesterol is synthesized from acetyl- CoA through a series of ~30 reactions using 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and squalene monooxygenase (SM) (also called squalene epoxidase) as the rate-limiting enzymes (highlighted in red). In addition to *de novo* biosynthesis, cholesterol carried by low-density lipoprotein (LDL) particles in the blood can be taken up by LDL receptor (LDLR) at the basal surface of polarized cells (such as enterocytes or hepatocytes). Free cholesterol can also be absorbed from dietary sources by enterocytes in the intestine and from bile in the biliary ducts by hepatocytes in the liver. This absorption is mediated by Niemann–Pick type C1-like 1 (NPC1L1) and the associated flotillins present on the apical surface of these cells. Excess cholesterol is exported to the blood by ATP- binding cassette subfamily A member 1 (ABCA1) or the homodimer of ATP-binding cassette subfamily G member 1 (ABCG1), or to the intestinal lumen and bile ducts by the ABCG5 and ABCG8 heterodimer. Cholesterol can also be converted to cholesteryl ester (CE) by acyl-coenzyme A:cholesterol acyltransferase (ACAT) for storage in lipid droplets or secretion as lipoproteins. CoA, coenzyme A; PP, pyrophosphate. Adapted from Luo et al. Nat Rev molecular cell biology 2019.

1.5.3 Increased cholesterol biosynthesis and uptake

Increased cholesterol biosynthesis is a hallmark of many cancers. In situations in which lipids and/or oxygen are limited, such as in the glioblastoma microenvironment, the master transcription factor sterol regulatory element-binding protein 2 (SREBP2) and its downstream targets, including

mevalonate-pathway enzymes, are significantly upregulated in tumors (169). Compared with time- and energy-consuming *de novo* cholesterol synthesis, increasing cholesterol uptake might be more efficient for cancer cells. An extreme example is that of some anaplastic large cell lymphoma cells, which fully rely on cholesterol uptake to acquire cholesterol, owing to the loss of squalene epoxidase (SQLE), a rate-limiting enzyme in the cholesterol-biosynthesis pathway (170). These cancer cells actively upregulate the LDLR, which takes up exogenous cholesterol as an alternative strategy to support proliferation. In contrast, lower levels of LDLR but higher levels of SQLE are expressed in advanced-stage prostate cancer, thus indicating a greater reliance on cholesterol synthesis than uptake (171). In the case of intestinal tumorigenesis, both cholesterol synthesis and uptake contribute (172). Although cholesterol uptake is an important source of cholesterol for cancer cells, how cells orchestrate cholesterol biosynthesis and uptake during cancer progression is complex and remains to be clarified. As mentioned previously, excessive cholesterol in normal cells is esterified and stored in LDs same occurs in cancer cells, thus high LDs and stored-cholesteryl ester content in tumors (173-176) are now considered as hallmarks of cancer aggressiveness (175, 177-179). Colon cancer stem cells showed a higher LD amount than their differentiated counterparts, as revealed by Raman spectroscopy imaging (180). Moreover, LD-rich cancer cells are more resistant to chemotherapy (173). In addition, LD content, especially cholesteryl ester, is mobilized by pancreatic cancer cells under a restricted cholesterol-rich LDL supply (176), and limiting LDL uptake reduces the oncogenic properties of pancreatic cancer cells and rendered them more sensitive to cytotoxic drugs (176).

Incorporating dietary interventions into cancer therapy holds promise as means to enhance the efficacy of either existing or novel cancer therapies and to reduce certain treatment-emergent side effects (4, 69, 181). Several studies show that periodic cycles of fasting or modified fasting

increase the activity of chemotherapeutics, tyrosine kinase inhibitors, and immune checkpoint inhibitors in mice (16, 69). The lab of Prof. Nencioni has previously shown that combining endocrine therapy (ET) for hormone receptor-positive (HR+) breast cancer (BC) with fasting or MF also makes these agents more active (by lowering circulating insulin, leptin, and IGF1) and delays acquired resistance to them (182, 183). Besides being studied as a complement for standard therapeutics, fasting/MF has also been used to enhance the antitumor properties of drugs that are normally used for non-oncological indications: this is the case of the antidiabetic drug, metformin, and vitamin C, suggesting that this dietary approach could also hold value for drug repurposing in oncology (184-186).

2. PURPOSE OF THE STUDY

Our study aims at repurposing FDA-approved, non-oncological, drugs via fasting or STS in gastrointestinal cancers and identifying the proposed mechanism of action behind the expected sensitization. In this context, using high-throughput drug screens, we have identified inhibitors of cholesterol biosynthesis (including several antifungal agents classified as azoles) as agents that become cytotoxic to cancer cells under fasting conditions or STS (low-glucose (0.5 g/L) DMEM medium with 1 % FBS). Based on our results, we hypothesized that fasting conditions could cooperate with agents, other than azoles, that inhibit cholesterol production to achieve synergistic anticancer effects. Since water-only fasting or modified diets inhibits AKT-mTOR signaling and reduce circulating growth factors such as insulin, IGF1, and leptin (16, 29, 182, 187). Therefore, we hypothesized that these fasting could be used to enhance the activity of cholesterol biosynthesis inhibitors through the inhibition of AKT signaling pathway and modulation of these circulating growth factors.

3. METHODS

Unless mentioned, all the experiments were performed in the lab of *Prof.* Alessio Nencioni located in Università di Genova - Di.M.I. - Dipartimento di Medicina Interna e Specialità Mediche, Genoa, Italy, by the doctoral candidate.

Cell Lines and Reagents

Capan-1 (PDAC) and HCT116 (CRC) cell lines were used in most of the experiments because they were the most sensitive to the combined treatments compared to single treatments, and they are considered as good *in vivo* animal xenograft models. Other cell lines from different cancer origins were used as proof of the concept that fasting conditions or STS cooperate with cholesterol biosynthesis inhibitors to kill different types of cancer cells originating from different tumor types. PK9, Capan-1, MIA PaCa-2, Panc-1, BxBC3, HCT116, HT-29, CT26, N87, PC3, OVCAR5, OVCAR8, MCF7, MDA-MB-231, SKBR3, 4T1, H1975, and A549 cell lines were either purchased from the ATCC (LGC Standards S.r.l., Milan, Italy) or provided and certified as mycoplasma-free by the cell bank of the IRCCS Ospedale Policlinico San Martino (Blood Transfusion Centre, B. Parodi, Genoa, Italy), while ID8 was purchased from Sigma Aldrich S.r.l. Cells were authenticated by DNA fingerprinting and isozyme detection. Cells were passaged for less than 6 months before their resuscitation for this study. All of our cell lines were routinely tested for mycoplasma contamination by Mycoalert Kit (Promega). HT-29 cell line was maintained in high glucose DMEM medium (LifeTechnologies, Italy) supplemented with 10% FBS, penicillin (50 units/ml), and streptomycin (50 µg/ml) (LifeTechnologies, Italy). ID8 cell line was maintained in high glucose DMEM (LifeTechnologies, Italy), 4% FBS, 5 µg/mL insulin prescribed as Humulin® R (Pharmacy of the IRCCS Ospedale Policlinico San Martino), 5 µg/mL transferrin (Sigma Aldrich S.r.l.) and 5 ng/mL sodium selenite (Sigma Aldrich S.r.l.) and

penicillin (50 units/ml), and streptomycin (50 µg/ml) (LifeTechnologies, Italy). All other cell lines were maintained in RPMI1640 medium (LifeTechnologies, Italy) supplemented with 10% FBS, penicillin (50 units/ml), and streptomycin (50 µg/ml) (LifeTechnologies, Italy). Recombinant human IGF1 and recombinant human leptin were purchased from Peprotech. Insulin (Humulin® R) was obtained from the Pharmacy of the IRCCS Ospedale Policlinico San Martino. Puromycin, cholesterol-water soluble (cholesterol), methyl-β-cyclodextrin (MβCD), protease/phosphatase inhibitor cocktail, sulforhodamine B, oxiconazole, miconazole nitrate salt (miconazole), and clotrimazole were purchased from Sigma Aldrich S.r.l. terbinafine hydrochloride (terbinafine) was purchased from Abcam. Simvastatin was purchased from Targetmol. Itraconazole was purchased from Selleck Chemicals. Low density lipoprotein from human plasma (LDL) was purchased from Thermo Fisher Scientific.

Library Screening

$2.4x10^3$ PK9 or $8x10^2$ A549 cells were plated in 96 well plates in their regular medium. 24h later, the cell medium was removed and cells were washed with phosphate buffer saline (PBS) and incubated either in DMEM no glucose medium (LifeTechnologies, Italy) with 10% FBS, 1g/L glucose (CTR medium) or in DMEM no glucose medium with 1% FBS, 0.5 g/L glucose short term starvation medium (STS). The day after, cells were treated with Microsource Spectrum Collection and Selleck Preclinical/Clinical Compound Library at a dose of 10 µM. Viability was determined 72h later by CellTiter96 Aqueous One assay (Promega) according to the manufacturer's instructions.

Cell Viability Assays

Cancer cells were plated in 96-well plates in their regular medium. After 24 h, the medium was removed and cells were washed with PBS and incubated in either CTR or STS medium. Where

indicated, cells were supplemented with insulin (500 pM), IGF1 (5 ng/ml), leptin (50 ng/ml), 3h MβCD incubation (3.5 mM), or cholesterol (5 μg/ml). After a further 24 h, cells were stimulated with or without miconazole, clotrimazole, oxiconazole, itraconazole, simvastatin, or terbinafine in CTR or STS medium at the indicated concentrations. Viability was determined 72 h later by CellTiter 96 Aqueous One assay (Promega) according to the manufacturer's instructions.

Organoid Culture and Viability Assays

Our collaborator Roberto Benelli in the lab of Prof. Alessandro Poggi (Unità di Oncologia Molecolare e Angiogenesi, Ospedale San Martino, Genoa, Italy) cultured human colorectal cancer-derived organoids OMCR15-045TK as previously described (188, 189). The growth of CRC organoids in different conditions was monitored in 96-wells plates, by the live-imager JuLI-Stage (Nano-Entek, Waltham, MA) for 8 days. Tripsinized organoids were included in single 3μl Geltrex (Gibco-Thermo) drops, in the center of each well, and allowed to recover in complete medium for 48 h and treated with or without clotrimazole and terbinafine in regular or STS medium (diluted culture medium 1:10 in DMEM/F12 without B27 and EGF) at the indicated concentrations for 192 h. Ten wells for each condition were live-monitored after treatment. The mean area of organoids was calculated from each image by Image-J. The time points of each well were normalized against time 0, to compensate for plating efficiency variability, and plotted in excel. Student *t*-test was used to assess the statistical significance of the variation of any experimental condition, compared to the same time point of controls.

Retroviral Transduction

pBABE-puro (PBP), and PBP-myr-AKT were purchased from Addgene (Cambridge, MA, USA). 1×10^6 Phoenix cells were plated in 60 mm Petri dishes and allowed to adhere for 24 h. Thereafter, cells were transfected with 4 μg plasmid DNA using TransIT-293 (Mirus Bio, Madison, WI)

according to the manufacturer's instructions. Viral supernatants were harvested after 36, 48, 60, and 72 h and used to infect Capan-1 (5×10^5), Panc-1 (4.5×10^5), and MCF7 (5×10^5) cells in 100 mm Petri dishes in the presence of 5 μg/ml protamine sulfate. Successfully infected cells were selected using 1 μg/ml puromycin.

Immunoblotting

For protein lysate generation from cultured cells, 5×10^5 Capan-1, or 3×10^5 MIA PaCa-2 cells were plated in 100 mm Petri dishes in their regular medium. After 24 h, the medium was removed, and cells were washed with PBS and incubated in either CTR or STS medium. After a further 24 h, cells were stimulated with or without clotrimazole (15 μM) or terbinafine (30 μM). 24 h later, cells were washed and protein lysates were generated using the following protocol. Cells were washed twice with cold PBS and then manually scraped in the presence of 50-200 μl lysis buffer (25mM Tris-phosphate, pH 7.8; 2mM DTT; 2mM 1,2-diaminocyclohexane-N,N,N´,N´-tetraacetic acid; 10% glycerol; 1% Triton X-100). Cell lysates were incubated on ice for 15 min with 10 sec vortex shaking every five min. Finally, lysates were spun at 10.000 g for 2 min at 4°C. Supernatants were recovered and either used immediately or stored for subsequent use (16, 190). Protein lysates from primary tumors were obtained by adding a 400μL mixture of RIPA buffer, anti-protease and anti-phosphatase cocktails to tumor masses then homogenized using TissueRuptor (Qiagen). After clear homogenization samples were washed twice in cold PBS and the pellets were used for lysate preparation. Protein concentration was determined according to standard Bradford assay. 35μg of proteins (for protein lysate obtained from *in vitro* cell culture) or 20μg of proteins (for protein lysate obtained from *in vivo* xenografts) were separated by SDS–PAGE, transferred to a PVDF membrane (Immobilon-P, Millipore S.p.A.) and detected with the following antibodies: anti-phospho-AKT (Ser473; #4058), anti-AKT (#9272), anti-phospho-p70S6kinase (Thr389; #9206),

anti p70S6kinase (#9202), anti-phospho-mTOR (Ser2448; #5536), anti-mTOR (#2983), anti-phospho-4E-BP1(Thr37/46; #2855), anti-4E-BP1 (#4923), all from Cell Signaling Technology; vinculin, and anti-β-actin from Santa Cruz Biotechnology. Band intensities were quantified with Quantity One SW software (Bio-Rad Laboratories, Inc.) using standard enhanced chemiluminescence.

Colony Formation Assays

1×10^3 Capan–1 cells were plated in 6-well plates in a regular medium. 24 h later, the cell medium was removed. Cells were washed twice with PBS and were incubated in either CTR or STS medium. The next day, cells were treated with 10 μM clotrimazole, or 20 μM terbinafine for 24 h. Then cell medium was removed and cells were cultured for 2 additional weeks in a regular culture medium. Thereafter, the culture plates were fixed with cold 3% trichloroacetic acid at 4°C for 30 minutes, washed with cold water, and dried overnight. Finally, the plates were stained with 0.4% sulforodhamine B (SRB) in 1% acetic acid, washed four times with 1% acetic acid to remove the unbound dye, dried overnight, and then colonies were counted.

***In vivo* Therapy**

All mouse experiments were performed in accordance with the relevant laws and institutional guidelines for animal care and use established in the Principles of Laboratory Animal Care (directive 86 /609 /EEC). Animal work was only started upon approval by the Italian Istituto Superiore di Sanità (ISS). 6–8-week-old female BALB/c athymic (nu+/nu+) (purchased from Envigo) were used in the experiments at the Animal Facility of the IRCCS Ospedale Policlinico San Martino. These animals were maintained in air-filtered laminar flow cabinets with a 12-h light cycle and food and water *ad libitum*. Mice were acclimatized for 1 week. 2×10^6 Capan-1 or HCT116 cells were injected subcutaneously (SC) into either one or both flanks of the mouse. Treatment was initiated when the tumors appeared as established palpable masses (~2 weeks after

cell injection). In each experiment, mice were randomly assigned to one of the following arms: control (*ad libitum* diet); clotrimazole (60 mg/kg twice a week in peanut oil, intraperitoneal (i.p) injections (191)); terbinafine (40 mg/kg/d in 5% of absolute ethanol + 95% of 0.5% methylcellulose by oral gavage (192)); LDL (0.25 mg/mouse (193, 194), i.p, twice a week coupled with fasting); fasting (water only, for 48 h every week for 4 weeks (68, 182)); or combinations of these treatments as indicated. Mice were housed in a clean, new cage to reduce coprophagy and residual chow. Body weight was measured immediately before, during, and after fasting. Fasting cycles were repeated every 7 d to obtain complete recovery of body weight before a new cycle. Tumor volume was calculated using the formula: tumor volume = $(w2 \times W) \times \pi/6$, where "w" and "W" are "minor side" and "major side" (in mm), respectively. The maximal tumor volume that was permitted by our Institutional Animal Care and Use Committee (IACUC) was 1,500 mm^3, and in none of the experiments were these limits exceeded. Tumor masses were always isolated at the end of the last fasting cycle, weighed, divided into two parts, and stored in liquid nitrogen for subsequent protein extraction and cholesterol quantification.

Sample size estimation was performed using PS (Power and Sample size calculation) software (Vanderbilt University). By this approach, we estimated that the number of mice that were assigned to each treatment group would reach a power of 0.85. The Type I error probability associated with our tests of the null hypothesis was 0.05. Mice were assigned to the different experimental groups in a random fashion. Operators were unblinded, blinding during animal experiments was not possible because mice were subject to a specific diet supply and daily treatment.

Mice Serum Assays

To measure the changes in serum IGF1, C-peptide, and leptin concentrations we inoculated female BALB/c nude mice with HCT116 then treated with fasting (or *ad libitum* diet) with or without clotrimazole or clotrimazole plus fasting plus i.p. injection of human plasma LDL, mice whole blood was collected in Eppendorf tubes before randomization and at the end of the experiment. Afterwards, serum was allowed to coagulate for 2 h at room temperature, centrifuged for 20 min at 4,000 rpm, and then it was stored in aliquots in PCR tubes at −80 °C until subsequent use. The serum parameters were analyzed at Istituto San Raffaele (Milan, Italy) using certified kits on the Ilab-650 automatic instrument (Werfen Italy). Each analysis was preceded by the measurement of certified internal quality controls purchased directly from the manufacturer.

Cholesterol Quantification

To measure total cholesterol concentration in cancer cells we plated 5×10^5 Capan-1 PDAC cells in 100 mm Petri dishes in their regular medium. After 24 h, the medium was removed, and cells were washed with PBS and incubated in either CTR or STS medium. After a further 24 h, cells were stimulated with or without clotrimazole (20 μM) or terbinafine (50 μM). Meanwhile to measure total cholesterol concentration in tumor masses female nude mice were SC inoculated with Capan-1 or HCT116 cells. When tumors became palpable, mice were randomized to be treated with *ad libitum* diet, terbinafine, weekly 48h fasting, or terbinafine + fasting (Capan-1 xenografts) or *ad libitum* diet, clotrimazole, fasting, clotrimazole + fasting, or clotrimazole + fasting + i.p. human plasma LDL (HCT116 xenografts). At the end of the experiment (24 h after treatment regarding the cells or 4 weeks of treatment regarding mice xenografts), 10^6 cells or 10 mg tumor masses were washed with cold PBS. Lipids were extracted by resuspending the samples in 200 μL of Chloroform: Isopropanol: NP-40 (7:11:0.1) using the TissueRuptor (Qiagen). The

extract was centrifuged for 5 – 10 minutes at 15,000 xg in a micro centrifuge. Organic phase (supernatant) was transferred, avoiding the pellet, to a new tube, air dried at 50°C to remove chloroform and subjected to vacuum for 30 minutes to remove trace organic solvent using vacuum concentrator. Dried lipids were dissolved by vortexing with 200 µL of Assay Buffer. 50 µl of total cholesterol reaction mixture were added to samples and standards mixed well and incubated at 37 °C for 30 minutes protected from light. Then absorbance was measured at OD570 nm.

Tumor masses preparations and OXPHOS Assays and ATP/AMP measurements

The following experiments were performed by our collaborator Silvia Ravera in Dipartimento di medicina sperimental (DIMES), University of Genoa, Italy.

Tissue Homogenate Preparation for OXPHOS Assays and ATP /AMP Measurements

To determine oxidative phosphorylation (OXPHOS) assays and ATP /AMP measurements female nude mice were SC inoculated with Capan-1 or HCT116 cells. When tumors became palpable, mice were randomized to be treated with *ad libitum* diet, terbinafine, weekly 48h fasting, or terbinafine + fasting (Capan-1 xenografts) or *ad libitum* diet, clotrimazole, fasting, clotrimazole + fasting, or clotrimazole + fasting + i.p. human plasma LDL (HCT116 xenografts). Mice were sacrificed at the end of the fourth fasting cycle and 40 mg of tumor masses were washed in PBS and homogenized by a Potter–Elvehjem system in 1 mL of PBS plus protease inhibitors. All operations have been performed on ice. Total proteins were estimated by the Bradford method (195).

Oxygen Consumption Rate Evaluation

Oxygen consumption rate (OCR) was measured at 25°C in a closed chamber, using an amperometric electrode (Unisense-Microrespiration, Unisense A/S, Denmark). 50 µg of total proteins were used for each experiment. To stimulate the pathways composed of Complexes I, III,

and IV, 10 mM pyruvate plus 5 mM malate were added; to activate the Complexes II, III, and IV pathways, 20 mM succinate was employed (196).

Evaluation of OXPHOS Efficiency

The OXPHOS efficiency was calculated as the ratio between the concentration of the produced ATP and the amount of consumed oxygen in the presence of respiring substrate and ADP, obtaining the phosphate/oxygen (P/O) ratio. In coupled conditions, this value is around 2.5 or 1.5 in the presence of pyruvate + malate or succinate, respectively. Conversely, in the uncoupled status, this value decreases proportionally to the grade of the OXPHOS inefficiency (197).

Assay of ATP synthesis through FoF1-ATP synthase activity

The assay was conducted at 25°C by measuring formed ATP from added ADP. 50 µg of total proteins were added to the incubation medium containing: 10 mM Tris-HCl pH 7.4, 50 mM KCl, 1 mM EGTA, 2 mM EDTA, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 0.6 mM ouabain, 0.040 mg/ml ampicillin, 0.2 mM di-adenosine-5'penta-phosphate, and the respiratory substrates: 10 mM pyruvate plus 5 mM malate or 20 mM succinate. Tissue homogenates were incubated in the assay medium for 5 min at 25°C, then ATP synthesis was induced by the addition of 0.2 mM ADP and monitored by the luciferin/luciferase ATP bioluminescence assay kit CLSII (Roche, Basel, Switzerland), on a Luminometer (GloMax® 20/20 Luminometer – Promega, Wisconsin, USA), for two minutes, every thirty seconds. ATP standard solutions (Roche, Basel, Switzerland) in the concentration range 10-10 - 10-7 M was used for calibration (196).

Evaluation of intracellular ATP and AMP content and calculation of ATP/AMP ratio

ATP and AMP intracellular content was evaluated by the enzyme coupling method, as previously described (196). For each assay, 50 µg of total protein was employed. ATP was assayed, following NADP reduction, at 340 nm. The assay medium contained: 50 mM Tris-HCl pH 8.0, 1 mM NADP,

10 mM MgCl2, and 5 mM glucose. Samples were analyzed spectrophotometrically before and after the addition of 4 µg of purified hexokinase plus glucose-6-phosphate dehydrogenase. AMP was assayed following the NADH oxidation, at 340 nm. The reaction medium contained: 100 mM Tris-HCl pH 8.0, 75 mM KCl, 5 mM $MgCl_2$, 0.2 mM ATP, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 10 IU adenylate kinase, 25 IU pyruvate kinase, and 15 IU of lactate dehydrogenase. The ATP/AMP value was calculated as the ratio between the intracellular concentration of ATP and AMP, expressed in mM.

Statistical Analysis

The cooperative index (CI) in library screening was calculated using effect-based strategy (response additivity approach) (198) as the sum of the specific cell deaths induced by the single agents divided by the specific cell death in response to the combination. CI values <1, =1, and >1 indicate a synergistic, additive, or infra-additive effect, respectively. No significant validation was applied in the drug screening since we used only one replica for each compound. The positive control used in our drug screening was the effect of starvation conditions alone on the cells which is considered to be nearly the same in each experiment. Z-score was not estimated in the drug screening experiments preformed in the current thesis since I calculated one single raw score per experiment. Compounds with a CI<0,8 were selected for the retesting and statistical analysis was applied. All statistical analyses were performed using GraphPad Prism 8.0. Data are presented as mean ± SEM. The results were analyzed by two-tailed Student's *t*-test and P values less than 0.05 were considered to be statistically significant (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, and ****, $P < 0.0001$; n.s., not significant).

4. RESULTS

Starvation enhances the anti-tumor effects of cholesterol biosynthesis inhibitors in pancreatic ductal adenocarcinoma (PDAC), colorectal cancer (CRC) cells, and CRC tumor organoids as well as various types of cancer cells from different origins.

We screened over 800 approved drugs in pancreatic cancer cells (PK9), searching for agents whose antitumor activity would be synergistically increased by starvation conditions (STS). By this approach, we identified three fungicidal agents (imidazole derivatives), i.e. clotrimazole, miconazole, and oxiconazole, which all became strongly cytotoxic against PK9 cancer cells when combined with starvation conditions (Fig.4a). Aside from azoles, other screen hits were identified that resulted in synergistic anticancer effects when combined with STS. Nevertheless, they were not utilized in this study since they were not from the same drug class. Since azoles inhibit 14α-demethylase, which is a key enzyme for cholesterol biosynthesis (199), we reasoned that other agents targeting this metabolic route (Fig.4f) may also acquire antitumor effects through fasting. Consistent with this notion, in subsequent experiments, we found that itraconazole, a triazole derivative, simvastatin, an inhibitor of cholesterol rate-limiting enzyme HMG-CoA reductase, and terbinafine, an inhibitor of the cholesterol-producing enzyme SQLE, also had their antitumor effects strongly enhanced by starvation in PK9 and Capan-1 pancreatic ductal adenocarcinoma (PDAC) cells (Fig.4b) and HCT116 (colorectal cancer) CRC cells (Fig.4c). STS also increased the anti-tumor activity of terbinafine and clotrimazole in tumor organoids from patients with CRC (Fig.4d). STS cooperated with clotrimazole and terbinafine to reduce intracellular cholesterol in cultured Capan-1 cells (Fig.4e). Methyl-β-cyclodextrin (MβCD), a compound that acutely depletes intracellular cholesterol (200), showed a synergistic interaction (in terms of anti-cancer activity) with starvation conditions in Capan-1 (Fig.4g) and in

MIA PaCa-2 cells (not shown) and when added to combined azoles (clotrimazole or terbinafine) and starvation, it further enhanced PDAC cell demise. Culture media supplementation with a water-soluble cholesterol formulation, cholesterol–MβCD, (cholesterol add-back) prevented the synergistic interaction between STS, and cholesterol biosynthesis inhibitors (Fig. 4h). Subsequently, we screened the same library in A549 lung cancer cell line in an attempt to identify whether the screen hits, identified azoles, would result in a synergistic anti-tumor effect when combined with STS similar to those observed in the screening of PK9 cell line. Surprisingly, we were able to obtain a cooperative index (CI) of the previously identified imidazole derivatives less than 0.8 in A549 lung cancer cell line (Fig. 5a), much the same as the results of PK9 screening. In subsequent experiments, we found STS strongly enhanced the anticancer activity of cholesterol biosynthesis inhibitors in various cancer cell lines from different origins (Fig. 5b-g). In addition, combined treatment reduced the number of Capan-1 cell colonies compared to other treatment groups (Fig. 5h). Taken together, these data suggest a potential antitumor effect of this combined therapy *in vivo*.

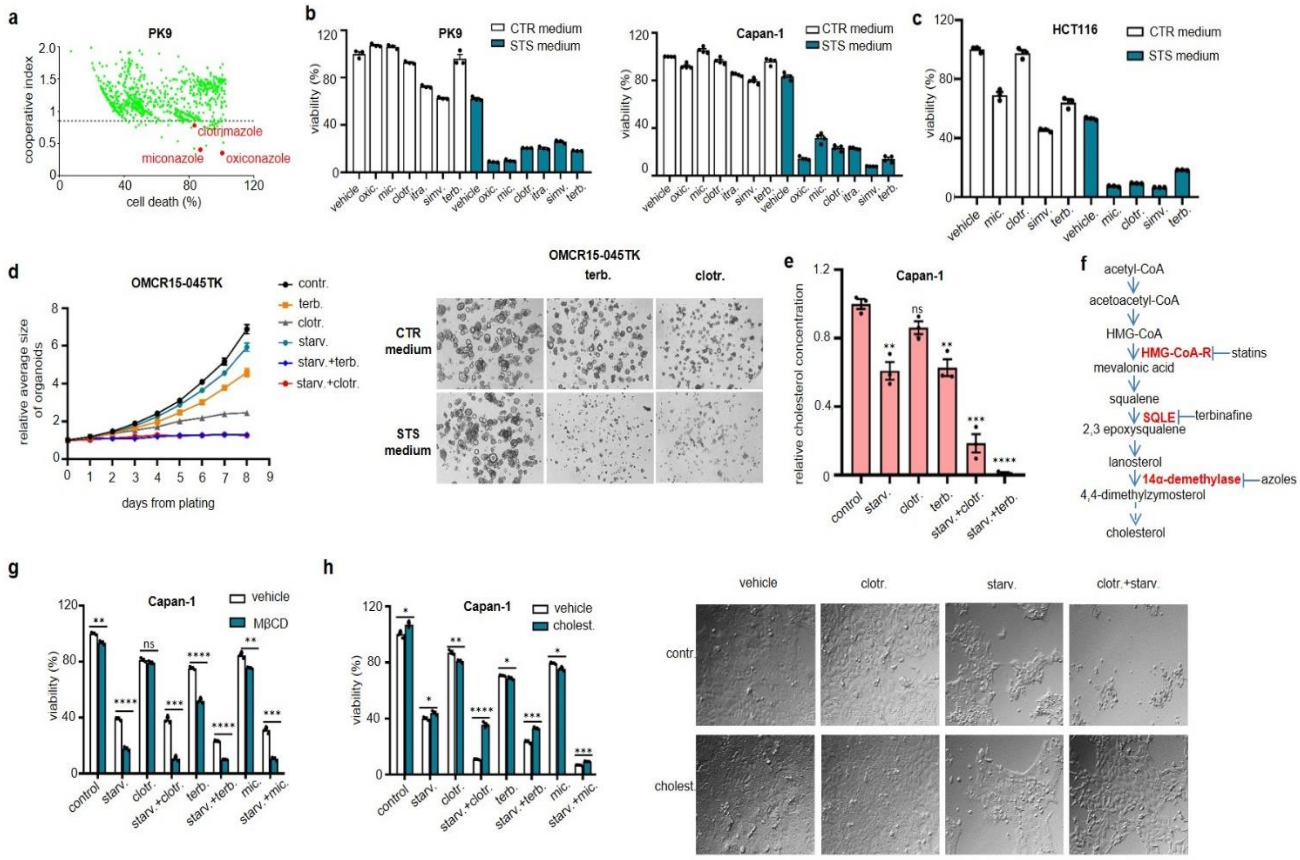

Fig. 4 | Cholesterol biosynthesis inhibitors cooperate with starvation to kill pancreatic cancer cells. **a,** Screens for compounds whose antitumor activity is enhanced by starvation (i.e. 1% FBS and 0.5 g/l glucose) in PK9 pancreatic cancer cells (Microsource Spectrum Collection and Selleck Preclinical/Clinical Compound Library). Cooperative index (CI) is the sum of the specific cell death induced by the single agents divided by the specific cell death in response to the combination. While CI values <1 are considered to be indicative of a synergistic effect; we set our threshold for hit validation at 0.8 to increase stringency. **b,** PK9 and Capan-1 (PDAC cell lines) cell viability [quantified by CellTiter96 Aqueous1 (Promega)] after a 72h treatment with the indicated drugs (oxiconazole (oxic.), miconazole (mic.), and clotrimazole (clotr.) 10 μM; itraconazole (itra.) 1 μM; simvastatin (simv.) and terbinafine (terb.) 50 μM), w/ or w/o starvation. **c,** HCT116 CRC cells cell viability [quantified by CellTiter96 Aqueous One assay (Promega)] after a 72h treatment with the indicated drugs (mic. and clotr. 15 μM; simv. 100 μM). **d,** Tumor organoids from patients with CRC were cultured w/ or w/o terb. 20 μM. or clotr. 15 μM. under control or STS conditions for 192 h, and then were imaged. **e,** Capan-1 cells were treated for 24h w/ or w/o 20 μM clotr. or 50 μM terb. under control or starvation conditions or the combination of each drug with starvation. Thereafter, cellular cholesterol was measured by Cholesterol/ Cholesteryl Ester Quantitation Assay Kit (Abcam). **f,** Cholesterol biosynthesis pathway. **g,** Capan-1 cells were plated in 96-well plates and treated for 96h w/ or w/o clotr. (10 μM), terb. (50 μM), mic. (10 μM) or a 3h methyl-β-cyclodextrin (MβCD) incubation (3.5 mM) under control or starvation conditions, or the combination of each drug with starvation. Thereafter, cell viability was quantified by CellTiter96 Aqueous One assay (Promega). **h,** Capan-1 cells were plated in 96-well plates and treated for 96h w/ or w/o clotr. (10 μM), terb. (30 μM), mic. (10 μM) under control or starvation conditions or combined drugs with starvation in the presence or absence

of 5 µg/ml cholesterol-methyl-β-cyclodextrin. Afterward, cells were imaged by phase contrast microscopy and cell viability was quantified as described above. All experiments were performed in at least triplicates. Data are presented as mean ± SEM. Data were analyzed by two-tailed Student's *t*-test and statistical analysis was applied with *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, ****: $p<0.0001$: n.s., not significant compared to control treatment.

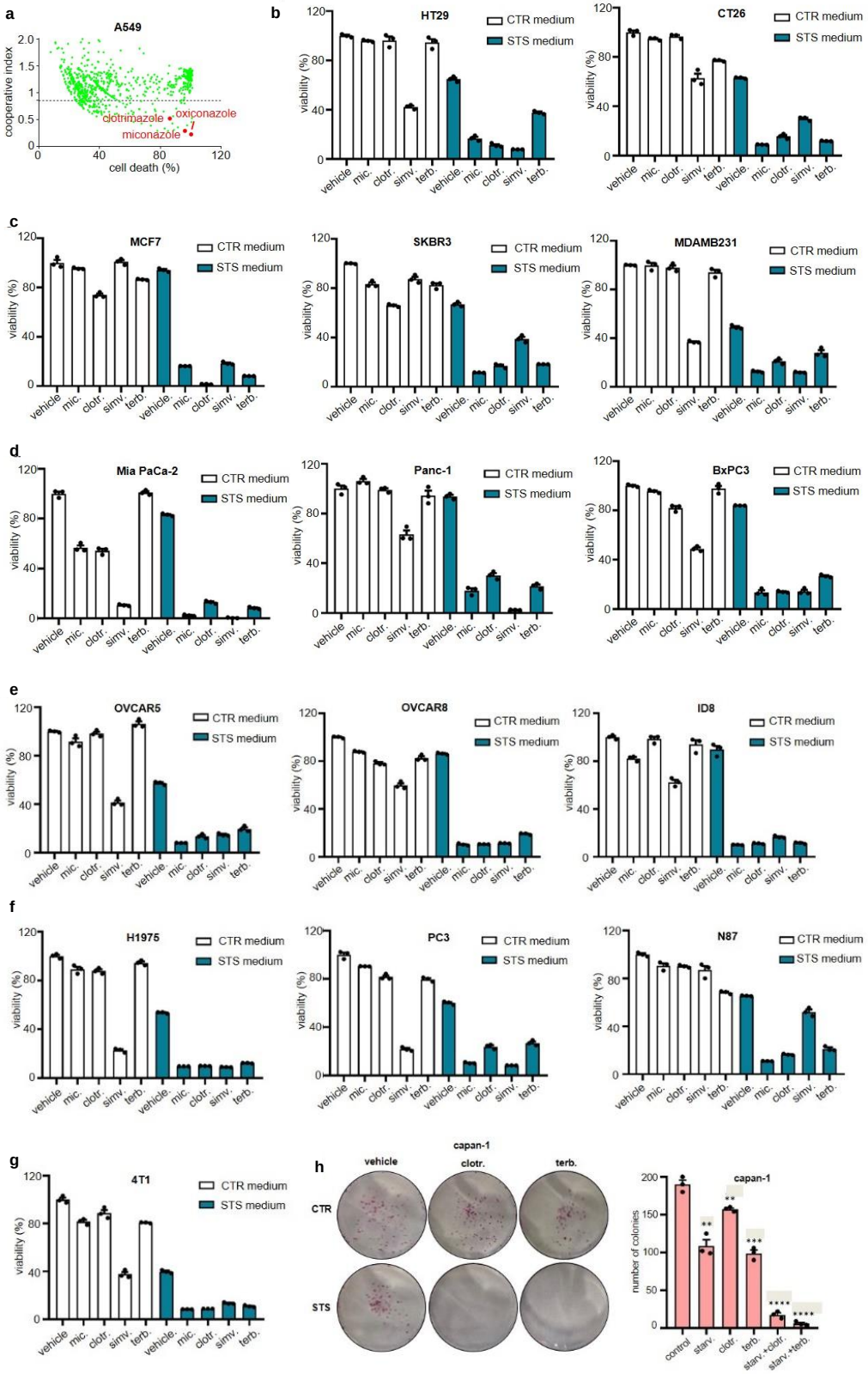
a
A549
cooperative index
cell death (%)
clotrimazole
oxiconazole
miconazole
b
HT29
CT26
c
MCF7
SKBR3
MDAMB231
d
Mia PaCa-2
Panc-1
BxPC3
e
OVCAR5
OVCAR8
ID8
f
H1975
PC3
N87
g
4T1
h
capan-1
vehicle
clotr.
terb.
CTR
STS
number of colonies
control
starv.
clotr.
terb.
starv.+clotr.
starv.+terb.
CTR medium
STS medium
viability (%)
vehicle
mic.
clotr.
simv.
terb.

Fig. 5 | Cholesterol biosynthesis inhibitors cooperate with starvation to kill various cancer cells. a, Screens for compounds whose antitumor activity is enhanced by starvation (i.e. 1% FBS and 0.5 g/l glucose) in A548 lung cancer cells (Microsource Spectrum Collection and Selleck Preclinical/Clinical Compound Library). Cooperative index (CI) is the sum of the specific cell death induced by the single agents divided by the specific cell death in response to the combination. While CI values <1 are considered to be indicative of a synergistic effect; we set our threshold for hit validation at 0.8 to increase stringency. **b,** HT29 (CRC human cell line) and CT26 (CRC murine cell line) cell viability after a 72h treatment with the indicated drugs (mic. and clotr. 15 μM; simv. 100 μM (in HT29 cells) or simv. 10 μM (in CT26 cells) and terb. 30 μM), w/ or w/o starvation). **c,** MCF7, SKBR3, and MDAMB231 (BC human cell lines) cell viability after a 72h treatment with the indicated drugs (mic. and clotr. 15 μM; simv. 30 μM (in MCF7 and SKBR3 cells) or simv. 100 μM (in MDAMB231 cells) and terb. 30 μM), w/ or w/o starvation. **d,** MIA PaCa-2, Panc-1 and BxPC3 (PDAC cell lines) cell viability after a 72h treatment with the indicated drugs (mic. and clotr. 15 μM; simv. 100 μM and terb. 30 μM), w/ or w/o starvation. **e,** OVCAR5 and OVCAR8 (ovarian human cancer cell lines) and ID8 (ovarian murine cancer cell line) cell viability after a 72h treatment with the indicated drugs (mic. and clotr. 15 μM; simv. 30 μM (in OVCAR5 and ID8 cells) or simv. 100 μM (in OVCAR8 cells) and terb. 30 μM), w/ or w/o starvation. **f,** H1975 (lung human cancer cell line), PC3 (prostate human cancer cell line), and N87 (gastric human cancer cell line) cell viability after a 72h treatment with the indicated drugs (miconazole (mic.) and clotrimazole (clotr.) 15 μM; simvastatin (simv.) 100 μM (in H1975 and PC3) or simv. 30 μM (in 4T1) and terbinafine (terb.) 30 μM), w/ or w/o starvation. **g,** 4T1 (BC murine cell line) cell viability after a 72h treatment with the indicated drugs (mic. and clotr. 15 μM; simv. 10 μM (in 4T1) and terb. 30 μM), w/ or w/o starvation. Thereafter, cell viability was quantified by CellTiter96 Aqueous One assay (Promega). **h,** Capan-1 cells were seeded in 6-well plates and cultured with or without STS, 10 μM clotr., 20 μM terb., or their combinations for 24 h. Thereafter, cells were cultured in a regular medium for an additional 10 days. Finally, cells were fixed and stained with sulforhodamine B and cell colonies were counted. All experiments were performed in at least triplicates. Data are presented as mean ± SEM. Data were analyzed by two-tailed Student's *t*-test and statistical analysis was applied with *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, ****: $p<0.0001$: n.s., not significant compared to control treatment.

Fasting plus inhibitors of *de novo* cholesterol biosynthesis pathway reduce tumor growth in PDAC (Capan-1) xenografts

To further investigate the cooperative role of fasting and cholesterol biosynthesis inhibitors against PDAC, we employed two *in vivo* experiments using Capan-1 cells which were injected into female nude mice that were treated with *ad libitum* (*ad lib*.) diet, weekly 48h every week fasting (starv.), or clotrimazole (clotr.) or combined fasting plus clotr. or terbinafine (terb.) or combined fasting plus terb. Weekly cycles of fasting were found to enhance clotrimazole and terbinafine antitumor effects in Capan-1 xenograft-bearing nude mice (Fig.6a, b). To determine whether azoles or terbinafine synergize with fasting by disrupting the intratumor cholesterol production, we determined total cholesterol content using Cholesterol/ Cholesteryl Ester Quantitation Assay Kit in isolated tumor masses. Consistent with the hypothesis that cholesterol biosynthesis inhibitors cooperate with fasting by targeting cholesterol production in cancer cells, we found that terbinafine plus fasting also blunt the cholesterol levels of Capan-1 xenografts (Fig.6b). In addition, combined fasting and terbinafine lowered circulating LDLs, whereas increased HDL cholesterol and reduced triglycerides as compared to terbinafine treatment alone) (Fig.6c). Collectively, these results point to that the demonstrated anticancer effects of this combined therapy may rely, at least in part, upon intracellular and tumor mass cholesterol levels.

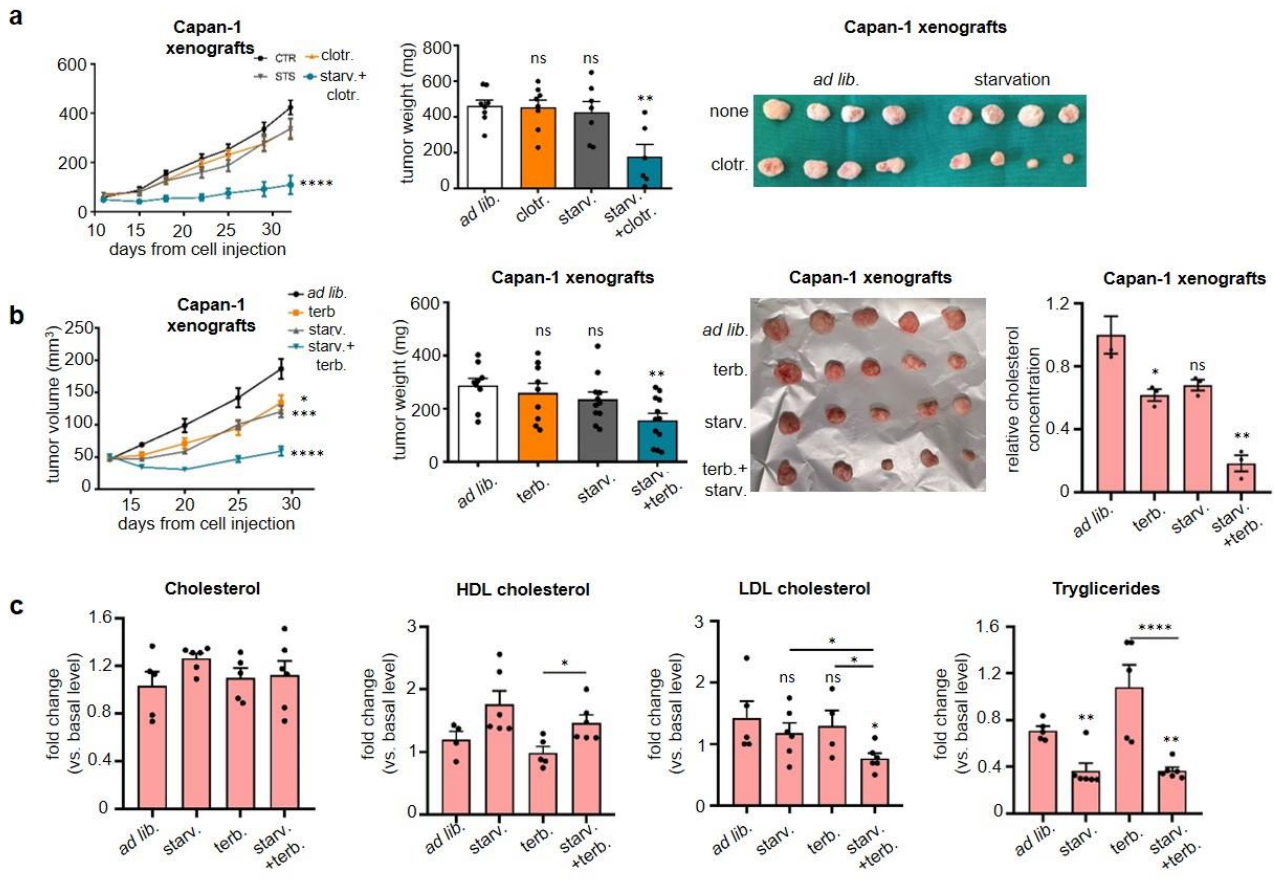

Fig. 6 | Fasting and inhibitors of cholesterol biosynthesis cooperate *in vivo* to slow tumor growth and reduce intratumor cholesterol and circulating LDL cholesterol. Growth of 2 × 10^6 Capan-1 subcutaneous xenografts (SC) in BALB/c female nude mice treated with *ad libitum* diet (*n*=6), weekly 48h fasting (starv.) (*n*=7), clotrimazole (clotr.) (60 mg/kg twice a week, i.p) (*n*=8) or combined starv.+clotr. (*n*=6) **(a)** *ad libitum* diet (*n*=9), weekly 48h fasting (starv.) (*n*=11), terbinafine (terb.) (40 mg/kg/d, orally) (*n*=11) or combined starv.+terb. (*n*=12) **(b)**. At the end of the experiment, tumors were weighed and imaged. In addition, in animals fed *ad libitum* or undergoing weekly fasting and treated w/ or w/o terb. tumor masses and peripheral blood were collected at the end of the experiment. Intratumor cholesterol content was measured by Cholesterol/ Cholesteryl Ester Quantitation Assay Kit (Abcam) normalized with the protein content of the respective tissue specimen. (b, panel on the right). **c,** total cholesterol, HDL and LDL cholesterol, and triglycerides were detected by standard chemistry (Animal biochemistry, Istituto San Raffaele, Milan). All experiments were performed in at least triplicates. Data are presented as mean ± SEM. Data were analyzed by two-tailed Student's *t*-test and statistical analysis was applied with *: p<0.05; **: p<0.01; ***: p<0.001, ****: p<0.0001: n.s., not significant compared to control treatment.

Exogenous administration of human plasma LDL counteracts the antitumor effect exerted by fasting plus clotrimazole in HCT116 xenografts

To test the hypothesis that exogenous administration of LDL cholesterol can restrain the previously shown antitumor effect of fasting plus cholesterol biosynthesis inhibitors, we injected BALB/c nude mice with HCT116 colorectal cancer (CRC) cell line and provided either *ad libitum* diet, or weekly 48h fasting, or clotrimazole, or clotrimazole plus weekly 48h fasting, or clotrimazole plus weekly 48h fasting plus intraperitoneal (i.p.) injection of human plasma LDL (twice a week coupled with the 48h of fasting). As anticipated, adding back LDL, which is referred to as bad cholesterol, in mice bearing HCT116 xenografts that were treated with clotrimazole plus fasting was sufficient to revert the fasting-induced enhancement of clotrimazole activity (Fig. 7a). We obtained similar results in cultured HCT116 cells when its culture media were supplemented with water-soluble cholesterol formulation and treated with clotrimazole plus STS (Fig. 7d). In addition, administering LDL to combined fasting and clotrimazole not only abrogated clotrimazole reduction of the intratumor cholesterol content of HCT116 xenografts via fasting but also increased circulating triglycerides as compared to fasting and clotrimazole treatment alone (Fig.7b and 7c respectively). These data suggest that exogenous cholesterol injection, in the form of LDL, impedes the antitumor effect produced by starvation plus cholesterol biosynthesis inhibitors.

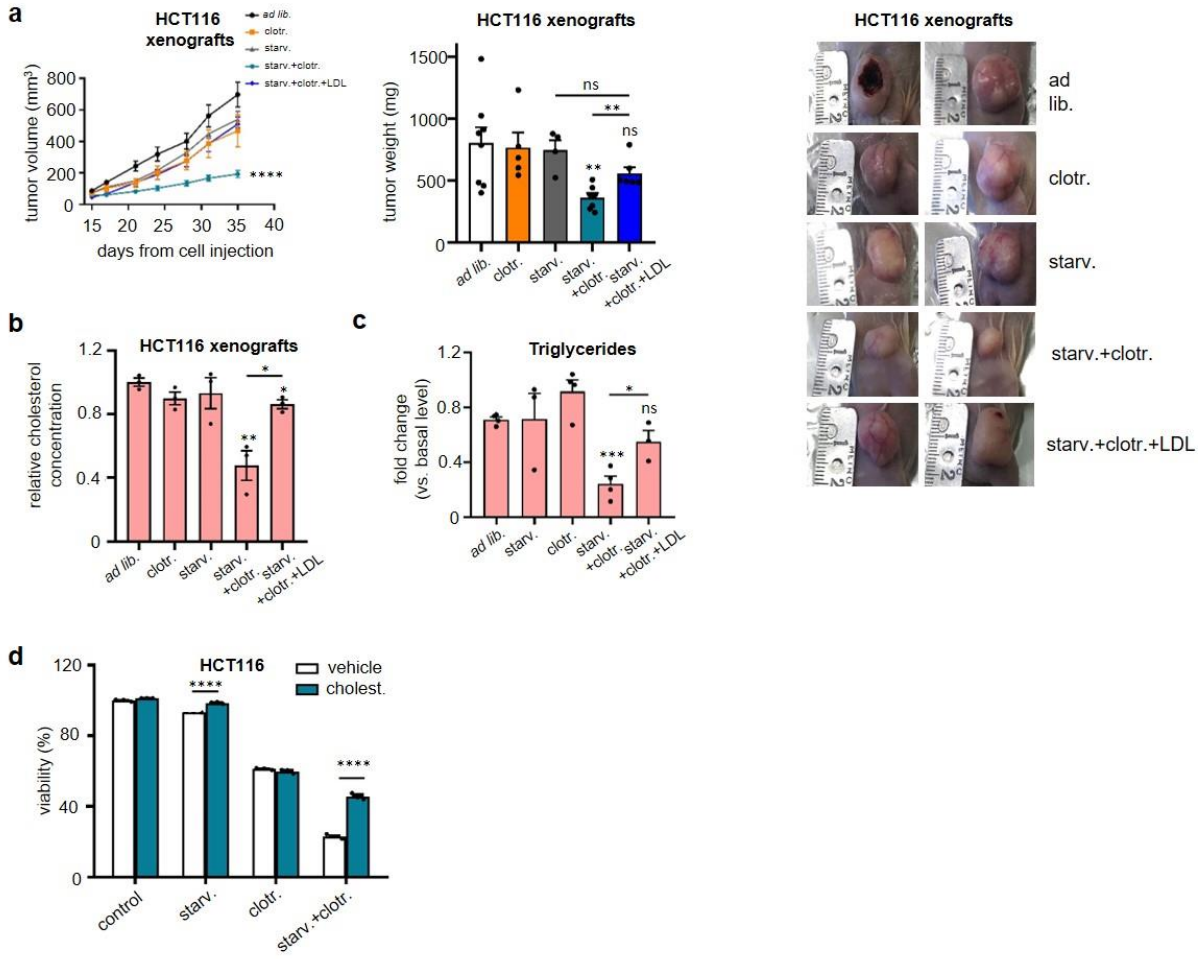

Fig. 7 | Adding back LDL reverts the fasting enhancement of clotrimazole antitumor activity *in vivo* and increases intratumor cholesterol and circulating triglycerides. a, Growth of 2 × 10^6 HCT116 SC xenografts in BALB/c female nude mice treated with *ad libitum* diet (*n*=8), or weekly 48h fasting (starv.) (*n*=4), or clotrimazole (clotr.) (60 mg/kg twice a week, i.p) (*n*=5) or combined starv.+clotr. (*n*=7), or clotr. +starv.+ i.p. injection of human plasma LDL (0.25 mg/mouse, twice a week coupled with starv.) (*n*=6). At the end of the experiment, tumors were weighted and imaged. **b,** Intratumor cholesterol content was measured by Cholesterol/ Cholesteryl Ester Quantitation Assay Kit (Abcam) normalized with the protein content of the respective tissue specimen. **c,** Peripheral blood was collected at the end of the experiment, and triglycerides were detected by standard chemistry (Animal biochemistry, Istituto San Raffaele, Milan). **d,** HCT116 cells were plated in 96-well plates and treated for 96h w/ or w/o clotr. (10 μM) under control or starvation conditions or combined drugs with starvation in the presence or absence of 5 μg/ml cholesterol-methyl-β-cyclodextrin. Thereafter, cell viability was quantified by CellTiter96 Aqueous One assay (Promega). Experiments in **b, c, d,** were performed in triplicates. Data are presented as mean ± SEM. Data were analyzed by two-tailed Student's *t*-test and statistical analysis was applied with *: p<0.05; **: p<0.01; ***: p<0.001, ****: p<0.0001: n.s., not significant compared to control treatment.

Fasting potentiates the activity of cholesterol biosynthesis inhibitors in gastrointestinal cancers through AKT inhibition and reducing circulating growth-promoting factors

Cholesterol is an essential component of mammalian cells, being the main lipid constituent of the plasma membrane (and being also abundant in most organelle membranes). At the plasma membrane level, cholesterol helps maintain membrane fluidity and is essential for the formation of liquid-ordered domains known as lipid rafts (201). The latter, in turn, harbor critical pro-survival signaling cascades, including IGF1/phosphatidylinositol 3-kinase (PI3K)/AKT signaling (201). Thus, we reasoned that the cooperation between cholesterol biosynthesis inhibiting agents and starvation conditions could rely, at least in part, on AKT inhibition. Consistent with this hypothesis, we found that combined clotrimazole or terbinafine and starvation strongly downregulated phosphorylated AKT in MIA PaCa-2 (PDAC) cells (Fig.8a), where, in addition to low AKT phosphorylation in response to the combination of miconazole or clotrimazole with starvation, reduced mTOR, p70S6K, and 4E-BP1 phosphorylations were also detected (which is consistent with dampened mTOR activity downstream of AKT). Similar results were obtained in Capan-1 cells (Fig. 8b). Consistent with this notion, tumors isolated from mice (in the experiment with Capan-1 xenografts) treated with terbinafine plus fasting showed reduced phosphorylation of AKT, whereas tumors from mice that were given human plasma LDL (in the experiment with HCT116 xenografts) during treatment with clotrimazole plus fasting showed AKT phosphorylation levels comparable to those in mice undergoing only fasting (Fig.8c) indicating that adding back bad cholesterol could revert the reduced AKT phosphorylation induced by cholesterol biosynthesis inhibitors plus fasting. Capan-1 cell transduction with myristoylated AKT conferred significant protection from combined terbinafine or clotrimazole and starvation (Fig.8d) in culture, similar results were obtained in MCF7 (HR+ BC) and Panc-1 (PDAC) cells when

transduced with myristoylated AKT (Fig. 9a, b), indicating that AKT inhibition via these combined interventions is indeed likely to play a role in this synergistic interaction. In subsequent experiments, we were able to prevent terbinafine potentiation by starvation through simultaneous supplementation with IGF1, insulin, and leptin (circulating growth-promoting factors) (Fig.8e), which are downregulated during fasting and to mediate fasting-dependent antitumor effects (16, 29, 69). In addition, mice treated with fasting plus clotrimazole had a significant decrease in serum C-peptide (a proxy of endogenous insulin production), circulating insulin-like growth factor 1 (IGF1), and leptin (Fig.8f) compared to single treatment (except mice undergoing only starvation). Thus, these data are strongly suggestive of a non-redundant role of pAKT and these three factors in mediating the observed antitumor synergy.

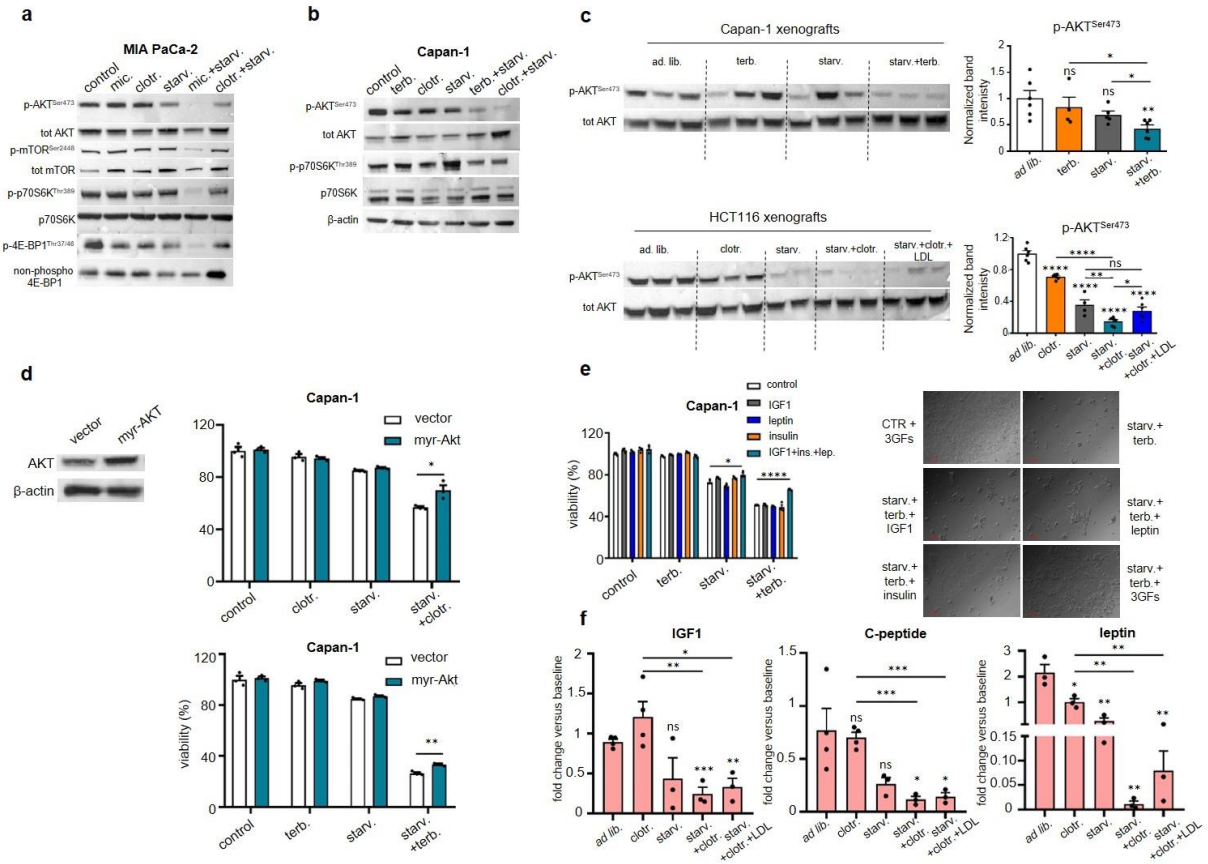

Fig. 8 | Fasting and inhibitors of the cholesterol biosynthesis pathway cooperate through AKT inhibition and via insulin, IGF1 and leptin reduction. **a,** MIA PaCa-2 (PDAC) cells were treated for 24h w/ or w/o 10 μM clotr. or 10 μM mic. under control or STS conditions. Thereafter, protein lysates were generated and total and phosphorylated AKT, mTOR, p-p70S6K, p70S6K, p-4E-BP1, and non-phospho 4E-BP1 were revealed by immunoblotting. **b,** Capan-1 cells were treated for 24h w/ or w/o 10 μM clotr. or 25 μM terb. under control or STS conditions. Thereafter, cells were used for protein lysate generation, and total and phosphorylated AKT, p-p70S6K, and p70S6K were detected by immunoblotting. **c,** Female nude mice were SC inoculated with Capan-1 or HCT116 cells. When tumors became palpable, mice were randomized to be treated with *ad libitum* diet, terbinafine (terb.), weekly 48h fasting (starv.), or terb. + starv. (Capan-1 xenografts) or *ad libitum* diet, clotrimazole (clotr.), starv., clotr. + starv., or clotr. + starv. + i.p. human plasma LDL (HCT116 xenografts). At the end of the experiment, mice were sacrificed, and tumor protein lysates were obtained. Left, phosphorylated (Ser473 for AKT) and total AKT (on the same gel) in the tumors were detected by immunoblotting. Right, phosphorylated AKT bands were quantified and normalized to total AKT (data points are biological replicates). **d,** Capan-1 (PDAC) cells were transduced with myristoilated AKT (or with a control vector). Cells were then treated w/ or w/o 5 μM clotr. or 10 μM terb. under control or STS conditions for 96h. Thereafter cell viability was determined by CellTiter96 Aqueous One assay (Promega) (data points are biological replicates). **e,** Capan-1 cells were treated for 96h with terb. (25 μM), STS conditions or their combination in the presence or absence of IGF1 (5 ng/ml), leptin (50 ng/ml), insulin (500 pM), or IGF1+leptin+insulin. Thereafter, cell viability was determined by CellTiter96 Aqueous One assay

(Promega) (data points are biological replicates). **f,** Changes in serum IGF1, C-peptide, and leptin concentration in female BALB/c nude mice that were inoculated with HCT116 then treated with starv. (or *ad libitum* diet) w/ or w/o clotr. or clotr. plus starv. plus i.p. injection of human plasma LDL (data points are biological replicates). Data are presented as mean ± SEM. Data were analyzed by two-tailed Student's *t*-test and statistical analysis was applied with *: p<0.05; **: p<0.01; ***: p<0.001, ****: p<0.0001: n.s., not significant compared to control treatment.

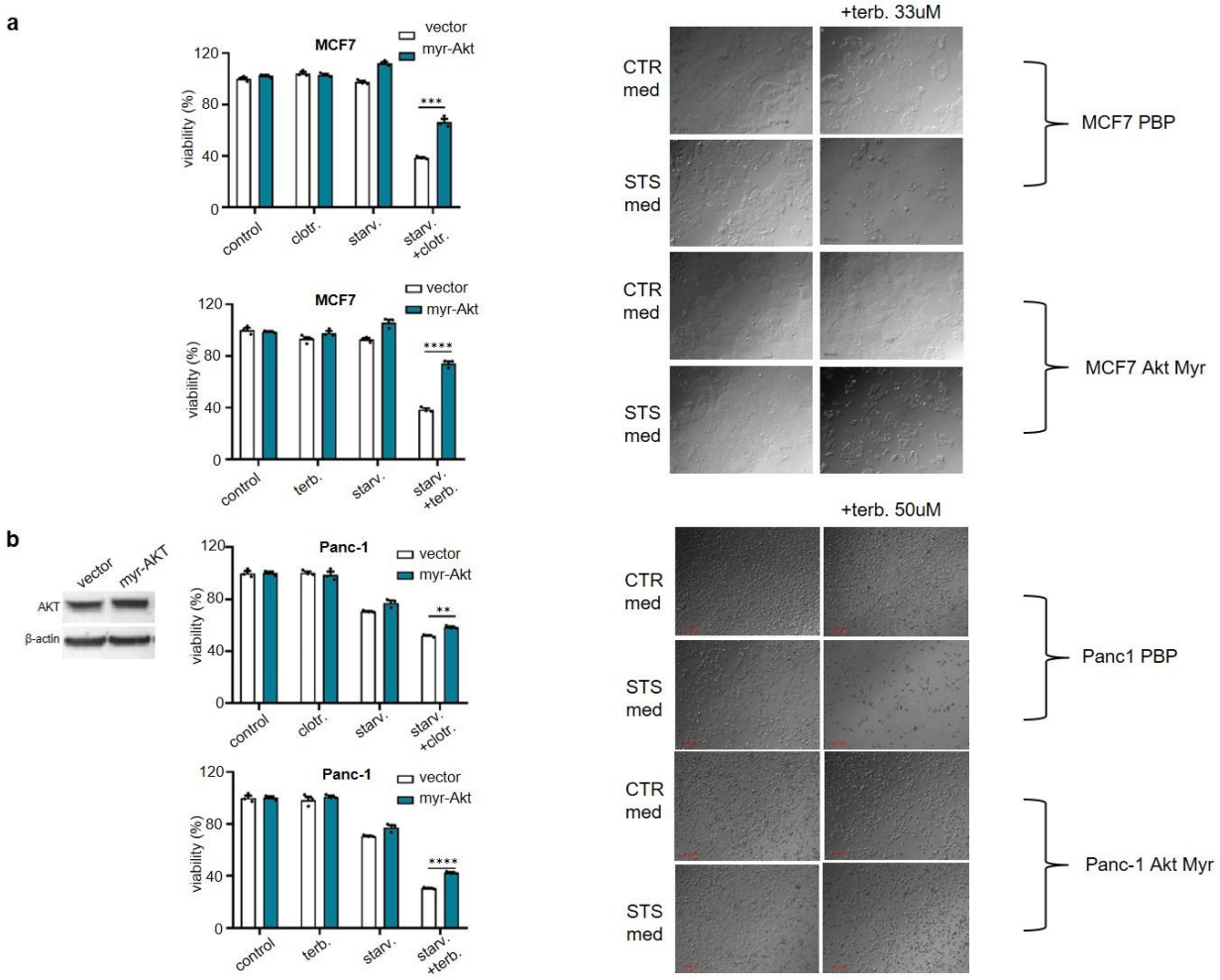

Fig. 9 | Myr Akt reduced the sensitivity of MCF7 and Panc-1 cells to combined starvation and inhibitors of the cholesterol biosynthesis pathway. a, MCF7 (HR+ BC) and **b,** Panc-1 (PDAC) cells were transduced with myristoilated AKT (or with a control vector). Cells were then treated w/ or w/o 5 µM clotrimazole (clotr.) or 10 µM terbinafine (terb.) under control or starvation conditions for 96h before cell viability was quantified by CellTiter96 Aqueous One assay (Promega). All experiments were performed in at least triplicates. Data are presented as mean ± SEM. Data were analyzed by two-tailed Student's *t*-test and statistical analysis was applied with *: p<0.05; **: p<0.01; ***: p<0.001, ****: p<0.0001: n.s., not significant compared to control treatment.

The cooperative effect of fasting and cholesterol biosynthesis inhibitors decreases mitochondrial oxidative phosphorylation (OXPHOS) in mouse gastrointestinal tumors

Mitochondrial oxidative phosphorylation (OXPHOS) is highly active and plays a pivotal role in cancer progression, survival, and metastasis also metabolic reprogramming is essential for tumor growth to reinforce cancer cell development (202, 203). Previous studies suggested that OXPHOS may be affected by cholesterol levels and its biosynthesis enzymes (204-206). Thus, we hypothesized that our combined therapy, which has previously shown blunt cholesterol levels *in vitro* and *in vivo*, could affect OXPHOS and cellular energy status in gastrointestinal tumors obtained from nude mice. To address this hypothesis, we subcutaneously implanted Capan-1 and HCT116 cells in BALB/c nude mice and subjected them to *ad libitum* diet, weekly cycles of fasting, terbinafine, or combined terbinafine and fasting, in case of Capan-1 xenografts, or *ad libitum* diet, fasting, clotrimazole, or combined clotrimazole and fasting, or clotrimazole and fasting+LDL in the HCT116 xenografts. Tumor masses were washed and homogenized, and total protein concentrations were estimated to determine OXPHOS assays and ATP /AMP measurements. As predicted, combined therapy diminished the activity of phosphate/oxygen ratio (P/O), O2 consumption, and ATP synthesis, determining a consistent decrease in the ATP/ AMP ratio (Figs. 10a–d) in the tumor masses of both experiments. Interestingly, LDL when added to the combined therapy opposed this effect. Overall, these findings indicate that cholesterol depletion, as a result of combining fasting and cholesterol biosynthesis inhibitors, might play a key role in reducing OXPHOS and energy status in gastrointestinal tumor xenografts, whereas cholesterol restoration abolishes this effect.

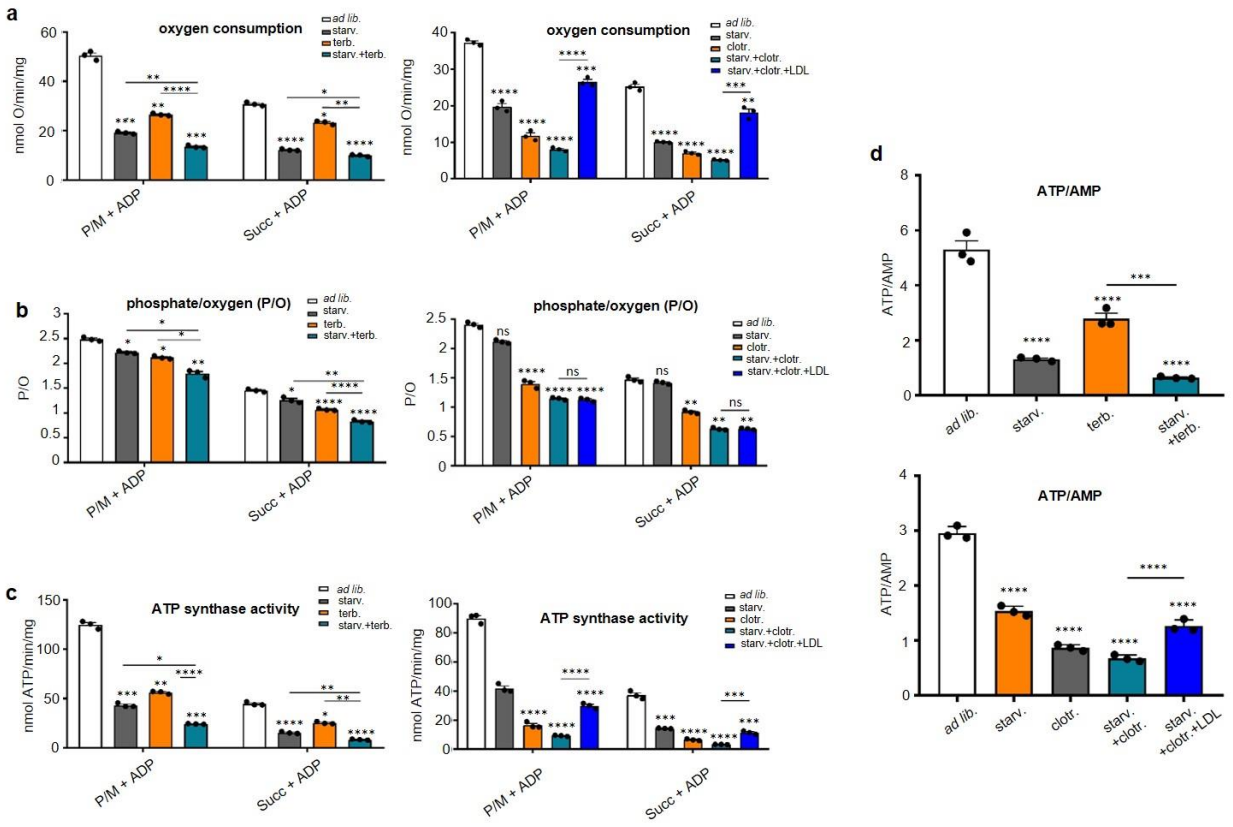

Fig. 10. cholesterol biosynthesis inhibitors and fasting diminish mitochondrial oxidative phosphorylation (OXPHOS) and energy status in mouse gastrointestinal tumors while LDL add-back enhances it. Female nude mice were inoculated with Capan-1 and HCT116 cells. When tumors became palpable, mice were randomized to be treated with *ad libitum* diet, terbinafine (terb.), weekly 48h fasting (starv.) or terb. + starv. (Capan-1 xenograft experiment) or *ad libitum* diet, clotrimazole (clotr.), starv., clotr. + starv., or clotr. + starv. + i.p. human plasma LDL (HCT116 xenograft experiment). Mice were sacrificed at the end of the fourth fasting cycle. Tumor masses were washed and homogenized, and total protein concentrations were estimated by Bradford assay to determine the following parameters. In **a,** oxygen consumption. **b,** phosphate/oxygen ratio. **c,** activity of Fo-F1 ATP synthase. In **a, b,** and **c** (left) is data from Capan-1 xenograft experiment while (right) is data from HCT116 xenograft experiment. **d,** and energy status, expressed as ATP/AMP ratio. In **d** (upper panel) is data from Capan-1 xenograft experiment while (lower panel) is data from HCT116 xenograft experiment. Data points are biological replicates. Data are presented as mean ± SEM. Data were analyzed by two-tailed Student's *t*-test and statistical analysis was applied with *: p<0.05; **: p<0.01; ***: p<0.001, ****: p<0.0001: n.s., not significant compared to control treatment.

5. DISCUSSION AND CONCLUSION

Considering the laborious, highly expensive costs, increase risk of failure, and slow pace of drug discovery, repurposing of available drugs to treat cancer, is increasingly becoming a promising approach since it requires the use of FDA-approved drugs, with shorter development timelines and potentially lower overall development costs (1, 3). In addition to being studied as a complement to standard therapeutics, periodic fasting has also been able to increase the antitumor effects of metformin and vitamin C (184-186), suggesting a promising key role of this dietary approach in the repositioning of drugs in oncology. Here we were able to obtain a powerful antitumor effect of cholesterol biosynthesis inhibitors once these were combined with periodic fasting in several cancer types from different origins (Fig. 5 and 6).

Multiple mechanisms promoting deregulation of cholesterol homeostasis can promote tumor development and progression. Recently, Riscal and colleagues reported that high dietary cholesterol promotes cancer cell progression and survival either *in vitro* or tumor growth *in vivo* in clear cell renal cell carcinoma and vice versa (207). Recent *in vitro* and *in vivo* studies on pancreatic ductal adenocarcinoma (PDAC) demonstrated that upregulation of the cholesterol synthesis pathway plays a crucial role in maintaining its growth and metastasis (86). Furthermore, upregulation of this pathway has been also found across clinical specimens (86). Besides, the study by McGregor and colleagues shows that PDAC cells acquire cholesterol from their extracellular environment, and maintain cholesterol homeostasis by inducing *de novo* synthesis when extracellular availability is limited (87). Thus, targeting the *de novo* cholesterol biosynthesis pathway represents an attractive therapeutic proposition. Several enzymes involved in this pathway are deregulated in cancer cells (208, 209), including but not limited to, the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), an enzyme targeted by statins

(86), squalene epoxidase (SQLE) [a cholesterol-producing enzyme with pro-oncogenic effects which is inhibited by terbinafine] (192, 210), and cytochrome p450 enzyme lanosterol 14α-demethylase CYP51, a key enzyme involved primarily in cholesterol biosynthesis which is obstructed by the antifungal drugs, azoles (199). Moreover, cholesterol accumulates in membrane domains and stabilizes them by interacting with other lipids and a certain class of raft proteins to form liquid-ordered domains known as lipid rafts (211), which, regulate numerous cellular signaling pathways related to cell proliferation, such as IGF1/phosphatidylinositol 3-kinase (PI3K)/AKT signaling (201, 212). We show here that fasting enhances the antitumor effect of cholesterol biosynthesis inhibitors, at least in part, by targeting intracellular cholesterol either *in vitro* or *in vivo* in gastrointestinal tumors. Consistent with this data, adding back cholesterol or LDL to the combined intervention resulted in cancer survival both *in vitro* and *in vivo*.

A recent study also reported that modified fasting (through L-Nutra's "FMD") reduces circulating growth-promoting factors in cancer patients (213), which may point out the important role of these factors in the cooperative antitumor effect of our combined intervention. In the line with this study, we found that supplementing circulating growth-promoting factors simultaneously was able to revert terbinafine potentiation induced by starvation in Capan-1 cells. In addition, these circulating growth factors were previously reported to induce cancer development and have overlapping effects in activating signaling cascades such as the PI3K–AKT–mTOR pathway in gastrointestinal tumors (214-218). Moreover, the lab of *Prof.* Nencioni has previously shown that periodic fasting or modified fasting (by the FMD) enhances the anticancer activity of hormone therapy through reducing blood insulin, IGF1, and leptin besides inhibition of the PI3K–AKT–mTOR pathway in HR+ BC (182). Here we demonstrate that exposure to fasting or STS and inhibitors of cholesterol

biosynthesis dramatically reduces pAKT activation in gastrointestinal tumors *in vitro* and *in vivo*, which is partially restored by the addition of LDL to the combined therapy *in vivo*.

An increasing body of evidence demonstrates that OXPHOS is upregulated in gastrointestinal cancers (219-221). For instance, mitochondrial genome sequence analysis from The Cancer Genome Atlas (TCGA) revealed deleterious mitochondrial DNA mutations in 53% of colon adenocarcinomas, potentially affecting OXPHOS level (222). Additionally, transcriptomic and metabolic analyses of PDAC cells revealed the reliance of the latter on mitochondrial respiration for cell survival (223). A previously published study by Zhou and colleagues demonstrated that HSP60, a mitochondrial protein, plays a fundamental role in the growth of PDAC cells (224). Furthermore, preclinical studies showed that cholesterol-lowering drugs reduced mitochondrial respiration in cancer cells or tumor xenografts (87, 225). Accordingly, in line with these data, our observation that fasting combined with inhibitors of cholesterol biosynthesis reduces mitochondrial respiration in tumor xenografts supports the hypothesis that this combined intervention might eradicate gastrointestinal tumor growth by targeting OXPHOS.

In conclusion, a pivotal cause for the enhancement of cholesterol biosynthesis inhibitors antitumor activity by fasting or STS appears to be through modulation of the metabolic biological mediators, insulin, IGF1, and leptin, with the consequent inhibition of the PI3K–AKT–mTOR pathway. This inhibition is likely the result of the disruption of cell membrane lipid rafts (where AKT binds, and whose integrity is essential for the activation of PI3K-AKT signaling) (137, 226) achieved, at least in part, through targeting intracellular cholesterol in gastrointestinal tumors. Taken together, these findings support the rationale for conducting clinical studies to assess the safety, feasibility, and activity of combining periodic cycles of fasting with inhibitors of cholesterol biosynthesis in cancer patients.

6. REFERENCES

1. Ashburn TT, Thor KB. Drug repositioning: identifying and developing new uses for existing drugs. Nature reviews Drug discovery. 2004;3(8):673-83.
2. Prasad V, De Jesús K, Mailankody S. The high price of anticancer drugs: origins, implications, barriers, solutions. Nature reviews Clinical oncology. 2017;14(6):381-90.
3. Pushpakom S, Iorio F, Eyers PA, Escott KJ, Hopper S, Wells A, et al. Drug repurposing: progress, challenges and recommendations. Nature reviews Drug discovery. 2019;18(1):41-58.
4. Lien EC, Vander Heiden MG. A framework for examining how diet impacts tumour metabolism. Nature Reviews Cancer. 2019;19(11):651-61.
5. Kerr J, Anderson C, Lippman SM. Physical activity, sedentary behaviour, diet, and cancer: an update and emerging new evidence. The Lancet Oncology. 2017;18(8):e457-e71.
6. Emmons KM, Colditz GA. Realizing the potential of cancer prevention—the role of implementation science. The New England journal of medicine. 2017;376(10):986.
7. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of US adults. New England Journal of Medicine. 2003;348(17):1625-38.
8. Kushi LH, Doyle C, McCullough M, Rock CL, Demark-Wahnefried W, Bandera EV, et al. American Cancer Society Guidelines on nutrition and physical activity for cancer prevention: reducing the risk of cancer with healthy food choices and physical activity. CA: a cancer journal for clinicians. 2012;62(1):30-67.
9. Ziegler RG, Hoover RN, Pike MC, Hildesheim A, Nomura AM, West DW, et al. Migration patterns and breast cancer risk in Asian-American women. JNCI: Journal of the National Cancer Institute. 1993;85(22):1819-27.
10. Nuotio J, Laitinen TT, Sinaiko AR, Woo JG, Urbina EM, Jacobs DR, et al. Obesity during childhood is associated with higher cancer mortality rate during adulthood: the i3C Consortium. International Journal of Obesity. 2022;46(2):393-9.
11. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. cell. 2011;144(5):646-74.
12. Lee C, Raffaghello L, Brandhorst S, Safdie FM, Bianchi G, Martin-Montalvo A, et al. Fasting cycles retard growth of tumors and sensitize a range of cancer cell types to chemotherapy. Science translational medicine. 2012;4(124):124ra27-ra27.
13. Raffaghello L, Lee C, Safdie FM, Wei M, Madia F, Bianchi G, et al. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. Proceedings of the National Academy of Sciences. 2008;105(24):8215-20.
14. Laviano A, Rossi Fanelli F. Toxicity in chemotherapy—when less is more. New England Journal of Medicine. 2012;366(24):2319-20.
15. Chabner BA, Roberts TG. Chemotherapy and the war on cancer. Nature Reviews Cancer. 2005;5(1):65-72.
16. Caffa I, D'Agostino V, Damonte P, Soncini D, Cea M, Monacelli F, et al. Fasting potentiates the anticancer activity of tyrosine kinase inhibitors by strengthening MAPK signaling inhibition. Oncotarget. 2015;6(14):11820.
17. Cheng C-W, Adams GB, Perin L, Wei M, Zhou X, Lam BS, et al. Prolonged fasting reduces IGF-1/PKA to promote hematopoietic-stem-cell-based regeneration and reverse immunosuppression. Cell stem cell. 2014;14(6):810-23.

18. de Groot S, Vreeswijk MP, Welters MJ, Gravesteijn G, Boei JJ, Jochems A, et al. The effects of short-term fasting on tolerance to (neo) adjuvant chemotherapy in HER2-negative breast cancer patients: a randomized pilot study. BMC cancer. 2015;15(1):1-9.
19. Dorff TB, Groshen S, Garcia A, Shah M, Tsao-Wei D, Pham H, et al. Safety and feasibility of fasting in combination with platinum-based chemotherapy. BMC cancer. 2016;16(1):1-9.
20. Bauersfeld SP, Kessler CS, Wischnewsky M, Jaensch A, Steckhan N, Stange R, et al. The effects of short-term fasting on quality of life and tolerance to chemotherapy in patients with breast and ovarian cancer: a randomized cross-over pilot study. BMC cancer. 2018;18(1):1-10.
21. Wei M, Brandhorst S, Shelehchi M, Mirzaei H, Cheng CW, Budniak J, et al. Fasting-mimicking diet and markers/risk factors for aging, diabetes, cancer, and cardiovascular disease. Science translational medicine. 2017;9(377):eaai8700.
22. Safdie FM, Dorff T, Quinn D, Fontana L, Wei M, Lee C, et al. Fasting and cancer treatment in humans: A case series report. Aging (Albany NY). 2009;1(12):988.
23. Koppold-Liebscher D, Kessler CS, Steckhan N, Bähr V, Kempter C, Wischnewsky M, et al. Short-Term fasting accompanying chemotherapy as a supportive therapy in gynecological cancer: protocol for a multicenter randomized controlled clinical trial. Trials. 2020;21(1):1-12.
24. Harvie M, Pegington M, Howell SJ, Bundred N, Foden P, Adams J, et al. Randomised controlled trial of intermittent vs continuous energy restriction during chemotherapy for early breast cancer. British journal of cancer. 2021:1-11.
25. Martin K, Jackson CF, Levy RG, Cooper PN. Ketogenic diet and other dietary treatments for epilepsy. Cochrane Database of Systematic Reviews. 2016(2).
26. Oliveira CL, Mattingly S, Schirrmacher R, Sawyer MB, Fine EJ, Prado CM. A nutritional perspective of ketogenic diet in cancer: a narrative review. Journal of the Academy of Nutrition and Dietetics. 2018;118(4):668-88.
27. Masood W, Annamaraju P, Uppaluri KR. Ketogenic diet. StatPearls [Internet]. 2020.
28. Urbain P, Strom L, Morawski L, Wehrle A, Deibert P, Bertz H. Impact of a 6-week non-energy-restricted ketogenic diet on physical fitness, body composition and biochemical parameters in healthy adults. Nutrition & metabolism. 2017;14(1):1-11.
29. Hopkins BD, Pauli C, Du X, Wang DG, Li X, Wu D, et al. Suppression of insulin feedback enhances the efficacy of PI3K inhibitors. Nature. 2018;560(7719):499-503.
30. Abdelwahab MG, Fenton KE, Preul MC, Rho JM, Lynch A, Stafford P, et al. The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma. PloS one. 2012;7(5):e36197.
31. Allen BG, Bhatia SK, Buatti JM, Brandt KE, Lindholm KE, Button AM, et al. Ketogenic diets enhance oxidative stress and radio-chemo-therapy responses in lung cancer xenografts. Clinical Cancer Research. 2013;19(14):3905-13.
32. Aminzadeh-Gohari S, Feichtinger RG, Vidali S, Locker F, Rutherford T, O'Donnel M, et al. A ketogenic diet supplemented with medium-chain triglycerides enhances the anti-tumor and anti-angiogenic efficacy of chemotherapy on neuroblastoma xenografts in a CD1-nu mouse model. Oncotarget. 2017;8(39):64728.
33. Pomatto-Watson LC, Bodogai M, Bosompra O, Kato J, Wong S, Carpenter M, et al. Daily caloric restriction limits tumor growth more effectively than caloric cycling regardless of dietary composition. Nature communications. 2021;12(1):1-17.
34. Morscher RJ, Aminzadeh-Gohari S, Feichtinger RG, Mayr JA, Lang R, Neureiter D, et al. Inhibition of neuroblastoma tumor growth by ketogenic diet and/or calorie restriction in a CD1-Nu mouse model. PloS one. 2015;10(6):e0129802.

35. Zahra A, Fath MA, Opat E, Mapuskar KA, Bhatia SK, Ma DC, et al. Consuming a ketogenic diet while receiving radiation and chemotherapy for locally advanced lung cancer and pancreatic cancer: the University of Iowa experience of two phase 1 clinical trials. Radiation research. 2017;187(6):743-54.
36. Maeyama M, Tanaka K, Nishihara M, Irino Y, Shinohara M, Nagashima H, et al. Metabolic changes and anti-tumor effects of a ketogenic diet combined with anti-angiogenic therapy in a glioblastoma mouse model. Scientific reports. 2021;11(1):1-11.
37. Liśkiewicz A, Właszczuk A, Gendosz D, Larysz-Brysz M, Kapustka B, Łączyński M, et al. Sciatic nerve regeneration in rats subjected to ketogenic diet. Nutritional Neuroscience. 2016;19(3):116-24.
38. Linard B, Ferrandon A, Koning E, Nehlig A, Raffo E. Ketogenic diet exhibits neuroprotective effects in hippocampus but fails to prevent epileptogenesis in the lithium-pilocarpine model of mesial temporal lobe epilepsy in adult rats. Epilepsia. 2010;51(9):1829-36.
39. Brandhorst S, Choi IY, Wei M, Cheng CW, Sedrakyan S, Navarrete G, et al. A periodic diet that mimics fasting promotes multi-system regeneration, enhanced cognitive performance, and healthspan. Cell metabolism. 2015;22(1):86-99.
40. Fontana L, Partridge L. Promoting health and longevity through diet: from model organisms to humans. Cell. 2015;161(1):106-18.
41. Holloszy JO, Fontana L. Caloric restriction in humans. Experimental gerontology. 2007;42(8):709-12.
42. Chen Y, Ling L, Su G, Han M, Fan X, Xun P, et al. Effect of intermittent versus chronic calorie restriction on tumor incidence: a systematic review and meta-analysis of animal studies. Scientific reports. 2016;6(1):1-11.
43. Harvey AE, Lashinger LM, Hays D, Harrison LM, Lewis K, Fischer SM, et al. Calorie restriction decreases murine and human pancreatic tumor cell growth, nuclear factor-κB activation, and inflammation-related gene expression in an insulin-like growth factor-1− dependent manner. PLoS One. 2014;9(5):e94151.
44. Berrigan D, Perkins SN, Haines DC, Hursting SD. Adult-onset calorie restriction and fasting delay spontaneous tumorigenesis in p53-deficient mice. Carcinogenesis. 2002;23(5):817-22.
45. Hursting SD, Dunlap SM, Ford NA, Hursting MJ, Lashinger LM. Calorie restriction and cancer prevention: a mechanistic perspective. Cancer & metabolism. 2013;1(1):1-11.
46. Rossi EL, Dunlap SM, Bowers LW, Khatib SA, Doerstling SS, Smith LA, et al. Energy balance modulation impacts epigenetic reprogramming, ERα and ERβ expression, and mammary tumor development in MMTV-neu transgenic mice. Cancer research. 2017;77(9):2500-11.
47. Maddocks OD, Berkers CR, Mason SM, Zheng L, Blyth K, Gottlieb E, et al. Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. Nature. 2013;493(7433):542-6.
48. Hens J, Sinha I, Perodin F, Cooper T, Sinha R, Plummer J, et al. Methionine-restricted diet inhibits growth of MCF10AT1-derived mammary tumors by increasing cell cycle inhibitors in athymic nude mice. BMC cancer. 2016;16(1):1-13.
49. Alidadi M, Banach M, Guest PC, Bo S, Jamialahmadi T, Sahebkar A, editors. The effect of caloric restriction and fasting on cancer. Seminars in Cancer Biology; 2021: Elsevier.
50. Sadeghian M, Rahmani S, Khalesi S, Hejazi E. A review of fasting effects on the response of cancer to chemotherapy. Clinical Nutrition. 2021;40(4):1669-81.

51. Fontana L, Partridge L, Longo VD. Extending healthy life span—from yeast to humans. science. 2010;328(5976):321-6.
52. Omodei D, Fontana L. Calorie restriction and prevention of age-associated chronic disease. FEBS letters. 2011;585(11):1537-42.
53. Arends J, Baracos V, Bertz H, Bozzetti F, Calder P, Deutz N, et al. ESPEN expert group recommendations for action against cancer-related malnutrition. Clinical nutrition. 2017;36(5):1187-96.
54. Khurana I. Essentials of medical physiology. Intia: Elsevier India. 2008.
55. Di Biase S, Shim HS, Kim KH, Vinciguerra M, Rappa F, Wei M, et al. Fasting regulates EGR1 and protects from glucose-and dexamethasone-dependent sensitization to chemotherapy. PLoS biology. 2017;15(3):e2001951.
56. Das SK, Roberts SB, Bhapkar MV, Villareal DT, Fontana L, Martin CK, et al. Body-composition changes in the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE)-2 study: a 2-y randomized controlled trial of calorie restriction in nonobese humans. The American journal of clinical nutrition. 2017;105(4):913-27.
57. Dirks AJ, Leeuwenburgh C. Caloric restriction in humans: potential pitfalls and health concerns. Mechanisms of ageing and development. 2006;127(1):1-7.
58. Fontana L, Weiss EP, Villareal DT, Klein S, Holloszy JO. Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. Aging cell. 2008;7(5):681-7.
59. Yilmaz ÖH, Katajisto P, Lamming DW, Gültekin Y, Bauer-Rowe KE, Sengupta S, et al. mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. Nature. 2012;486(7404):490-5.
60. Yousefi M, Nakauka-Ddamba A, Berry CT, Li N, Schoenberger J, Simeonov KP, et al. Calorie restriction governs intestinal epithelial regeneration through cell-autonomous regulation of mTORC1 in reserve stem cells. Stem Cell Reports. 2018;10(3):703-11.
61. Brennan AM, Mantzoros CS. Drug Insight: the role of leptin in human physiology and pathophysiology—emerging clinical applications. Nature clinical practice Endocrinology & metabolism. 2006;2(6):318-27.
62. Pollak M. The insulin and insulin-like growth factor receptor family in neoplasia: an update. Nature Reviews Cancer. 2012;12(3):159-69.
63. Jardé T, Perrier S, Vasson M-P, Caldefie-Chézet F. Molecular mechanisms of leptin and adiponectin in breast cancer. European journal of cancer. 2011;47(1):33-43.
64. Newman JC, Verdin E. Ketone bodies as signaling metabolites. Trends in Endocrinology & Metabolism. 2014;25(1):42-52.
65. Xia S, Lin R, Jin L, Zhao L, Kang H-B, Pan Y, et al. Prevention of dietary-fat-fueled ketogenesis attenuates BRAF V600E tumor growth. Cell metabolism. 2017;25(2):358-73.
66. Lee C, Safdie FM, Raffaghello L, Wei M, Madia F, Parrella E, et al. Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index. Cancer research. 2010;70(4):1564-72.
67. Pietrocola F, Pol J, Vacchelli E, Rao S, Enot DP, Baracco EE, et al. Caloric restriction mimetics enhance anticancer immunosurveillance. Cancer cell. 2016;30(1):147-60.
68. Di Biase S, Lee C, Brandhorst S, Manes B, Buono R, Cheng C-W, et al. Fasting-mimicking diet reduces HO-1 to promote T cell-mediated tumor cytotoxicity. Cancer cell. 2016;30(1):136-46.

69. Nencioni A, Caffa I, Cortellino S, Longo VD. Fasting and cancer: molecular mechanisms and clinical application. Nature Reviews Cancer. 2018;18(11):707-19.
70. Ibrahim EM, Al-Foheidi MH, Al-Mansour MM. Energy and caloric restriction, and fasting and cancer: a narrative review. Supportive Care in Cancer. 2021;29(5):2299-304.
71. Bianchi G, Martella R, Ravera S, Marini C, Capitanio S, Orengo A, et al. Fasting induces anti-Warburg effect that increases respiration but reduces ATP-synthesis to promote apoptosis in colon cancer models. Oncotarget. 2015;6(14):11806.
72. Lo Re O, Panebianco C, Porto S, Cervi C, Rappa F, Di Biase S, et al. Fasting inhibits hepatic stellate cells activation and potentiates anti-cancer activity of Sorafenib in hepatocellular cancer cells. Journal of cellular physiology. 2018;233(2):1202-12.
73. Lu Z, Xie J, Wu G, Shen J, Collins R, Chen W, et al. Fasting selectively blocks development of acute lymphoblastic leukemia via leptin-receptor upregulation. Nature medicine. 2017;23(1):79-90.
74. D'Aronzo M, Vinciguerra M, Mazza T, Panebianco C, Saracino C, Pereira SP, et al. Fasting cycles potentiate the efficacy of gemcitabine treatment in in vitro and in vivo pancreatic cancer models. Oncotarget. 2015;6(21):18545.
75. Strickaert A, Saiselet M, Dom G, De Deken X, Dumont JE, Feron O, et al. Cancer heterogeneity is not compatible with one unique cancer cell metabolic map. Oncogene. 2017;36(19):2637-42.
76. Shim HS, Wei M, Brandhorst S, Longo VD. Starvation promotes REV1 SUMOylation and p53-dependent sensitization of melanoma and breast cancer cells. Cancer research. 2015;75(6):1056-67.
77. Chen GG, Liu Z, Vlantis A, Tse G, Leung B, Van Hasselt C. Heme oxygenase-1 protects against apoptosis induced by tumor necrosis factor-α and cycloheximide in papillary thyroid carcinoma cells. Journal of cellular biochemistry. 2004;92(6):1246-56.
78. Sun P, Wang H, He Z, Chen X, Wu Q, Chen W, et al. Fasting inhibits colorectal cancer growth by reducing M2 polarization of tumor-associated macrophages. Oncotarget. 2017;8(43):74649.
79. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin. 2020;70(1):7-30.
80. Arnold M, Abnet CC, Neale RE, Vignat J, Giovannucci EL, McGlynn KA, et al. Global Burden of 5 Major Types of Gastrointestinal Cancer. Gastroenterology. 2020;159(1):335-49.e15.
81. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
82. Araghi M, Soerjomataram I, Jenkins M, Brierley J, Morris E, Bray F, et al. Global trends in colorectal cancer mortality: projections to the year 2035. International journal of cancer. 2019;144(12):2992-3000.
83. Cao C, Yan TD, Black D, Morris DL. A systematic review and meta-analysis of cytoreductive surgery with perioperative intraperitoneal chemotherapy for peritoneal carcinomatosis of colorectal origin. Annals of surgical oncology. 2009;16(8):2152-65.
84. Are C, Chowdhury S, Ahmad H, Ravipati A, Song T, Shrikandhe S, et al. Predictive global trends in the incidence and mortality of pancreatic cancer based on geographic location, socio-economic status, and demographic shift. Journal of surgical oncology. 2016;114(6):736-42.
85. Dekker E, Tanis PJ, Vleugels JLA, Kasi PM, Wallace MB. Colorectal cancer. Lancet (London, England). 2019;394(10207):1467-80.

86. Gunda V, Genaro-Mattos TC, Kaushal JB, Chirravuri-Venkata R, Natarajan G, Mallya K, et al. Ubiquitous Aberration in Cholesterol Metabolism across Pancreatic Ductal Adenocarcinoma. Metabolites. 2022;12(1):47.
87. McGregor GH, Campbell AD, Fey SK, Tumanov S, Sumpton D, Blanco GR, et al. Targeting the metabolic response to statin-mediated oxidative stress produces a synergistic antitumor response. Cancer research. 2020;80(2):175-88.
88. Wu C, Wang M, Shi H. Cholesterol Promotes Colorectal Cancer Growth by Activating the PI3K/AKT Pathway. Journal of Oncology. 2022;2022:1515416.
89. Wang C, Li P, Xuan J, Zhu C, Liu J, Shan L, et al. Cholesterol Enhances Colorectal Cancer Progression via ROS Elevation and MAPK Signaling Pathway Activation. Cellular Physiology and Biochemistry. 2017;42(2):729-42.
90. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer research. 2014;74(11):2913-21.
91. Bouvier A-M, Uhry Z, Jooste V, Drouillard A, Remontet L, Launoy G, et al. Focus on an unusual rise in pancreatic cancer incidence in France. International journal of epidemiology. 2017;46(6):1764-72.
92. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. New England Journal of Medicine. 2014;371(11):1039-49.
93. Neoptolemos JP, Kleeff J, Michl P, Costello E, Greenhalf W, Palmer DH. Therapeutic developments in pancreatic cancer: current and future perspectives. Nature reviews Gastroenterology & hepatology. 2018;15(6):333-48.
94. Principe DR, Underwood PW, Korc M, Trevino JG, Munshi HG, Rana A. The Current Treatment Paradigm for Pancreatic Ductal Adenocarcinoma and Barriers to Therapeutic Efficacy. Frontiers in Oncology. 2021:2773.
95. Kleeff J, Korc M, Apte M, La Vecchia C, Johnson CD, Biankin AV, et al. Pancreatic cancer. Nature reviews Disease primers. 2016;2:16022.
96. Midha S, Chawla S, Garg PK. Modifiable and non-modifiable risk factors for pancreatic cancer: A review. Cancer letters. 2016;381(1):269-77.
97. Sarantis P, Koustas E, Papadimitropoulou A, Papavassiliou AG, Karamouzis MV. Pancreatic ductal adenocarcinoma: Treatment hurdles, tumor microenvironment and immunotherapy. World journal of gastrointestinal oncology. 2020;12(2):173.
98. Tesfaye AA, Philip PA. Adjuvant treatment of surgically resectable pancreatic ductal adenocarcinoma. Clin Adv Hematol Oncol. 2019;17(1):54-63.
99. Ducreux M, Boige V, Malka D, editors. Treatment of advanced pancreatic cancer. Seminars in oncology; 2007: Elsevier.
100. Conroy T, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul J-L, et al. FOLFIRINOX or gemcitabine as adjuvant therapy for pancreatic cancer. New England Journal of Medicine. 2018;379(25):2395-406.
101. Carrière C, Young AL, Gunn JR, Longnecker DS, Korc M. Acute pancreatitis markedly accelerates pancreatic cancer progression in mice expressing oncogenic Kras. Biochemical and biophysical research communications. 2009;382(3):561-5.
102. Philip B, Roland CL, Daniluk J, Liu Y, Chatterjee D, Gomez SB, et al. A high-fat diet activates oncogenic Kras and COX2 to induce development of pancreatic ductal adenocarcinoma in mice. Gastroenterology. 2013;145(6):1449-58.

103. Guerra C, Schuhmacher AJ, Cañamero M, Grippo PJ, Verdaguer L, Pérez-Gallego L, et al. Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. Cancer cell. 2007;11(3):291-302.
104. Chang H-H, Moro A, Chou CEN, Dawson DW, French S, Schmidt AI, et al. Metformin decreases the incidence of pancreatic ductal adenocarcinoma promoted by diet-induced obesity in the conditional KrasG12D mouse model. Scientific reports. 2018;8(1):1-10.
105. Yuan C, Bao Y, Wu C, Kraft P, Ogino S, Ng K, et al. Prediagnostic body mass index and pancreatic cancer survival. Journal of Clinical Oncology. 2013;31(33):4229.
106. Duan L, Yang W, Wang X, Zhou W, Zhang Y, Liu J, et al. Advances in prognostic markers for colorectal cancer. Expert Review of Molecular Diagnostics. 2019;19(4):313-24.
107. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin. 2019;69(1):7-34.
108. Bailey CE, Hu CY, You YN, Bednarski BK, Rodriguez-Bigas MA, Skibber JM, et al. Increasing disparities in the age-related incidences of colon and rectal cancers in the United States, 1975-2010. JAMA surgery. 2015;150(1):17-22.
109. Bardou M, Barkun AN, Martel M. Obesity and colorectal cancer. Gut. 2013;62(6):933-47.
110. Amersi F, Agustin M, Ko CY. Colorectal cancer: epidemiology, risk factors, and health services. Clinics in colon and rectal surgery. 2005;18(3):133-40.
111. Castejón M, Plaza A, Martinez-Romero J, Fernandez-Marcos PJ, Cabo R, Diaz-Ruiz A. Energy Restriction and Colorectal Cancer: A Call for Additional Research. Nutrients. 2020;12(1).
112. Thanikachalam K, Khan G. Colorectal Cancer and Nutrition. Nutrients. 2019;11(1):164.
113. DeSantis CE, Miller KD, Dale W, Mohile SG, Cohen HJ, Leach CR, et al. Cancer statistics for adults aged 85 years and older, 2019. CA Cancer J Clin. 2019;69(6):452-67.
114. Biller LH, Schrag D. Diagnosis and Treatment of Metastatic Colorectal Cancer: A Review. Jama. 2021;325(7):669-85.
115. Bellio H, Fumet JD, Ghiringhelli F. Targeting BRAF and RAS in Colorectal Cancer. Cancers. 2021;13(9).
116. Xie YH, Chen YX, Fang JY. Comprehensive review of targeted therapy for colorectal cancer. Signal transduction and targeted therapy. 2020;5(1):22.
117. Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, et al. The consensus molecular subtypes of colorectal cancer. Nat Med. 2015;21(11):1350-6.
118. Thanki K, Nicholls ME, Gajjar A, Senagore AJ, Qiu S, Szabo C, et al. Consensus Molecular Subtypes of Colorectal Cancer and their Clinical Implications. International biological and biomedical journal. 2017;3(3):105-11.
119. Valenzuela G, Canepa J, Simonetti C, Solo de Zaldívar L, Marcelain K, González-Montero J. Consensus molecular subtypes of colorectal cancer in clinical practice: A translational approach. World journal of clinical oncology. 2021;12(11):1000-8.
120. Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boelens PG, et al. Colorectal cancer. Nature reviews Disease primers. 2015;1:15065.
121. Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. Nature reviews Gastroenterology & hepatology. 2019;16(12):713-32.
122. Sánchez-Gundín J, Fernández-Carballido AM, Martínez-Valdivieso L, Barreda-Hernández D, Torres-Suárez AI. New Trends in the Therapeutic Approach to Metastatic Colorectal Cancer. International journal of medical sciences. 2018;15(7):659-65.

123. Wolf AMD, Fontham ETH, Church TR, Flowers CR, Guerra CE, LaMonte SJ, et al. Colorectal cancer screening for average-risk adults: 2018 guideline update from the American Cancer Society. CA Cancer J Clin. 2018;68(4):250-81.
124. van der Stok EP, Spaander MCW, Grünhagen DJ, Verhoef C, Kuipers EJ. Surveillance after curative treatment for colorectal cancer. Nature reviews Clinical oncology. 2017;14(5):297-315.
125. Veettil SK, Wong TY, Loo YS, Playdon MC, Lai NM, Giovannucci EL, et al. Role of Diet in Colorectal Cancer Incidence: Umbrella Review of Meta-analyses of Prospective Observational Studies. JAMA network open. 2021;4(2):e2037341.
126. Weng ML, Chen WK, Chen XY, Lu H, Sun ZR, Yu Q, et al. Fasting inhibits aerobic glycolysis and proliferation in colorectal cancer via the Fdft1-mediated AKT/mTOR/HIF1α pathway suppression. Nat Commun. 2020;11(1):1869.
127. Alhourani AH, Tidwell TR, Bokil AA, Røsland GV, Tronstad KJ, Søreide K, et al. Metformin treatment response is dependent on glucose growth conditions and metabolic phenotype in colorectal cancer cells. Scientific Reports. 2021;11(1):10487.
128. Simons K, Ikonen E. How cells handle cholesterol. Science. 2000;290(5497):1721-6.
129. Farese Jr RV, Herz J. Cholesterol metabolism and embryogenesis. Trends in genetics. 1998;14(3):115-20.
130. Ikonen E. Cellular cholesterol trafficking and compartmentalization. Nature reviews Molecular cell biology. 2008;9(2):125-38.
131. Goldstein JL, Brown MS. The LDL receptor. Arteriosclerosis, thrombosis, and vascular biology. 2009;29(4):431-8.
132. Munro S. Lipid rafts: elusive or illusive? Cell. 2003;115(4):377-88.
133. Simons K, Ehehalt R. Cholesterol, lipid rafts, and disease. The Journal of clinical investigation. 2002;110(5):597-603.
134. Sezgin E, Levental I, Mayor S, Eggeling C. The mystery of membrane organization: composition, regulation and roles of lipid rafts. Nature reviews Molecular cell biology. 2017;18(6):361-74.
135. Simons K, Toomre D. Lipid rafts and signal transduction. Nature reviews Molecular cell biology. 2000;1(1):31-9.
136. Alikhani N, Ferguson RD, Novosyadlyy R, Gallagher EJ, Scheinman EJ, Yakar S, et al. Mammary tumor growth and pulmonary metastasis are enhanced in a hyperlipidemic mouse model. Oncogene. 2013;32(8):961-7.
137. Mollinedo F, Gajate C. Lipid rafts as major platforms for signaling regulation in cancer. Advances in biological regulation. 2015;57:130-46.
138. Zhuang L, Lin J, Lu ML, Solomon KR, Freeman MR. Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. Cancer Res. 2002;62(8):2227-31.
139. Martins IJ, Hone E, Foster JK, Sünram-Lea SI, Gnjec A, Fuller SJ, et al. Apolipoprotein E, cholesterol metabolism, diabetes, and the convergence of risk factors for Alzheimer's disease and cardiovascular disease. Molecular psychiatry. 2006;11(8):721-36.
140. van der Kant R, Goldstein LSB, Ossenkoppele R. Amyloid-β-independent regulators of tau pathology in Alzheimer disease. Nature reviews Neuroscience. 2020;21(1):21-35.
141. Haase CL, Tybjærg-Hansen A, Nordestgaard BG, Frikke-Schmidt R. HDL Cholesterol and Risk of Type 2 Diabetes: A Mendelian Randomization Study. Diabetes. 2015;64(9):3328-33.
142. Silvente-Poirot S, Poirot M. Cancer. Cholesterol and cancer, in the balance. Science. 2014;343(6178):1445-6.

143. Kuzu OF, Noory MA, Robertson GP. The Role of Cholesterol in Cancer. Cancer Res. 2016;76(8):2063-70.
144. Repa JJ, Mangelsdorf DJ. The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. Annual review of cell and developmental biology. 2000;16:459-81.
145. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science. 1986;232(4746):34-47.
146. Altmann SW, Davis HR, Jr., Zhu LJ, Yao X, Hoos LM, Tetzloff G, et al. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science. 2004;303(5661):1201-4.
147. Luo J, Yang H, Song BL. Mechanisms and regulation of cholesterol homeostasis. Nature reviews Molecular cell biology. 2020;21(4):225-45.
148. Maxfield FR, van Meer G. Cholesterol, the central lipid of mammalian cells. Current opinion in cell biology. 2010;22(4):422-9.
149. Huang B, Song B-l, Xu C. Cholesterol metabolism in cancer: mechanisms and therapeutic opportunities. Nature Metabolism. 2020;2(2):132-41.
150. Rosenson RS, Brewer Jr HB, Davidson WS, Fayad ZA, Fuster V, Goldstein J, et al. Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. Circulation. 2012;125(15):1905-19.
151. Gelissen IC, Harris M, Rye KA, Quinn C, Brown AJ, Kockx M, et al. ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. Arterioscler Thromb Vasc Biol. 2006;26(3):534-40.
152. Phillips MC. Molecular mechanisms of cellular cholesterol efflux. The Journal of biological chemistry. 2014;289(35):24020-9.
153. Chang TY, Li BL, Chang CC, Urano Y. Acyl-coenzyme A:cholesterol acyltransferases. American journal of physiology Endocrinology and metabolism. 2009;297(1):E1-9.
154. Petan T, Jarc E, Jusović M. Lipid droplets in cancer: guardians of fat in a stressful world. Molecules. 2018;23(8):1941.
155. Miyazaki A, Sakashita N, Lee O, Takahashi K, Horiuchi S, Hakamata H, et al. Expression of ACAT-1 protein in human atherosclerotic lesions and cultured human monocytes-macrophages. Arterioscler Thromb Vasc Biol. 1998;18(10):1568-74.
156. Gorin A, Gabitova L, Astsaturov I. Regulation of cholesterol biosynthesis and cancer signaling. Current opinion in pharmacology. 2012;12(6):710-6.
157. Poirot M, Soules R, Mallinger A, Dalenc F, Silvente-Poirot S. Chemistry, biochemistry, metabolic fate and mechanism of action of 6-oxo-cholestan-3β,5α-diol (OCDO), a tumor promoter and cholesterol metabolite. Biochimie. 2018;153:139-49.
158. Kuzu OF, Gowda R, Noory MA, Robertson GP. Modulating cancer cell survival by targeting intracellular cholesterol transport. Br J Cancer. 2017;117(4):513-24.
159. Gu L, Saha ST, Thomas J, Kaur M. Targeting cellular cholesterol for anticancer therapy. The FEBS journal. 2019;286(21):4192-208.
160. Li YC, Park MJ, Ye SK, Kim CW, Kim YN. Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents. The American journal of pathology. 2006;168(4):1107-18; quiz 404-5.
161. Liu Z, Liu X, Liu S, Cao Q. Cholesterol promotes the migration and invasion of renal carcinoma cells by regulating the KLF5/miR-27a/FBXW7 pathway. Biochem Biophys Res Commun. 2018;502(1):69-75.

162. Chimento A, Casaburi I, Avena P, Trotta F, De Luca A, Rago V, et al. Cholesterol and its metabolites in tumor growth: therapeutic potential of statins in cancer treatment. Frontiers in endocrinology. 2019:807.
163. Ding X, Zhang W, Li S, Yang H. The role of cholesterol metabolism in cancer. American journal of cancer research. 2019;9(2):219.
164. Wang Y, Liu C, Hu L. Cholesterol regulates cell proliferation and apoptosis of colorectal cancer by modulating miR-33a-PIM3 pathway. Biochemical and biophysical research communications. 2019;511(3):685-92.
165. Costa GA, de Souza SB, da Silva Teixeira LR, Okorokov LA, Arnholdt ACV, Okorokova-Façanha AL, et al. Tumor cell cholesterol depletion and V-ATPase inhibition as an inhibitory mechanism to prevent cell migration and invasiveness in melanoma. Biochimica et Biophysica Acta (BBA)-General Subjects. 2018;1862(3):684-91.
166. Lyu J, Yang EJ, Head SA, Ai N, Zhang B, Wu C, et al. Pharmacological blockade of cholesterol trafficking by cepharanthine in endothelial cells suppresses angiogenesis and tumor growth. Cancer letters. 2017;409:91-103.
167. Wen Y-A, Xiong X, Zaytseva YY, Napier DL, Vallee E, Li AT, et al. Downregulation of SREBP inhibits tumor growth and initiation by altering cellular metabolism in colon cancer. Cell death & disease. 2018;9(3):1-14.
168. Li N, Zhou ZS, Shen Y, Xu J, Miao HH, Xiong Y, et al. Inhibition of the sterol regulatory element-binding protein pathway suppresses hepatocellular carcinoma by repressing inflammation in mice. Hepatology. 2017;65(6):1936-47.
169. Lewis C, Brault C, Peck B, Bensaad K, Griffiths B, Mitter R, et al. SREBP maintains lipid biosynthesis and viability of cancer cells under lipid-and oxygen-deprived conditions and defines a gene signature associated with poor survival in glioblastoma multiforme. Oncogene. 2015;34(40):5128-40.
170. Garcia-Bermudez J, Baudrier L, Bayraktar EC, Shen Y, La K, Guarecuco R, et al. Squalene accumulation in cholesterol auxotrophic lymphomas prevents oxidative cell death. Nature. 2019;567(7746):118-22.
171. Stopsack KH, Gerke TA, Andrén O, Andersson S-O, Giovannucci EL, Mucci LA, et al. Cholesterol uptake and regulation in high-grade and lethal prostate cancers. Carcinogenesis. 2017;38(8):806-11.
172. Wang B, Rong X, Palladino EN, Wang J, Fogelman AM, Martín MG, et al. Phospholipid remodeling and cholesterol availability regulate intestinal stemness and tumorigenesis. Cell stem cell. 2018;22(2):206-20. e4.
173. Qiu B, Ackerman D, Sanchez DJ, Li B, Ochocki JD, Grazioli A, et al. HIF2α-dependent lipid storage promotes endoplasmic reticulum homeostasis in clear-cell renal cell carcinoma. Cancer discovery. 2015;5(6):652-67.
174. Accioly MT, Pacheco P, Maya-Monteiro CM, Carrossini N, Robbs BK, Oliveira SS, et al. Lipid bodies are reservoirs of cyclooxygenase-2 and sites of prostaglandin-E2 synthesis in colon cancer cells. Cancer research. 2008;68(6):1732-40.
175. Yue S, Li J, Lee S-Y, Lee HJ, Shao T, Song B, et al. Cholesteryl ester accumulation induced by PTEN loss and PI3K/AKT activation underlies human prostate cancer aggressiveness. Cell metabolism. 2014;19(3):393-406.
176. Guillaumond F, Bidaut G, Ouaissi M, Servais S, Gouirand V, Olivares O, et al. Cholesterol uptake disruption, in association with chemotherapy, is a promising combined metabolic therapy

for pancreatic adenocarcinoma. Proceedings of the National Academy of Sciences. 2015;112(8):2473-8.
177. Bozza PT, Viola JP. Lipid droplets in inflammation and cancer. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA). 2010;82(4-6):243-50.
178. de Gonzalo-Calvo D, López-Vilaró L, Nasarre L, Perez-Olabarria M, Vázquez T, Escuin D, et al. Intratumor cholesteryl ester accumulation is associated with human breast cancer proliferation and aggressive potential: a molecular and clinicopathological study. BMC cancer. 2015;15(1):1-14.
179. Abramczyk H, Surmacki J, Kopeć M, Olejnik AK, Lubecka-Pietruszewska K, Fabianowska-Majewska K. The role of lipid droplets and adipocytes in cancer. Raman imaging of cell cultures: MCF10A, MCF7, and MDA-MB-231 compared to adipocytes in cancerous human breast tissue. Analyst. 2015;140(7):2224-35.
180. Tirinato L, Liberale C, Di Franco S, Candeloro P, Benfante A, La Rocca R, et al. Lipid droplets: a new player in colorectal cancer stem cells unveiled by spectroscopic imaging. Stem cells. 2015;33(1):35-44.
181. Goncalves MD, Cantley LC. A 'fast'er way to treat breast cancer. Nature Metabolism. 2020;2(7):559-60.
182. Caffa I, Spagnolo V, Vernieri C, Valdemarin F, Becherini P, Wei M, et al. Fasting-mimicking diet and hormone therapy induce breast cancer regression. Nature. 2020;583(7817):620-4.
183. Caffa I, Nencioni A. Enhancing endocrine therapy activity via fasting cycles: biological rationale and clinical feasibility. Molecular & Cellular Oncology. 2021;8(1):1853492.
184. Elgendy M, Cirò M, Hosseini A, Weiszmann J, Mazzarella L, Ferrari E, et al. Combination of hypoglycemia and metformin impairs tumor metabolic plasticity and growth by modulating the PP2A-GSK3β-MCL-1 axis. Cancer cell. 2019;35(5):798-815. e5.
185. Di Tano M, Raucci F, Vernieri C, Caffa I, Buono R, Fanti M, et al. Synergistic effect of fasting-mimicking diet and vitamin C against KRAS mutated cancers. Nature communications. 2020;11(1):1-11.
186. Marini C, Bianchi G, Buschiazzo A, Ravera S, Martella R, Bottoni G, et al. Divergent targets of glycolysis and oxidative phosphorylation result in additive effects of metformin and starvation in colon and breast cancer. Scientific reports. 2016;6(1):1-13.
187. Araki K, Miyoshi Y. Mechanism of resistance to endocrine therapy in breast cancer: the important role of PI3K/Akt/mTOR in estrogen receptor-positive, HER2-negative breast cancer. Breast cancer (Tokyo, Japan). 2018;25(4):392-401.
188. Saint-Martin A, Martínez-Ríos J, Castañeda-Patlán MC, Sarabia-Sánchez MA, Tejeda-Muñoz N, Chinney-Herrera A, et al. Functional Interaction of Hypoxia-Inducible Factor 2-Alpha and Autophagy Mediates Drug Resistance in Colon Cancer Cells. Cancers. 2019;11(6):755.
189. Di Mascolo D, Varesano S, Benelli R, Mollica H, Salis A, Zocchi MR, et al. Nanoformulated Zoledronic Acid Boosts the Vδ2 T Cell Immunotherapeutic Potential in Colorectal Cancer. Cancers. 2020;12(1):104.
190. Piacente F, Caffa I, Ravera S, Sociali G, Passalacqua M, Vellone VG, et al. Nicotinic acid phosphoribosyltransferase regulates cancer cell metabolism, susceptibility to NAMPT inhibitors, and DNA repair. Cancer Research. 2017;77(14):3857-69.
191. Wang J, Jia L, Kuang Z, Wu T, Hong Y, Chen X, et al. The in vitro and in vivo antitumor effects of clotrimazole on oral squamous cell carcinoma. PloS one. 2014;9(6):e98885.

192. Liu D, Wong CC, Fu L, Chen H, Zhao L, Li C, et al. Squalene epoxidase drives NAFLD-induced hepatocellular carcinoma and is a pharmaceutical target. Science translational medicine. 2018;10(437).
193. Furumaki H, Matsuoka S, Sakurai T, Kohanawa M, Zhao S, Kuge Y, et al. A novel murine model for non-alcoholic steatohepatitis developed by combination of a high-fat diet and oxidized low-density lipoprotein. Laboratory investigation. 2012;92(2):265-81.
194. Yin L, Shi Y, Liu X, Zhang H, Gong Y, Gu Q, et al. A rat model for studying the biological effects of circulating LDL in the choriocapillaris-BrM-RPE complex. The American journal of pathology. 2012;180(2):541-9.
195. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976;72(1-2):248-54.
196. Becherini P, Caffa I, Piacente F, Damonte P, Vellone VG, Passalacqua M, et al. SIRT6 enhances oxidative phosphorylation in breast cancer and promotes mammary tumorigenesis in mice. Cancer & Metabolism. 2021;9(1):1-16.
197. Ravera S, Vigliarolo T, Bruno S, Morandi F, Marimpietri D, Sabatini F, et al. Identification of Biochemical and Molecular Markers of Early Aging in Childhood Cancer Survivors. bioRxiv. 2021.
198. Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape. Pharmacology research & perspectives. 2015;3(3):e00149.
199. Warrilow AG, Price CL, Parker JE, Rolley NJ, Smyrniotis CJ, Hughes DD, et al. Azole antifungal sensitivity of sterol 14α-demethylase (CYP51) and CYP5218 from Malassezia globosa. Scientific reports. 2016;6(1):1-10.
200. Mahammad S, Parmryd I. Cholesterol depletion using methyl-β-cyclodextrin. Methods in membrane lipids: Springer; 2015. p. 91-102.
201. Mollinedo F, Gajate C. Lipid rafts as major platforms for signaling regulation in cancer. Advances in biological regulation. 2015;57:130-46.
202. Zacksenhaus E, Shrestha M, Liu JC, Vorobieva I, Chung PE, Ju Y, et al. Mitochondrial OXPHOS induced by RB1 deficiency in breast cancer: implications for anabolic metabolism, stemness, and metastasis. Trends in cancer. 2017;3(11):768-79.
203. Jones RA, Robinson TJ, Liu JC, Shrestha M, Voisin V, Ju Y, et al. RB1 deficiency in triple-negative breast cancer induces mitochondrial protein translation. The Journal of clinical investigation. 2016;126(10):3739-57.
204. Xu Z, Huang L, Dai T, Pei X, Xia L, Zeng G, et al. SQLE Mediates Metabolic Reprogramming to Promote LN Metastasis in Castration-Resistant Prostate Cancer. OncoTargets and therapy. 2021;14:4285.
205. Yamamoto K, Nogimori Y, Imamura H, Ando J. Shear stress activates mitochondrial oxidative phosphorylation by reducing plasma membrane cholesterol in vascular endothelial cells. Proceedings of the National Academy of Sciences. 2020;117(52):33660-7.
206. Solsona-Vilarrasa E, Fucho R, Torres S, Nuñez S, Nuño-Lámbarri N, Enrich C, et al. Cholesterol enrichment in liver mitochondria impairs oxidative phosphorylation and disrupts the assembly of respiratory supercomplexes. Redox biology. 2019;24:101214.
207. Riscal R, Bull CJ, Mesaros C, Finan JM, Carens M, Ho ES, et al. Cholesterol auxotrophy as a targetable vulnerability in clear cell renal cell carcinoma. Cancer Discovery. 2021;11(12):3106-25.

208. Mullen PJ, Yu R, Longo J, Archer MC, Penn LZ. The interplay between cell signalling and the mevalonate pathway in cancer. Nature Reviews Cancer. 2016;16(11):718-31.
209. Zahra Bathaie S, Ashrafi M, Azizian M, Tamanoi F. Mevalonate pathway and human cancers. Current molecular pharmacology. 2017;10(2):77-85.
210. Brown DN, Caffa I, Cirmena G, Piras D, Garuti A, Gallo M, et al. Squalene epoxidase is a bona fide oncogene by amplification with clinical relevance in breast cancer. Scientific reports. 2016;6(1):1-13.
211. Simons K, Vaz WL. Model systems, lipid rafts, and cell membranes. Annu Rev Biophys Biomol Struct. 2004;33:269-95.
212. Zhuang L, Lin J, Lu ML, Solomon KR, Freeman MR. Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. Cancer research. 2002;62(8):2227-31.
213. Vernieri C, Fuca G, Ligorio F, Huber V, Vingiani A, Iannelli F, et al. Fasting-mimicking diet is safe and reshapes metabolism and antitumor immunity in cancer patients. Cancer Discovery. 2021.
214. Rieder S, W Michalski C, Friess H. Insulin-like growth factor signaling as a therapeutic target in pancreatic cancer. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2011;11(5):427-33.
215. Kasprzak A. Insulin-Like Growth Factor 1 (IGF-1) Signaling in Glucose Metabolism in Colorectal Cancer. International Journal of Molecular Sciences. 2021;22(12):6434.
216. Gutiérrez-Salmerón M, Lucena SR, Chocarro-Calvo A, García-Martínez JM, Orozco RMM, García-Jiménez C. Metabolic and hormonal remodeling of colorectal cancer cell signalling by diabetes. Endocrine-Related Cancer. 2021;28(6):R191-R206.
217. Klement R, Fink M. Dietary and pharmacological modification of the insulin/IGF-1 system: exploiting the full repertoire against cancer. Oncogenesis. 2016;5(2):e193-e.
218. Fenton JI, Hord NG, Lavigne JA, Perkins SN, Hursting SD. Leptin, insulin-like growth factor-1, and insulin-like growth factor-2 are mitogens in ApcMin/+ but not Apc+/+ colonic epithelial cell lines. Cancer Epidemiology and Prevention Biomarkers. 2005;14(7):1646-52.
219. Ashton TM, McKenna WG, Kunz-Schughart LA, Higgins GS. Oxidative phosphorylation as an emerging target in cancer therapy. Clinical Cancer Research. 2018;24(11):2482-90.
220. Yang J, Ren B, Yang G, Wang H, Chen G, You L, et al. The enhancement of glycolysis regulates pancreatic cancer metastasis. Cellular and Molecular Life Sciences. 2020;77(2):305-21.
221. Ghosh P, Vidal C, Dey S, Zhang L. Mitochondria targeting as an effective strategy for cancer therapy. International journal of molecular sciences. 2020;21(9):3363.
222. Larman TC, DePalma SR, Hadjipanayis AG, Protopopov A, Zhang J, Gabriel SB, et al. Spectrum of somatic mitochondrial mutations in five cancers. Proceedings of the National Academy of Sciences. 2012;109(35):14087-91.
223. Viale A, Pettazzoni P, Lyssiotis CA, Ying H, Sánchez N, Marchesini M, et al. Oncogene ablation-resistant pancreatic cancer cells depend on mitochondrial function. Nature. 2014;514(7524):628-32.
224. Zhou C, Sun H, Zheng C, Gao J, Fu Q, Hu N, et al. Oncogenic HSP60 regulates mitochondrial oxidative phosphorylation to support Erk1/2 activation during pancreatic cancer cell growth. Cell death & disease. 2018;9(2):1-14.
225. Mehibel M, Ortiz-Martinez F, Voelxen N, Boyers A, Chadwick A, Telfer BA, et al. Statin-induced metabolic reprogramming in head and neck cancer: a biomarker for targeting monocarboxylate transporters. Scientific reports. 2018;8(1):1-12.

226. Li YC, Park MJ, Ye S-K, Kim C-W, Kim Y-N. Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents. The American journal of pathology. 2006;168(4):1107-18.

1