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With this excitement we are ready for the next publications of **2024** and once again we need your precious help, to be able to grow even more ***ARO*** in the name of scientific excellence.

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RESEARCH ARTICLE

A PROSPECTIVE OBSERVATIONAL STUDY TO EVALUATE IMPACT OF ORAL SUPPLEMENTATION WITH AHCC AND *LACTOBACILLUS CRISPATUS M247* ON HPV CLEARANCE AND LOW-GRADE SQUAMOUS INTRAEPITHELIAL LESION REGRESSION

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ABSTRACT: Human papillomavirus (HPV), especially high-risk types like HPV 16 and 18, can progress from low-grade lesions (LSIL) to cancer. While HR-HPV and LSIL often regress naturally, some cases may advance to malignancy. Current treatments vary in efficacy and can have adverse effects. Emerging research on *Lactobacillus Crispatus M247* and Active Hexose Correlated Compound (AHCC) shows potential for enhancing HPV clearance and LSIL regression with minimal side effects. However, the precise impact of these treatments remains under study.

The primary endpoint is to evaluate the effectiveness of AHCC and *L. Crispatus M247* in treating women with chronic cervicitis or low-grade squamous intraepithelial lesions (L-SIL) caused by high-risk HPV. The secondary endpoint is to monitor any side effects and measure patient adherence to the treatment regimen.

This prospective observational cohort study followed 40 women with abnormal cervical cytology up to L-SIL and HR-HPV infection over 6 months. Cohort A (20 patients) underwent AHCC and *L. Crispatus* treatment, while Cohort B (20 patients), received regular follow-up without specific treatment. The study assessed the treatment's impact, controlling for age, BMI, sexual history, contraception use, and smoking habits. Key evaluations included molecular tests, colposcopy, and biopsy at the start and end of the study period, with additional monitoring of dropout and adherence rates and any side effects to determine the treatment's feasibility and safety.

With a 17.5% dropout rate (mostly COVID-related) from the initial 40 patients, no side effects were noted. HR-HPV clearance was achieved by 73.3% in Group A, versus 0% in Group B ($p < 0.001$) at the 6th month. L-SIL regressed to chronic cervicitis in 13% of Group A ($p = 0.048$), while 26.3% of Group B progressed to H-SIL, significantly differing from Group A ($p = 0.042$) at the 6th month. This observational cohort study confirms the feasibility and efficacy of AHCC and *L. Crispatus M247* supplementation for improving HR-HPV clearance and L-SIL regression, with no side effects and good adherence. Results support further investigation through randomized controlled trials and studies on the vaginal microbiota's role in cancer prevention.

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Impact statement: Combining AHCC and *L. Crispatus M247* significantly enhances HR-HPV clearance and LSIL regression, potentially reducing future oncogenesis risks in patients without adverse effects.

Key words: *Human papillomavirus (HPV); Lactobacillus Crispatus M247; Active Hexose Correlated Compound (AHCC); LSIL regression; HPV clearance.*

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INTRODUCTION

Background

HPV is a group of more than 200 related viruses, of which at least 14 are cancer-causing (also known as high-risk types). HPV types 16 and 18 are the most dangerous being responsible for most cervical cancer cases. These viruses infect the epithelial cells of the cervix (1, 2) and induce oncogenesis by two mechanisms: the E6 protein degrades the p53 tumor suppressor, while the E7 protein impairs the retinoblastoma (Rb) protein leading to the accumulation of mutations and ultimately result in malignant cell transformation (3). Low-Grade Squamous Intraepithelial Lesion (LSIL) indicates mild abnormalities in cervical cells, often related to HPV infection. While some HR-HPV infections may spontaneously clear and LSIL can regress without intervention, other cases see the persistence of HPV and progression of LSIL to cancerous states. The contemporary presence of both HR-HPV infections and LSIL reduces the chance to clear the virus infection and the spontaneous regression of LSIL (4-7). At the same time, all LSIL treatments, such as cryotherapy and laser therapy, and excisional methods like Cold Knife Conization (CKC), Loop Electrical Excision Procedure (LEEP), and laser conization, have distinct side effects and may not clear HPV entirely. Ablative methods lack specimen for further study, potentially causing discomfort and requiring multiple sessions while excisional methods vary in side effect rates, with CKC leading to 2-17% major bleeding and a 16% chance of premature birth. While laser conization and LEEP show lower rates of premature birth, it is not 0%. Equipment costs and the need for specialized training can also limit access to these treatments. This uncertainty complicates decision-making regarding treatment, especially considering that no conclusive treatment exists for the complete eradication of HR-HPV infection, which poses a risk of driving malignant transformation in other area of the cervix and genital areas (8, 9). The necessity for a new treatment approach is evident, particularly for patients newly diagnosed with HR-HPV infection, with or without LSIL, where the likelihood but not the certainty of spontaneous resolution argues against immediate, aggressive treatment due to the risk of outweighing potential benefits. The spontaneous clearance rate of high-risk HPV (HR-HPV) without any treatment varies across studies, underscoring the body's innate ability to clear HPV infections and our incomplete understanding of this process. Reported clearance rates vary, ranging from 43% after 6 months to 65% after 18 months (10, 11). Conversely, half of HR-HPV-positive women cleared the virus in a mere 7.5 months (12). Additionally, an observational study involving 1079 women showed that 46.3% cleared HPV, with a notably quicker clearance in the biopsy cohort (68.7%) compared to the baseline. This indicates that conservative management, when excluding high-grade disease via biopsy, is effective for HPV clearance and diagnostic procedures like biopsies do not impair, but rather may correlate with, an increased chance of HPV clearance (13). Existing literature identifies various factors associated with the progression and regression of HR-HPV and LSIL. However, despite the development of numerous nomograms and algorithms, there remains an inability to predict the progression of these conditions with absolute certainty. Persistent HR-HPV infection, particularly types 16 and 18, is a critical factor in the progression from LSIL to HSIL and cervical cancer (14). The immune system's response is central to controlling or eliminating HPV (15), with factors such as nutritional status, particularly levels of vitamins A, C, E, and folate, and zinc, by supporting the growth and normal functioning of lymphocytes, neutrophils, and macrophages, playing supportive roles (16). Lifestyle factors, including smoking, obesity, and physical inactivity, negatively impact immune efficiency and facilitate lesion progression (17, 18). Emerging evidence suggests strongly that HPV vaccination may encourage LSIL regression by enhancing immune response to the virus (19). Other influ-

ential factors include chronic stress, which weakens immune function (20), and specific conditions such as HIV/AID or those undergoing immunosuppressive therapy for any disease which compromise immune surveillance (21). Genetic predispositions (22) and additional cofactors like age, number of childbirths, prolonged oral contraceptive use, and co-infections with other sexually transmitted infections further affect the likelihood of LSIL progression to HSIL and cervical cancer (23). Recent studies have increasingly substantiated the significant role that vaginal microbiota plays in both the risk and progression of HPV infection, leading to HPV-related diseases. It has become apparent that the composition of the vaginal microbiota is a determinant in the trajectory of these diseases; a reduction in the prevalence of Lactobacillus species alongside a rise in microbial diversity correlates with sustained HPV infections and the development of cervical lesions. This association indicates a progression toward more severe HPV-related conditions (24, 25). On the contrary, the predominance of certain Lactobacillus strains, especially *L. Crispatus*, is associated with the regression and clearance of HPV, which suggests a protective effect against the progression of the disease (26). The *L. Crispatus M247* strain, which was isolated from the fecal material of a healthy child, is distinguished by its positive traits related to aggregation, colonization, and the modulation of inflammatory responses. The genome of this strain spans 2.1 Mbp and contains 2187 coding genes that are essential for various cellular processes, including carbohydrate and protein metabolism, DNA and RNA processing, and the biosynthesis of cell walls, capsules, and ribosomes. Remarkably, *L. Crispatus M247* includes genes that encode for Lactobacillus epithelium adhesin (LEA) and fibronectin, which are crucial for vaginal colonization and outcompeting pathogens, such as *Gardnerella vaginalis*. Additionally, this strain synthesizes exopolysaccharides (EPS) that facilitate bacterial adhesion, offer protection from environmental stressors, and display antibiotic properties. The potential of *L. Crispatus M247* for the colonization of vaginal tissues and the production of bacteriocins has been noted, particularly for its antagonistic action against specific strains of uropathogenic *Staphylococcus epidermidis* and *Escherichia coli* (27, 28). Based on current evidence, *L. Crispatus M247* is deemed safe, lacking virulence factors, and is unlikely to carry plasmids. This assessment aligns with the safety criteria established by the European Food Safety Authority (EFSA), confirming its phenotypic safety (29). According to Wan *et al.*, *Lactobacillus Crispatus* can cause cells to undergo apoptosis and inhibit the proliferation of the cervical precancerous cell line Ect1/E6E7 in a time-dependent way (30). *L. Crispatus M247* is a tested probiotic for oral administration shown to have fecal and vaginal colonizing properties (31, 32). Two clinical studies have investigated the effects of *L. Crispatus M247* on HPV clearance. The first, an open, non-controlled trial with 35 HPV-positive women, showed a 70% reduction in HPV positivity after a 90-day oral treatment with the probiotic. Notably, 94% of these women shifted to a healthier Community State Type I status (33). The second study, a randomized controlled trial with 160 women, compared the effects of long-term oral administration of *L. Crispatus M247* against a placebo. It was found that the group receiving *L. Crispatus M247* had a notably higher success rate in eliminating HPV-related cytological anomalies after six months, with 61.5% showing resolution as opposed to 41.3% in the placebo group ($p = 0.041$). Despite this, the rate of complete HPV-DNA clearance by the end of the study did not significantly differ between the two groups (34). Additionally, the study did not account for variables such as the vaccination history of the participants or whether they had single or multiple HPV genotype infections. Cultured *Lentinula edodes Mycelia* Extract also known as, ACTIVE HEXOSE CORRELATED COMPOUND, AHCC, Cultured *Lentinula edodes Mycelia* Extract, Cultured *Lentinula edodes Mycelia* Extract AHCC, ECLM, *Lentinula edodes Mycelia* Extract AHCC, *Lentinula edodes Mycelia* Extract Standardized Extract of Cultured *Lentinula edodes Mycelia* and, Yinuojin Ruanjiaonang is an orally bioavailable capsule-based formulation of a standardized extract of cultured *Lentinula edodes* (Shiitake mushroom) mycelia (ECLM), which is high in the polysaccharides beta- and alpha-glucans, with potential antioxidant, immunomodulating and antineoplastic activities. When ingested, the extract derived from cultured *Lentinula edodes Mycelia* initiates an immune response by interacting with toll-like receptors, particularly TLR-4. This interaction prompts the activation of various immune cells, including dendritic cells, natural killer cells, macrophages, and T-cells, and stimulates the production of cytokines, potentially leading to the elimination of HPV infections (35). AHCC has been the subject of research for its potential immune-modulating effects and its role in supporting cancer treatment,

improving immune responses, and possibly affecting the clearance of viral infections. A recent study by Smith *et al.* suggested that AHCC, a mushroom extract, may effectively clear high-risk HPV infections. The study included laboratory tests and animal studies, which supported the clearance of HPV, followed by two small pilot studies with women having persistent high-risk HPV. In one group, 6 out of 10 participants taking 3 grams of AHCC orally showed clearance after 3 to 6 months, while in another, 4 out of 9 participants taking 1 gram achieved clearance after 7 months. Low levels of Interferon-beta (IFNβ) were observed in those who cleared the virus, suggesting that AHCC might work by modifying IFNβ expression and signaling (36). Despite these encouraging results, the effectiveness of AHCC awaits confirmation from an ongoing phase II clinical trial and another study by Beihua Kong, as AHCC is not yet established as a definitive treatment for high-risk HPV infections (37). AHCC and *L. Crispatus M247*, both recognized for their potential benefits, have found their way into our gynecological practices due to their availability in the free market. The clinical challenge we face involves a specific group of patients: those HR-HPV infection, with or without LSIL. These individuals are not immediately recommended for conventional treatments because of the high likelihood of spontaneous regression. However, relying solely on follow-up care doesn't assure complete HPV clearance, and other treatments might be more harmful than beneficial due to their invasive nature.

Objectives

Our research seeks to address a critical question: can the combined therapy of AHCC and *L. Crispatus M247* enhance HR-HPV clearance and LSIL regression rates beyond what is seen with spontaneous recovery, without inducing side effects?

MATERIALS AND METHODS

Study design

This study is an observational, prospective, single-centre investigation. Our primary goal was to determine the effectiveness of the combined treatment of AHCC and *L. Crispatus M247*, in achieving HR-HPV clearance and in facilitating the regression of LSIL, while also closely monitoring for any progression towards more severe lesions. Additionally, we sought to assess the side effects associated with the treatment and gauge patient adherence to the regimen.

We observed the cohort of patients who decided to follow the combined treatment (Papion plus Crispact) and the matched cohort of patients who completed follow up only. To evaluate the effectiveness of this treatment, both groups underwent HPV-DNA testing, colposcopy, and biopsy after 6 months. Combined treatment included Papion 500mg capsules, derived from *Lentinula edodes*, two capsules in the morning, two after lunch, and two after dinner and Crispact, a sachet containing a minimum of 20 billion colony-forming units of *L. Crispatus M247* (IMG-P-23257), to be taken daily. Dosage and administration guidelines were established based on preliminary research and safety profiles. Written informed consent for observational studies was obtained from all participants; the protocol and other materials were in accord with the Helsinki Declaration of 1975 and approved by institutional review boards.

Setting

This study was conducted in the Eastern Sicily region, within the province of Messina, Southern Italy at a tertiary university hospital. The period of recruitment and data collection extended from July 2020 to July 2023. All procedures, ranging from data collection to diagnostic assessments were conducted at the Department of Gynecology and Obstetrics at the University Hospital "G. Martino" in Messina.

Participants

Participants for this observational prospective cohort study were recruited from women attending the outpatient clinic for cervical cancer prevention and colposcopy at our department. The inclusion criteria were limited to women diagnosed with HR-HPV and concurrent abnormal cervical cytology up to LSIL, who also consented to share their data for the observational study. Exclusion criteria were set for those presenting with HSIL, invasive cervical cancer, a history of surgical treatment for HSIL, immunosuppressive conditions, any oncological diseases, inflammatory disorders, a history of HPV vaccination, or those under 18 years old and pregnancy. To ensure a pure evaluation of the therapy's effectiveness, our study intentionally excludes individuals who have chosen to receive the HPV vaccine from both groups. This decision is based

on two considerations: the vaccine's potential to significantly boost HPV clearance and LSIL regression, and the variability of vaccine efficacy among patients due to age and comorbidities. From the eligible population, we selected two well-balanced cohorts, matched for confounding factors but significantly differing in one variable: the exposure factor, represented by the combined therapy of AHCC and *L. Crispatus M247*.

Variables

The primary endpoints were changes in HR-HPV status and cervical histological status between the two cohorts.

The secondary endpoints focused on the rate of side effects and patient compliance with the combined treatment.

Matching factors for the study consisted of age, BMI, sexual history (age at first intercourse and number of partners), use of contraception, and smoking habits (defined as more than 10 cigarettes per day).

Data Sources/Measurement

The changes in HR-HPV status and cervical histological status were determined using molecular tests, colposcopy, and biopsy. Following the detection of cytological abnormalities on routine PAP tests, HPV-DNA testing was conducted. Women with a single abnormal cytology result (including atypical squamous cells of undetermined significance (ASCUS), atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (ASC-H), or LSIL associated with an HR-HPV type) were referred for colposcopy. Cervical cells from each sample were collected by centrifugation, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Germany). This was followed by a PCR-based HPV-DNA assay and reverse dot blot genotyping (HPV-HS Bio plus HPV-strip or HPV-type, AB Analitica, Padova, Italy), which facilitated the identification of 11 low-risk HPV types (HPV-6, -11, -40, -42, -43, -44, -54, -61, -70, -72, -81) and 18 high-risk or probable high-risk HPV types (HPV-16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -68, -73, -82). Colposcopic examinations were performed by a single experienced colposcopist in accordance with the standards of the Italian Society of Colposcopy and Cervical-Vaginal Pathology (SICPCV) (38). The colposcopic findings were categorized as normal transformation zone (NTZ), abnormal transformation zone of low grade (ATZG1) with minor changes, and abnormal transformation zone of high grade (ATZG2) with major changes. Histological diagnoses were classified into three categories: chronic cervicitis (inflammatory/reactive lesions), LSIL, and HSIL, following the guidelines of the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology (38).

Bias

To mitigate selection bias, we deliberately chose a very specific population within a narrowly defined geographic area. Both the cohorts were matched for cofounding factors. This targeted approach was employed to enhance both the internal validity and the potential external validity of the data, ensuring that our findings could be more accurately generalized to similar contexts. To address measurement bias, we standardized our data collection methods and utilized validated measurement tools across the study. This included comprehensive training for all research staff involved in data collection to ensure consistency and accuracy in the measurements recorded. Regarding the classical observer bias typically associated with this kind of study, in our case, it was effectively nullified since all primary endpoints involved objective assessments. The HR-HPV status was determined using automated processes, eliminating the potential for subjective interpretation by researchers. Similarly, the histological status was assessed by an expert pathologist who conducted evaluations on biopsy samples obtained post-colposcopy. This pathologist was blinded to the study cohorts. To lessen attrition bias resulting from participant dropout, we implemented strategies aimed at maximizing retention, including regular follow-up reminders.

Study size

Considering the very specific and restricted nature of our target population, alongside the exploratory aims of our study, we opted to commence with a sample size of 20 patients in each group. This approach was chosen to ensure a precise and concentrated examination of the research questions within a manageable cohort, facilitating detailed insights into the initial outcomes.

Statistical methods

Numerical data were expressed as mean and standard deviation (S.D.) and categorical variables as absolute frequencies and percentage. Examined variables did not present normal distribution, as verified by the Kolmogorov Smirnov test; consequently, the non-parametric approach was used. Compari-

Table 1. *Descriptive analysis of the clinical characteristics of the 2 groups of patients.*

	GROUP A N.15	GROUP B N.19	P VALUE
Age	35.4 ± 10.7	37.3 ± 10.1	0.603
Body mass index	22.3 ± 2.1	23 ± 1.9	0.365
Smokers n. (%)	5 (33.3)	11 (57.9)	0.154
Age at first intercourse	17.8 ± 2.3	16.5 ± 1.9	0.104
N. of Partners	4.6 ± 3.2	3.6 ± 1.1	0.309
Estroprogestins use (%)	4 (26.7)	5 (26.3)	0.982

P values are typed bold-face if statistically significant (P <0.05 or lower) or bold-face in italics if borderline significant (P = 0.10 to 0.05).

son between group A and B was performed by using the Mann Whitney test for numerical variables and the Chi Square test for categorical variables. To evaluate, for each group examined, the existence of statistically significant differences in two different points (at baseline and after six months), the Mc Nemar test for the binary variables (HR-HPV and histological findings in group B) and the Wilcoxon test (for histological findings in group A) were applied. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 17 (Armonk, NY, IBM Corp.). A p-value smaller than 0.05 was considered statistically significant.

RESULTS

The comparative descriptive analysis, detailed in **Table 1**, reveals the clinical characteristics of the two patient groups, highlighting the lack of significant differences across the cofounding factors including age, Body Mass Index (BMI), smoking habits, age at first intercourse, number of sexual partners, and the use of estroprogestins. The average age in group A was 35 years, compared to 37 years in group B. The BMI was closely matched at 22.3 for group A and 23 for group B. Although the number of smokers in group B was higher compared to group A, this difference was not statistically significant (p-value = 0.154). This suggested that this discrepancy did not materially influence the comparative outcomes of the study. Additionally, the age at first intercourse was comparable (17.8 years for group A vs. 16.5 years for group B), as was the average number of sexual partners (4.6 for group A vs. 3.6 for group B). The use of estroprogestins was also similar, with a 4% usage rate in group A versus 5% in group B. This uniformity across both cohorts underscores

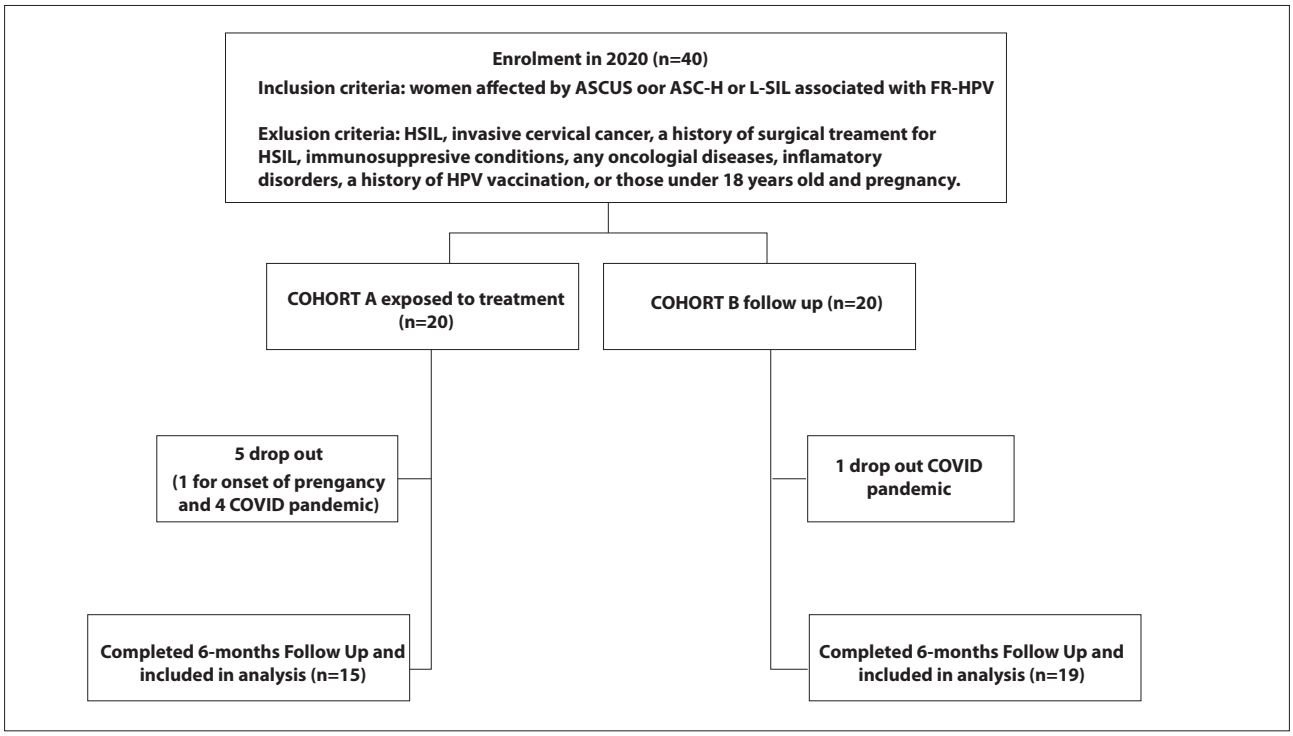

Figure 1. *STROBE flow chart.*

Table 2. *HPV type distribution in cervical samples detected by PCR in groups A and B at time 0 and after 6 months.*

HPV DETECTION	ALL N. (%)	GROUP A N. (%)		GROUP B N. (%)	
	Time 0	Time 0	Time 6	Time 0	Time 6
Single infections	22 (64.7)	9 (60)	0	13 (68.4)	14 (73.6)
Multiple infections	12 (35.3)	6 (40)	4 (26.6)	6 (31.5)	5 (26.3)
HR Type specific					
16	8 (23.5)	0	0	8 (42.1)	8 (42.1)
18	5 (14.7)	3 (20)	0	2 (10.5)	2 (10.5)
31	2 (5.8)	0	0	2 (10.5)	2 (10.5)
39	1 (2.9)	0	0	1 (5.2)	1 (5.2)
45	5 (14.7)	3 (20)	1 (6.6)	2 (10.5)	2 (10.5)
51	5 (14.7)	4 (26.6)	1 (6.6)	1 (5.3)	1 (5.3)
56	2 (5.8)	1 (6.6)	1 (6.6)	1 (5.2)	1 (5.2)
58	3 (8.8)	1 (6.6)	1 (6.6)	1 (5.2)	1 (5.2)
59	1 (2.9)	0	0	1 (5.2)	1 (5.2)
66	9 (26.4)	7 (46.6)	2 (13.3)	2 (10.5)	2 (10.5)
68	1 (2.9)	0	0	1 (5.2)	1 (5.2)
73	1 (2.9)	1 (6.6)	1 (6.6)	0	0
LR type specific					
42	2 (3.3)	0	0	2 (10.5)	2 (10.5)
61	3 (8.8)	3 (20)	3 (20)	0	0
87	1 (2.9)	0	0	1 (5.2)	

HPV: Human Papilloma virus; HR: high risk; LR: low risk. Due to multiple HPV infection, the overall percentage of HPV types exceeds 100%, because it expresses the HPV genotype-specific distribution in the studied population.

the balanced nature of the study's sample, facilitating a more reliable comparison of the treatment outcomes. Out of 40 patients enrolled, 34 completed the study. Among the 20 treated patients (group A), one was excluded because of the onset of pregnancy. Due to the COVID pandemic, 4 patients of group A and one of group B dropped out because of declining to continue follow up (**Figure 1** - **Appendix 1**). HPV type distribution in cervical samples is shown in **Table 2**. In our study the HPV-66 was found as the most common type (26.4.%) followed by HPV-16 (23.5%), -18, -45, -51 (14.7% respectively), -58 (8.8%), -31, -56 (5.8% respectively) and -39, -59, -68, -73 (2.9% respectively). Due to multiple HPV infection, the overall percentage of HPV types exceeds 100%, because it expresses the HPV genotype-specific distribution in the studied population.

The descriptive sub analysis of HR-HPV genotypes within each group revealed a non-uniform distribution across the different HPV types, as illustrated in the heatmap (**Figure 2**). Notably, HPV-16 was absent in group A (0%) but showed a significant presence in group B (42.1%). Conversely, HPV-18 appeared in 20% of cases in group A versus 10.5% in group B. Other types such as HPV-31, HPV-39, and HPV-68 were not found in group A but were present in group B at rates of 10.5%, 5.2%, and 5.2%, respectively. Meanwhile, HPV-45 and HPV-51 were more common in group A, at 20% and 26.6% respectively, compared to 10.5% and 5.3% in group B. HPV-56 and HPV-58 showed similar prevalence across groups, with a slight edge for group A. Significantly, HPV-66 was markedly more prevalent in group A (46.6%) than in group B (10.5%), while HPV-73 was found exclusively in group A (6.6%). The clinical findings after six months are shown in **Table 3**. The clearance of HR-HPV was significantly higher in group A, where 73.3% of the cases (11 out of 15) achieved clearance, compared to group B, which saw no cases of clearance among 19 patients, marking a statistically significant difference between the two groups ($p < 0.001$). While colposcopic findings remained largely unchanged after six months in both groups, the biopsy results revealed a notable contrast. On biopsy, a regression of L-SIL to chronic cervicitis was found in 2/15 (13%) of cases in group A compared to 0/19 (0%) of cases

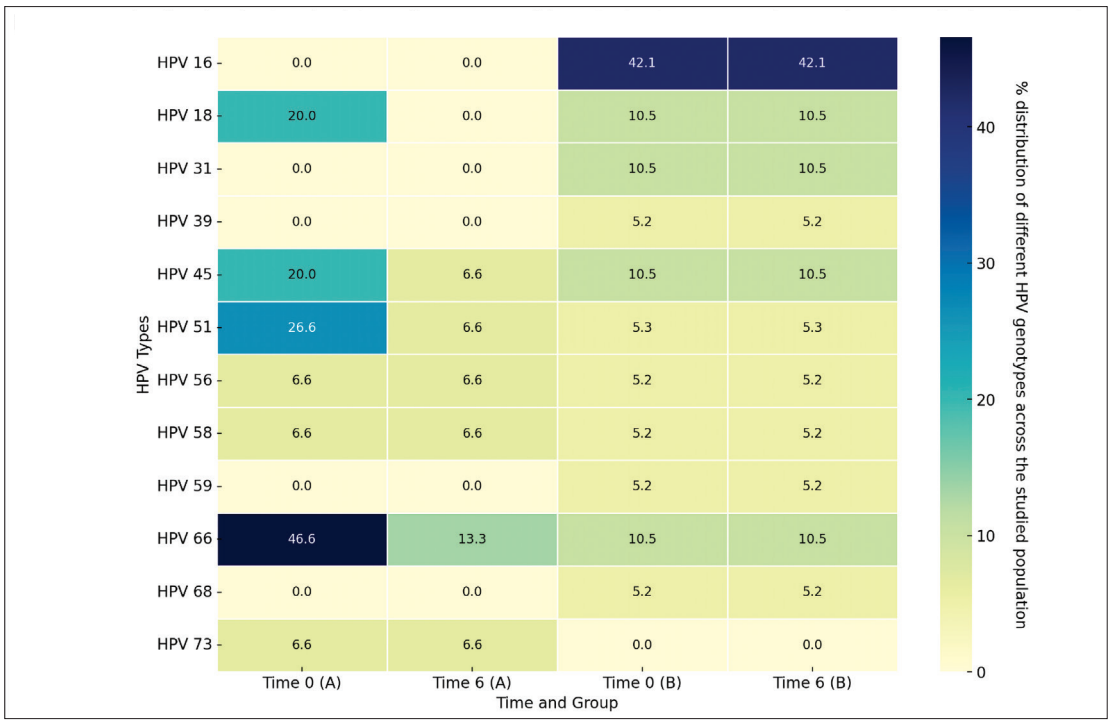

Figure 2. *HPV type distribution in cervical samples detected by PCR in groups A and B at time 0 and after 6 months.*

in group B with significant difference between the 2 groups (p = 0.048); on the other hand, there was no worsening of the histological finding in group A, while it was found a progression from LSIL to HSIL in 5/19 (26.3%) of cases in group B with significant difference between the 2 groups (p = 0.042) (**Figure 3**, **Figure 4**). There were no reported side effects, and patient compliance with the therapy reached 100%. It's important to note that any instances of dropout were attributed to reasons related to the COVID-19 pandemic, rather than any issues associated with the therapy itself. We acknowledge that the differential dropout rates in group A and B pose a limitation that is why our statistical analysis focused solely on participants who completed the study to maintain data integrity.

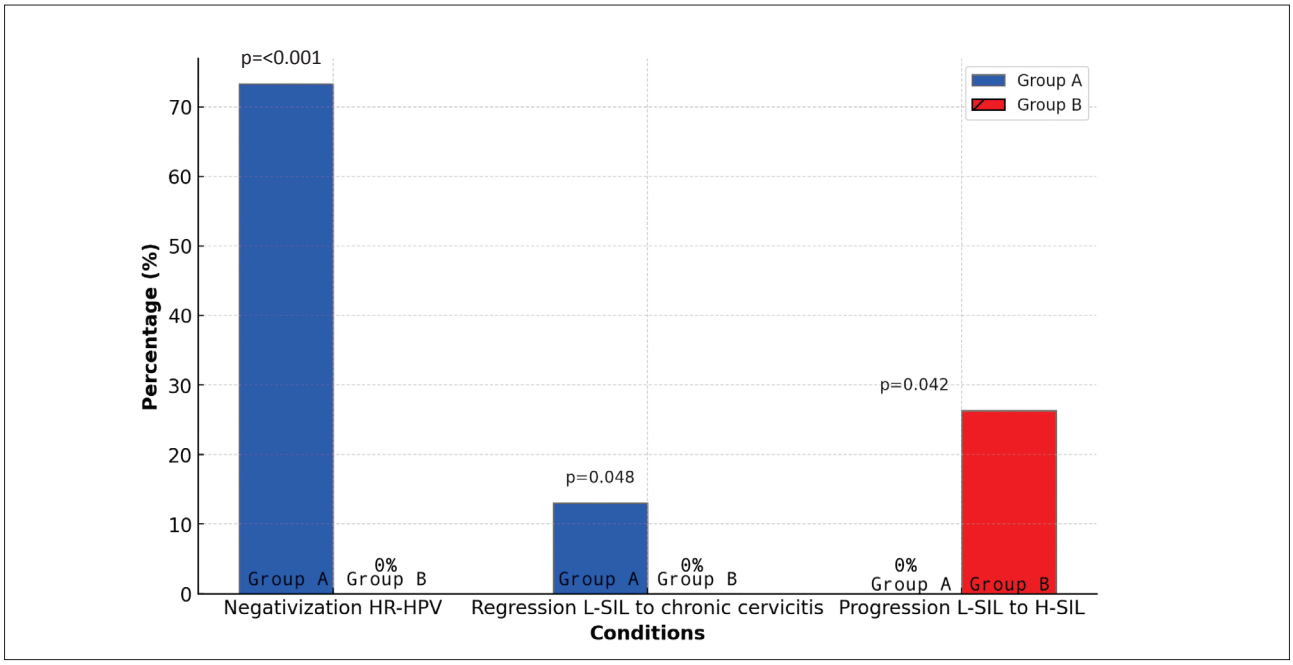

Figure 3. *HR-HPV and histological findings after 6 months in groups A and B (group B zeros highlighted).*

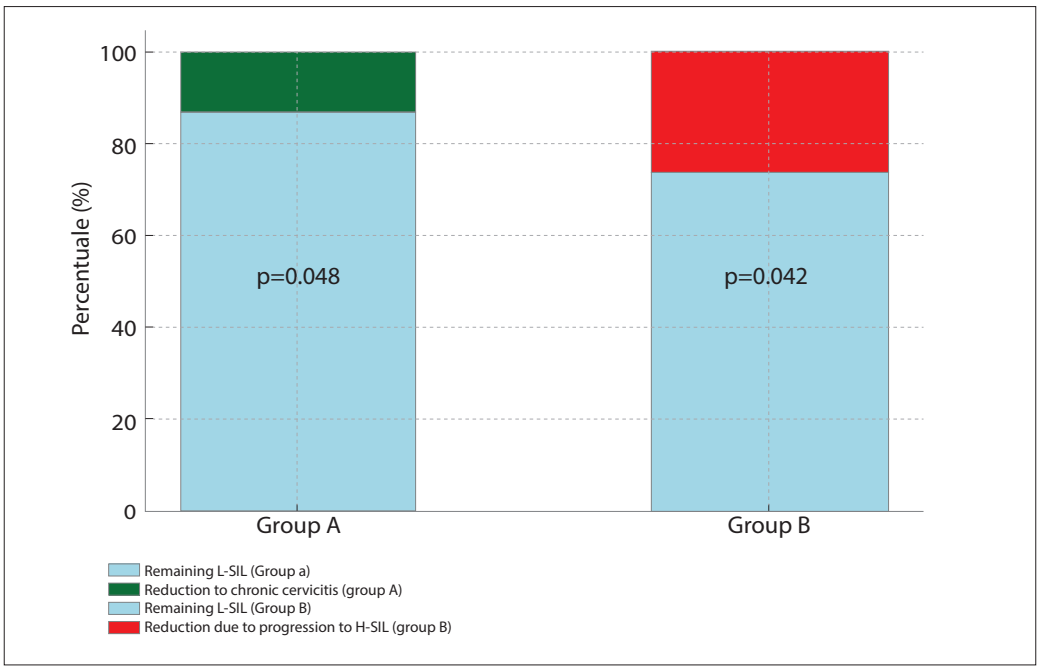

Figure 4. *Histological status changes in groups A and B after 6 months (adjusted).*

Table 3. *HR-HPV and histological findings after 6 months in groups A and B.*

	GROUP A N.15 (%)	GROUP B N.19 (%)	P VALUE
Negativization HR-HPV	11 (73.3)	0 (0)	<0.001
Regression L-SIL to chronic cervicitis	2 (13)	0 (0)	0.048
Progression L-SIL to H-SIL	0 (0)	5/19 (26.31)	0.042

HR-HPV: High Risk Human Papilloma virus; L-SIL: Low Grade Squamous Intraepithelial Lesion; H-SIL: High Grade Squamous Intraepithelial Lesion. P values are typed bold-face if statistically significant (P <0.05 or lower) or bold-face in italics if borderline significant (P = 0.10 to 0.05).

Subgroups analysis:

-differential clearance rates in single and multiple HR-HPV infections: the analysis of HR-HPV clearance rates reveals a marked contrast in the effectiveness of the treatment between patients with single versus multiple infections. Specifically, in group A, which received the combined therapy, patients with a single HR-HPV infection showed a 67% higher likelihood of achieving viral clearance compared to those with multiple infections, a difference that was statistically significant with a p-value of 0.01099. This suggests that the combined therapy was particularly effective for patients with single infections. Conversely, we observed, in group B, that only one patient with multiple infections spontaneously regressed to a single infection, while none of the patients with a single infection regressed to no infection. This light change was not statistically significant (0% clearance rate in "group B single infection" versus 16.6% clearance rate in "group B multiple infections"; p-value of 0.3157) (**Figure 5**). In summary, the findings strongly indicate that within group A, individuals with single HR-HPV infections responded more favorably to the treatment than those with multiple infections. However, it is crucial to interpret these results with an understanding of the limited sample size, which may constrain the extendibility of these conclusions to a wider population;

-dynamics of each HR-HPV genotype clearance: in group A, the therapy led to distinctive responses among various HR-HPV genotypes over the six-month period. Specifically, HPV 18 experienced the highest reduction at 100%, followed by HPV 51 with 75.19%, HPV 66 with 71.46%, and HPV 45 with 67%, while HPV 56, HPV 58, and HPV 73 showed no impact from the treatment. It's important to note that no increases in detection rates were observed for any of the genotypes, and crucially, no new HPV genotypes were identified at the six-month mark (**Figure 6**). In contrast, the

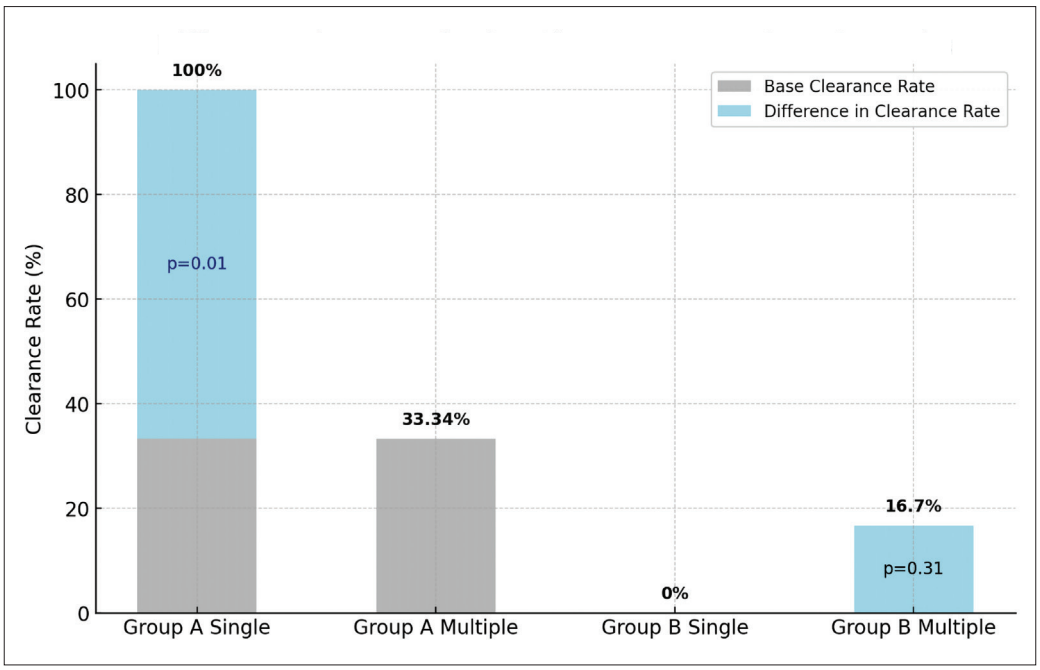

Figure 5. *Differences in HPV infection clearance rates over 6 months.*

HR-HPV genotype distribution in group B showed no change at the six-month mark.

DISCUSSION

In this prospective observational study, the combined therapy of AHCC and *L. Crispatus M 247* was associated for the first time with an improvement in HR-HPV clearance and the regression rate of LSIL, notably without any adverse effects and demonstrating excellent patient compliance. Literature review indicates the best performing spontaneous clearance rates for HR-HPV fluctuate around 65% (10, 11). These variations are influenced by demographic and geographical factors, highlighting the value of targeted studies for gaining insights into the disease's distribution and behavior across dif-

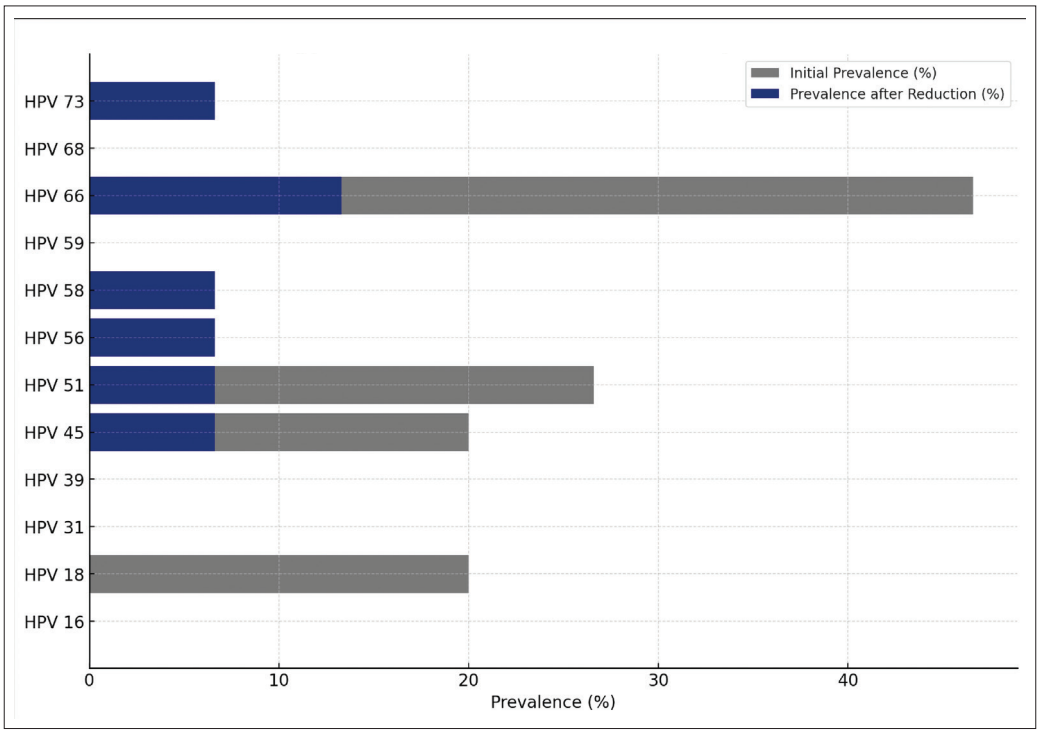

Figure 6. *HPV genotype prevalence and reduction in group A over 6 months.*

ferent populations. Given the high rates of spontaneous HR-HPV clearance, follow-up is often preferred over more invasive treatments due to the potential for adverse effects. However, about 30% of individuals may either retain the virus or experience progression to more severe preneoplastic stages, underlining the urgent need for effective and safe treatments. Observations from our cohort (group B) over six months revealed no instances of spontaneous HR-HPV clearance or LSIL regression; conversely, 19% of cases progressed to HSIL. Our findings from group B contrast sharply with those reported in the literature, often due to the absence of crucial data necessary for normalizing observed populations and drawing definitive conclusions. Factors such as the absence of HPV vaccination, short follow-up durations, and discrepancies in risk factor distribution relative to the general population are believed to explain these differences. Our cohorts, both group A and B, though comparable to each other, represented a high-risk segment for disease progression from HR-HPV infection, indicated by prevalent risk factors such as smoking (33.3%-57.9%), number of sexual partners (4.6-3.6), and age at first intercourse (16.5-17.8 years). Another variable that could influence the disparity between our observed population and the general population, or those in other studies, is the average age of the groups, which ranges from 35.4 to 37.3 years. It is well-documented that spontaneous LSIL regression or HR-HPV clearance is more likely at a younger age, specifically under 25 years old. Despite deviations from the general population, our cohorts were balanced and matched for confounding factors, leading to a significant statistical difference between the combined therapy and follow-up groups in a very short time. And, when comparing our group A, which received the combined therapy, to the observed groups from the literature that underwent only follow-up, still a notable difference emerges. Our group A exhibited a HR-HPV clearance rate of 73.3% within six months, surpassing the best average spontaneous HR-HPV clearance rate reported in the literature of 65%, which was achieved over a period of two years (10, 11). This aligns with other studies that underscore the effectiveness of such treatments. For instance, AHCC supplementation significantly boosted the immune response against persistent HPV infections, with 63.6% of patients (14 out of 22) testing negative for HPV RNA and DNA after six months (39). Oral *L. Crispatus M247* supplementation was linked to a higher resolution rate of HPV-related cytological abnormalities compared to follow-up care alone (60.5% vs. 41.3%, p = 0.05), even though complete HPV clearance was achieved in only 15.3% of treated patients versus 9.3% in untreated cases (34). Furthermore, in our sub-analysis focused on the significant contribution to clearance in group A, when we examine the two subgroups (single infection versus multiple infections, defined by the presence of more than one HPV type), a statistical difference is observed between them: patients with a single HR-HPV infection had a 67% greater likelihood of achieving viral clearance compared to those with multiple infections. This suggests that it is more feasible to reduce a single infection than multiple infections with this therapy within 6 months. One limitation arises from the non-homogeneous distribution of HR-HPV genotypes between the two cohort groups. Given this limitation, further randomized clinical studies are needed to randomize the two arms, ensuring a balance and match in the distribution of this variable that can influence the expected efficacy of the treatment outcome. Despite this limitation, it is noteworthy to report the differential response across all the different HPV genotypes underscores the importance of considering genotype-specific dynamics when assessing the efficacy of HPV treatments, as it highlights significant clearance in certain genotypes, preventing new genotype onset. In contrast, the HR-HPV genotype distribution in Group B remained unchanged at the six-month evaluation. This consistency contrasts with the dynamic changes observed in group A, where specific genotypes showed significant reductions in prevalence. While HPV vaccination and cervical screening have significantly lowered cervical cancer rates and preneoplastic lesions (40-42), thereby mitigating the disease's severe impact on women's survival and quality of life (43-46), global coverage remains low at only 21% in 2022 (47). This gap in prevention accentuates the urgent need to enhance HPV clearance and LSIL regression rates, particularly in populations with high-risk HPV infections. In light of this, our observational study targeted individuals with HR-HPV infection, with abnormal cervical cytology up to LSIL but not beyond. Considering the side effects of invasive treatments and the high rate of spontaneous resolution, current guidelines typically recommend regular follow-up. The overmentioned treatments, however, addressed the lesions without eliminating the HR-HPV infection itself, which could potentially lead to oncogenesis

in other genital areas in the future of the patient's medical history. Since our study demonstrated that the combination therapy of AHCC and *L. Crispatus M247* more effectively enhances HR-HPV clearance and LSIL regression compared to regular follow-up, without causing adverse effects, and potentially reduces oncogenesis risk by directly targeting HR-HPV, there is potential for prescribing this combination to patients with genital HPV infections independently from potential histological lesions of the cervix. This approach could complement existing therapies across various stages of HPV-related diseases, from preneoplastic lesions to cancer.

CONCLUSIONS

The observed HR-HPV clearance rate difference of 73.3% between group A and group B over six months, alongside the notable modification in histological status - with a 13% regression rate in Group A versus a 26.3% progression rate in Group B - highlights the potential of AHCC and *L. Crispatus* supplementation in the immunomodulation of cervical preneoplastic lesions. Despite the challenges in drawing definitive conclusions due to the small cohort size, this observational prospective study involving women at high risk of progression from Sicily, affected by HR-HPV infection with abnormal cervical cytology up to LSIL, suggests a promising avenue for enhancing HR-HPV clearance and preventing cervical preneoplastic lesions through oral supplementation of AHCC and *L. Crispatus*. These findings underscore the importance of conducting further in vivo clinical randomized studies to verify the efficacy of this supplementation in preventing cervical cancer in women with HR-HPV-related LSIL, thereby strengthening the case for such interventions in clinical practice.

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COMPLIANCE WITH ETHICAL STANDARDS

Fundings

No funding was utilized for this study as it comprised observational research incorporating routine clinical practices.

Conflict of interests

The authors declare that there are no conflicts of interest associated with this publication.

Availability of data and materials

The data supporting the findings of this study are available upon reasonable request to the corresponding author.

Authors' contributions

VS, AE, VI and CM: significantly contributed to the conception and design of the work; VS, LP, and RG: played a crucial role in data collection; AA and CM: were instrumental in the analysis and interpretation of the data; ML and LM: dedicated efforts to drafting the manuscript and critically revising it for important intellectual content; VS and AE: provided final approval for the version to be published; FC, FD, and CM: ensured the accuracy and integrity of the work, addressing any related questions thoroughly.

Ethical approval

This research adhered to the ethical standards of the World Medical Association's Declaration of Helsinki and complies with the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals, including the inclusion of diverse human populations in terms of sex, age, and ethnicity.

Human studies and subjects

Written informed consent was obtained from all participants prior to their involvement in the study. The study protocol, along with other relevant materials, adhered to the ethical standards as mentioned above. Approval for all research activities was granted by the institutional review board. As this was an observational study, researchers were limited to observing and analyzing data without interfering with the standard management of the patients. This approach ensured that the observational nature of the study did not impact the man-

agement or clinical care of the patients, who underwent the standard procedures and treatments necessary for their condition and their desire. All patient data were anonymized to protect privacy and confidentiality, maintaining the highest standards of data protection and ethical consideration.

Animal studies

N/A.

Publications ethics

The publication ethics followed by this study align with those outlined by the International Committee of Medical Journal Editors (ICMJE), regarding publishing and editorial issues in medical journals.

Plagiarism

The article provides a comprehensive review of the latest studies in the field, with accurate citations.

Data falsification and fabrication

The writing and contents of the article are entirely original and were developed entirely by the authors.

REFERENCES

1. zur Hausen H. Condylomata acuminata and human genital cancer. Cancer Res. 1976 Feb;36(2 pt 2):794.
2. Dürst M, Gissmann L, Ikenberg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc Natl Acad Sci U S A. 1983;80(12):3812-5. doi: 10.1073/pnas.80.12.3812.
3. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science. 1990;248(4951):76-9. doi: 10.1126/science.2157286.
4. Chen Y, Qiu X, Wang W, Li D, Wu A, Hong Z, et al. Human papillomavirus infection and cervical intraepithelial neoplasia progression are associated with increased vaginal microbiome diversity in a Chinese cohort. BMC Infect Dis. 2020;20(1):629. doi: 10.1186/s12879-020-05324-9.
5. Kang WD, Ju UC, Kim SM. Human papillomavirus genotyping for predicting disease progression in women with biopsy-negative or cervical intraepithelial neoplasia grade 1 of low-grade intraepithelial lesion cytology. Int J Gynecol Cancer. 2023:ijgc-2023-004902. doi: 10.1136/ijgc-2023-004902.
6. Al-Awadhi R, Alroomy M, Al-Waheeb S, Alwehaidah MS. Altered mitochondrial DNA copy number in cervical exfoliated cells among highrisk HPVpositive and HPVnegative women. Exp Ther Med. 2023;26(5):521. doi: 10.3892/etm.2023.12220.
7. Wang N, Li X, Liu X, Bian M, Hou Y, Zhou Y, et al. Clinical Efficacy and Safety of AdV-tk Gene Therapy for Patients with Cervical Squamous Intraepithelial Lesion: A Prospective Study. Hum Gene Ther. 2023;34(19-20):1033-40. doi: 10.1089/hum.2023.066.
8. Cooper DB, Carugno J, Dunton CJ, Menefee GW. Cold Knife Conization of the Cervix. 2023. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan–.
9. Athanasiou A, Veroniki AA, Efthimiou O, Kalliala I, Naci H, Bowden S, et al. Comparative effectiveness and risk of preterm birth of local treatments for cervical intraepithelial neoplasia and stage IA1 cervical cancer: a systematic review and network meta-analysis. Lancet Oncol. 2022;23(8):1097-108. doi: 10.1016/S1470-2045(22)00334-5.
10. Bulkmans NW, Berkhof J, Bulk S, Bleeker MCG, van Kemenade FJ, Rozendaal L, et al. High-risk HPV type-specific clearance rates in cervical screening. Br J Cancer 96. 2007; 1419–24. doi: 10.1038/sj.bjc.6603653.
11. Zhang WY, Ma CB, Xiao JY, Zhou HR. [Spontaneous clearance of high risk human papillomavirus infection]. Zhonghua Fu Chan Ke Za Zhi. 2010;45(7):515-8.
12. Dalstein V, Riethmuller D, Prétet JL, Le Bail Carval K, Sautière JL, Carbillet JP, et al. Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. Int J Cancer. 2003;106(3):396-403. doi: 10.1002/ijc.11222.
13. Petry KU, Horn J, Luyten A, Mikolajczyk RT. Punch biopsies shorten time to clearance of high-risk human papillomavirus infections of the uterine cervix. BMC Cancer. 2018;18(1):318. doi: 10.1186/s12885-018-4225-9.
14. Schiffman M, Boyle S, Raine-Bennett T, Katki HA, Gage JC, Wentzensen N, et al. The Role of Human Papillomavirus Genotyping in Cervical Cancer Screening: A Large-Scale Evaluation of the cobas HPV Test. Cancer Epidemiol Biomarkers Prev. 2015;24(9):1304-10. doi: 10.1158/1055-9965.EPI-14-1353.

15. Woodman CB, Collins SI, Young LS. The natural history of cervical HPV infection: unresolved issues. Nat Rev Cancer. 2007;7(1):11-22. doi: 10.1038/nrc2050.
16. Nunnari G, Coco C, Pinzone MR, Pavone P, Berretta M, Di Rosa M, et al. The role of micronutrients in the diet of HIV-1-infected individuals. Front Biosci (Elite Ed). 2012;4(7):2442-56. doi: 10.2741/e556. PMID: 22652651.
17. Plummer M, Herrero R, Franceschi S, Meijer CJ, Snijders P, Bosch FX, et al. Smoking and cervical cancer: pooled analysis of the IARC multi-centric case--control study. Cancer Causes Control. 2003;14(9):805-14. doi: 10.1023/b:caco.0000003811.98261.3e.
18. Lee JK, So KA, Piyathilake CJ, Kim MK. Mild obesity, physical activity, calorie intake, and the risks of cervical intraepithelial neoplasia and cervical cancer. PLoS One. 2013;8(6):e66555. doi: 10.1371/journal.pone.0066555.
19. Brown DR, Kjaer SK, Sigurdsson K, Iversen OE, Hernandez-Avila M, Wheeler CM, et al. The impact of quadrivalent human papillomavirus (HPV; types 6, 11, 16, and 18) L1 virus-like particle vaccine on infection and disease due to oncogenic nonvaccine HPV types in generally HPV-naive women aged 16-26 years. J Infect Dis. 2009;199(7):926-35. doi: 10.1086/597307.
20. Cohen S, Janicki-Deverts D, Miller GE. Psychological stress and disease. JAMA. 2007;298(14):1685-7. doi: 10.1001/jama.298.14.1685.
21. Clifford GM, Polesel J, Rickenbach M, Dal Maso L, Keiser O, Kofler A, et al. Cancer risk in the Swiss HIV Cohort Study: associations with immunodeficiency, smoking, and highly active antiretroviral therapy. J Natl Cancer Inst. 2005;97(6):425-32. doi: 10.1093/jnci/dji072.
22. Song S, Pitot HC, Lambert PF. The human papillomavirus type 16 E6 gene alone is sufficient to induce carcinomas in transgenic animals. J Virol. 1999;73(7):5887-93. doi: 10.1128/JVI.73.7.5887-5893.1999.
23. Smith JS, Green J, Berrington de Gonzalez A, Appleby P, Peto J, Plummer M, et al. Cervical cancer and use of hormonal contraceptives: a systematic review. Lancet. 2003;361(9364):1159-67. doi: 10.1016/s0140-6736(03)12949-2.
24. Lin W, Zhang Q, Chen Y, Dong B, Xue H, Lei H, et al. Changes of the vaginal microbiota in HPV infection and cervical intraepithelial neoplasia: a cross-sectional analysis. Sci Rep. 2022;12(1):2812. doi: 10.1038/s41598-022-06731-5.
25. Avsaroglu E, Kaleli B, Kilic D, Kaleli I, Guler T. A Decrease in Lactobacilli in the Vaginal Microbiota Is Independently Associated With HPV Persistence in Women With High-Risk HPV Infection. Cureus. 2023;15(12):e50907. doi: 10.7759/cureus.50907.
26. Li Y, Yu T, Yan H, Li D, Yu T, Yuan T, et al. Vaginal Microbiota and HPV Infection: Novel Mechanistic Insights and Therapeutic Strategies. Infect Drug Resist. 2020;13:1213-20. doi: 10.2147/IDR.S210615.
27. Bertuccioli A, Cardinali M, Zonzini G, Cazzaniga M, Di Pierro F. Lactobacillus crispatus M247: Characteristics of a Precision Probiotic Instrument for Gynecological and Urinary Well-Being. Microbiology Research. 2022; 13(4):963-71. doi: 10.3390/microbiolres13040069
28. Di Pierro F, Bertuccioli A, Cattivelli D, Soldi S, Elli M. Lactobacillus crispatus M247: a possible tool to counteract CST IV. Nutrafoods. 2018;17:169-72. doi: 10.17470/NF-018-0001-4.
29. Strus M, Chmielarczyk A, Kochan P, Adamski P, Chełmicki Z, Chełmicki A, et al. Studies on the effects of probiotic Lactobacillus mixture given orally on vaginal and rectal colonization and on parameters of vaginal health in women with intermediate vaginal flora. Eur J Obstet Gynecol Reprod Biol. 2012;163(2):210-5. doi: 10.1016/j.ejogrb.2012.05.001.
30. Wan B, Wei LJ, Tan TM, Qin L, Wang H. Inhibitory effect and mechanism of Lactobacillus crispatus on cervical precancerous cells Ect1/E6E7 and screening of early warning factors. Infect Agent Cancer. 2023;18(1):5. doi: 10.1186/s13027-023-00483-1.
31. Mitra A, MacIntyre DA, Marchesi JR, Lee YS, Bennett PR, Kyrgiou M. The vaginal microbiota, human papillomavirus infection and cervical intraepithelial neoplasia: what do we know and where are we going next? Microbiome. 2016;4(1):58. doi: 10.1186/s40168-016-0203-0.
32. Di Pierro F, Bertuccioli A, Saghеddu V, Cattivelli D, Soldi S, Elli M. Antibiotic resistance profile and adhesion properties of Lactobacillus crispatus M247. Nutrafoods. 2019; 89-94. doi: 10.17470/NF-019-0012.
33. DI Pierro F, Criscuolo AA, Dei Giudici A, Senatori R, Sesti F, Ciotti M, et al. Oral administration of Lactobacillus crispatus M247 to papillomavirus-infected women: results of a preliminary, uncontrolled, open trial. Minerva Obstet Gynecol. 2021;73(5):621-31. doi: 10.23736/S2724-606X.21.04752-7.

34. Dellino M, Cascardi E, Laganà AS, Di Vagno G, Malvasi A, Zaccaro R, et al. Lactobacillus crispatus M247 oral administration: Is it really an effective strategy in the management of papillomavirus-infected women? Infect Agent Cancer. 2022;17(1):53. doi: 10.1186/s13027-022-00465-9.
35. NCI. Cultured Lentinula edodes Mycelia Extract (Code C179283). In: NCI Thesaurus. Available from: https://ncit.nci.nih.gov/ncitbrowser/ConceptReport.jsp?dictionary=NCI%20Thesaurus&code=C179283. Accessed: Mar 8, 2024.
36. Smith JA, Mathew L, Gaikwad A, Rech B, Burney MN, Faro JP, et al. From Bench to Bedside: Evaluation of AHCC Supplementation to Modulate the Host Immunity to Clear High-Risk Human Papillomavirus Infections. Front Oncol. 2019;9:173. doi: 10.3389/fonc.2019.00173.
37. ClinicalTrials.gov. Evaluation of AHCC® for the Clearance of High Risk-HPV Infections in Chinese Female, Shandong University 2020. Available from: https://classic.clinicaltrials.gov/ct2/show/study/NCT04633330. Accessed: Mar 11, 2024.
38. Darragh TM, Colgan TJ, Cox JT, Heller DS, Henry MR, Luff RD, et al. The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology. Arch Pathol Lab Med. 2012;136(10):1266-97. doi: 10.5858/arpa.LGT200570.
39. Smith JA, Mathew L, Gaikwad A, Rech B, Burney MN, Faro JP, et al. From Bench to Bedside: Evaluation of AHCC Supplementation to Modulate the Host Immunity to Clear High-Risk Human Papillomavirus Infections. Front Oncol. 2019;9:173. doi: 10.3389/fonc.2019.00173.
40. Drolet M, Bénard É, Pérez N, Brisson M; HPV Vaccination Impact Study Group. Population-level impact and herd effects following the introduction of human papillomavirus vaccination programmes: updated systematic review and meta-analysis. Lancet. 2019;394(10197):497-509. doi: 10.1016/S0140-6736(19)30298-3.
41. Lei J, Ploner A, Elfström KM, Wang J, Roth A, Fang F, et al. HPV Vaccination and the Risk of Invasive Cervical Cancer. N Engl J Med. 2020;383(14):1340-8. doi: 10.1056/NEJMoa1917338.
42. Cancer Research UK: Cervical cancer statistics. Available from: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/cervical-cancer. Accessed: Mar 11, 2024.
43. SEER. Cervix Uteri Relative Survival Rates by Time Since Diagnosis. Available from: https://seer.cancer.gov/statfacts/html/corp.html. Accessed: Jan 12, 2024.
44. Shin W, Ham TY, Park YR, Lim MC, Won YJ. Comparing survival outcomes for cervical cancer based on the 2014 and 2018 International Federation of Gynecology and Obstetrics staging systems. Sci Rep. 2021; 11(6988). doi: 10.1038/s41598-021-86283-2.
45. Li Z, Lin Y, Cheng B, Zhang Q, Cai Y. Prognostic Model for Predicting Overall and Cancer-Specific Survival Among Patients With Cervical Squamous Cell Carcinoma: A SEER Based Study. Front Oncol. 2021;11:651975. doi: 10.3389/fonc.2021.651975.
46. Zhang J, Qin L, Chen HM, Hsu HC, Chuang CC, Chen D, et al. Overall survival, locoregional recurrence, and distant metastasis of definitive concurrent chemoradiotherapy for cervical squamous cell carcinoma and adenocarcinoma: before and after propensity score matching analysis of a cohort study. Am J Cancer Res. 2020;10(6):1808-20.
47. Suk R, Hong YR, Rajan SS, Xie Z, Zhu Y, Spencer JC. Assessment of US Preventive Services Task Force Guideline-Concordant Cervical Cancer Screening Rates and Reasons for Underscreening by Age, Race and Ethnicity, Sexual Orientation, Rurality, and Insurance, 2005 to 2019. JAMA Netw Open. 2022;5(1):e2143582. doi: 10.1001/jamanetworkopen.2021.43582.

APPENDIX 1

STROBE Statement-Checklist of items that should be included in reports of cohort studies

	ITEM NO	RECOMMENDATION	PAGE NO
Title and abstract	1	*(a)* Indicate the study's design with a commonly used term in the title or the abstract	3
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/ rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	6
Methods			
Study design	4	Present key elements of study design early in the paper	6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7
Bias	9	Describe any efforts to address potential sources of bias	7
Study size	10	Explain how the study size was arrived at	7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, explain how loss to follow-up was addressed	
		(*e*) Describe any sensitivity analyses	
Results			8
Participants	13*	(a) Report numbers of individuals at each stage of study-eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	8
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	8
Outcome data	15*	Report numbers of outcome events or summary measures over time	8
Main results	16	*(a)* Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included *(b)* Report category boundaries when continuous variables were categorized *(c)* If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	8
Other analyses	17	Report other analyses done-eg analyses of subgroups and interactions, and sensitivity analyses	11
DISCUSSION			
Key results	18	Summarise key results with reference to study objectives	12
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14
Generalisability	21	Discuss the generalisability (external validity) of the study results	14
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

**Give information separately for exposed and unexposed groups.*

An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at https://journals.plos.org/plosmedicine/, Annals of Internal Medicine at https://www.acpjournals.org/journal/aim, and Epidemiology at https://journals.lww.com/epidem/pages/default.aspx). Information on the STROBE Initiative is available at https://www.strobe-statement.org/).

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RESEARCH ARTICLE

AFLIBERCEPT PLUS FOLFIRI AS SECOND-LINE THERAPY IN METASTATIC COLORECTAL CANCER (MCRC) DURING PANDEMIC COVID-19: A REAL-WORLD EXPERIENCE

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ABSTRACT: The use of Aflibercept plus Folfiri represents the second-line chemotherapy in patients with metastatic colorectal cancer (mCRC) previously treated with Oxaliplatin. The outbreak of COVID-19 upset the standardized procedure of routinary access to the hospital with a lot of difficulty in administering chemotherapy. For this reason, we conducted this retrospective study on 78 enrolled patients who were diagnosed with mCRC, through the pandemic period. Primary endpoints were quality of life (QoL), progression-free survival (PFS) and overall response rate (ORR) in two patient groups treated before and after the onset of COVID-19, group A and group B, respectively. Secondary endpoints were tolerability profile, prognostic factors and carcinoembryonic antigen (CEA) reduction. The median age in all patients was 58 years old, and the median PFS was 6.2 months (95% CI: 5.1-7.2). A significant correlation was observed between decreased CEA levels and PFS with a P value of 0.63 (P = 0.009), which led to consequent improvement of QoL. The treatment was well tolerated, with good disease control and a manageable toxicity profile. Our survival analysis shows a non-significant difference in PFS in the two groups of patients treated before and after COVID-19 (6.1 versus 6.2 months). Furthermore, our analysis suggests left-side tumor site and wild-type RAS/BRAF status as potential prognostic factors for PFS and ORR. The results showed therapeutic benefits of AFL plus Folfiri as second-line therapy in mCRC patients previously treated with Oxaliplatin. The use of AFL plus Folfiri showed efficacy and safety, although the COVID-19 pandemic has affected the management of patients' care.

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Impact statement: AFL plus Folfiri showed therapeutic benefits in mCRC patients previously treated with Oxaliplatin. AFL influenced patient care management, maintaining good QoL.

Key words: *Aflibercept; chemotherapy; colorectal cancer; pandemic; covid-19; survival; quality of life.*

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INTRODUCTION

The outbreak of the coronavirus disease 2019 (COVID-19) has generated new challenges in cancer patients' care procedures, with a particular impact on the readjustment of the resources and the risk-benefit balance of cancer therapies (1). Cancer centers have developed new strategies and also preventive measures to deal with oncological patients during the COVID-19 pandemic (2). Such measures include the performance of oropharyngeal swabs, the administration of oral therapies instead of intravenous ones, and the spreading of remote multidisciplinary teams (general practitioners, psychologists, health professionals) to reduce the access of patients to the wards at a high risk of contagion (3, 4). The pandemic has called for a review of our daily medical practices, including our approach to colorectal cancer (CRC) management. Even during the COVID-19 pandemic, chemotherapy combined with target therapies remains an effective strategy to treat metastatic colorectal cancer (mCRC) (5, 6). Aflibercept (AFL) plus FOLFIRI (Fluorouracil, Leucovorin, and Irinotecan) has been shown effective in increasing the chances of survival of patients with advanced CRC, after previous treatments including oxaliplatin-based regimens with the addition of anti-VEGF (vascular endothelial growth factor) Bevacizumab monoclonal antibody or anti-EGFR (Epidermal growth factor receptor) Cetuximab or Panitumumab monoclonal antibodies, according to RAS/BRAF gene status (7-9). AFL is a second-generation antiangiogenic with a broader spectrum of action than Bevacizumab, as it can block both VEGF-A and PIGF, inhibiting the activity of VEGFR-1 and VEGFR-2 receptors by blocking tumor neoangiogenesis (10-12). VELOUR phase III randomised controlled trial (ClinicalTrials.gov NCT00561470) analyzed the effect of adding AFL to FOLFIRI, as a second-line option. Compared to FOLFIRI alone, the combination resulted in 1.5 month median overall survival (OS), (13.50 vs.12.06 respectively; HR: 0.817; 95% CI: 0.713-0.937; p = 0.0032) and median 2.2 months in PFS improvements (6.90 vs. 4.67 respectively; HR: 0.758; 95% CI: 0.661-0.869; p = 0.00007) (13, 14). The safety profile of this combination has been proven acceptable and manageable. Based on the results of the VELOUR study, we conducted a mono-institutional retrospective analysis, which embraced both the pre-pandemic and the pandemic periods, to evaluate the safety and efficacy of AFL in combination with FOLFIRI in patients with progressing mCRC previously treated with Oxaliplatin based regimens as routinely used in clinical practice (15, 16).

PATIENTS AND METHODS

Design and Participants

Between June 2016 and March 2022 a total of 78 patients were enrolled in the study. The inclusion criteria were: histologically confirmed diagnosis of mCRC; age ≥18 years old; Eastern Cooperative Oncology Group (ECOG) performance status from 0 to 2; measurable disease according to Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 (17, 18); progressive disease after first-line therapy with Oxaliplatin (according to RAS/BRAF gene status) regular heart function with left ventricular ejection fraction (LVEF) >50% and electrocardiogram (ECG) with sinus rhythm; adequate bone marrow, renal and hepatic functions; computerized tomography and/or magnetic resonance imaging of the central nervous system available for radiological review.

The exclusion criteria were: patient whit hypersensitivity to AFL, its excipients, or any other formulation components; no concomitant anticancer therapies were allowed, and radiotherapy at extracranial sites must have been stopped at least one month before starting the treatment.

Method of Administration

All patients received 4 mg/kg of AFL intravenously according to the treatment assignment, for more than an hour on day 1 every two weeks, immediately followed by the FOLFIRI regimen (Irinotecan 180 mg/m^2 IV for more than 90 minutes, with leucovorin 400 mg/m^2 IV for more than 2 hours, followed by FU 400 mg/m^2 bolus and FU 2400 mg/m^2 continuous infusion for more than 46 hours) (19). Patients were premedicated as indicated in routine clinical practice. Electrocardiogram (ECG) and echocardiogram were performed at baseline and every three months. Treatment was administered until disease progression or development of unacceptable toxicity (20). The outbreak of the COVID-19 pandemic has required a revision of routine medical care to minimize the risk of exposing patients to the virus infection.

Evaluation of Response and Toxicity

Dose interruption was allowed to manage treatment-related adverse events (TRAEs). FOLFIRI TRAEs and side effects were assessed at the end of each cycle and reported according to the Common Terminology Criteria for Adverse Events (CTCAE), Version 4.0. G3 or G4 toxic effects were managed with dose reduction or delay, in compliance with clinical practice procedures. Patient characteristics such as performance status, histopathological data, laboratory and radiological data (number of metastatic sites ≥2), and treatment outcomes were collected and reviewed to identify any prognostic factors to assess the best second-line therapy.

Quality of Life

QoL was regularly assessed by the psycho-oncologists, which provided all patients with the EORTC QLQ-C30 (European Organization for Research and Treatment of Cancer) questionnaire at the beginning of the treatment and after three months (21). The questionnaire is composed of both single-item and multi-item scales. The scaling is organized into five functional domains (physical, role, cognitive, emotional, and social), three-item symptom scales (fatigue, pain, and nausea and vomiting), a global health status/QoL scale, and six single-item scales (dyspnoea, loss of appetite, insomnia, constipation, diarrhoea and perceived financial impact of the disease). The score is evaluated according to a linear grading scale ranging from 0 to 100. A high score on a functional scale and global health status/QoL represents a high/healthy level of functioning, whereas a high score on a symptom scale/item represents a high level of symptomatology/problems.

Statistical Analysis

All statistical analyses ware performed using Statistical Package for Social Science (SPSS) software version 25. for Mac (IBM Corp., Armonk, NY, USA). Descriptive statistics were used to describe the baseline data of the patients, divided into two groups, respectively cancer treatment before COVID-19 (Group A) and after COVID-19 (Group B), with standard deviation or qualitative data being expressed as percentages. Proportions for categorical variables were compared using Student t tests. ORR is defined as the percentage of patients who achieved complete or partial response after treatment. PFS was calculated from the beginning of the treatment until progression, the PFS curve was assessed using the Kaplan-Meier method and the subgroup analysis of survival curves was performed with the Log-rank test. Bravais-Pearson's (r) linear correlation index was used to define the relation between PFS and CEA with a 95% confidence interval (CI). The statistical significance was defined as a p-value of less than 0.05. The last follow-up was in August 2022.

Endpoints

The main objective was to assess the impact of the COVID-19 pandemic on the management of patients. Primary endpoints were the comparison of the QoL, PFS and ORR in the two patient groups treated before and after the onset of COVID-19, group A and group B, respectively. Tolerability profile, prognostic factors and carcinoembryonic antigen (CEA) reduction were secondary endpoints.

Ethical Aspects

The study, approved by the Local Ethics Committee, Policlinico Palermo 1, was conducted in full compliance with the provisions of the Declaration of Helsinki as well as with the Good Clinical Practice guidelines. All patients provided written informed consent to be included in this study.

RESULTS

Descriptive Patients Characteristics

A total number of 78 patients were enrolled in this retrospective study, divided into two groups. Group A comprised all patients who underwent cancer treatment before the onset of the COVID-19 pandemic, between Jun 2016 and February 2020, while Group B included all those patients who were treated between March 2020 and March 2022. Demographic characteristics of the patients were well-balanced in all two cohorts (**Table 1**), in the Group A (cancer treatment before COVID-19) were 56 patients (29 male and 27 female), with a median age of 58 years old (range 49-68), in the Group B (cancer treatment after COVID-19) were 22 patient (12 male and 10 female) with a median age of 57 years old (range 51-62). All patients were previously treated with an Oxaliplatin first-line regimen. As for the ECOG performance status, in all 78 patients, 25 (32%) patients had ECOG 0, 43 (55%) had ECOG 1, and 10 (13%) had ECOG 2. Patients who had already undergone primary surgery were 65,

Table 1. *Baseline clinical and pathological characteristics (n. 78) divided in two groups (Group A and Group B).*

	GROUP A N = 56 (%)	GROUP B N = 22 (%)	*P* VALUE
Mean age (range)	58 (49-64)	57 (51-62)	0.02
Sex			
Male	29 (52%)	12 (58%)	1.93
Female	27 (48%)	10 (42%)	1.09
ECOG performance status			
0	18 (32%)	7 (31%)	0.78
1	32 (57%)	11 (50%)	1.45
2	6 (11%)	4 (18%)	0.08
Primary tumor location			
Single left-site	21 (38%)	14 (64%)	0.92
Single right-site	34 (61%)	8 (36%)	0.88
Single transverse-site	1 (1%)	-	
K-RAS and B-RAF status			
Wild-type	33 (59%)	16 (72%)	0.78
Mutant	23 (41%)	6 (28%)	0.98
Location of metastasis			
Liver	14 (25%)	8 (36%)	0.16
Lung	9 (16%)	5 (23%)	0.08
Lymph nodes	33 (59%)	9 (41%)	1.51

Group A: cancer treatment before COVID-19; Group B: cancer treatment after COVID-19; ECOG: Eastern Cooperative Oncology Group.

whilst 13 had concurrent hepatic metastasectomy. Anti-EGFR therapies were administered to 49 patients with wild-type RAS tumors, whilst anti-VEGF therapy with Bevacizumab was administered to 29 patients with mutant RAS/BRAF tumors. Furthermore, 17 patients (22%) were treated with FOLFOX and 61 patients (78%) with CAPOX. The primary tumor site was the left side in 35 patients, the right side in 42 patients, and the transverse colon in 1 patient only. The metastatic sites of the disease were the liver, lungs, and peritoneum. 16 patients had pre-treatment CEA values <10 mg/dl and only one metastatic site (liver or lung). **Table 1** shows the main characteristics of the study groups.

Clinical outcomes

On average, patients received 9 cycles of chemotherapy (range: 7-11). AFL plus FOLFIRI was well-tolerated, with a manageable toxicity profile. After a median follow-up of 12.6 months (range: 9.2-13.6) response rates according to RECIST criteria showed 1 (1%) complete response (CR); 15 (19%) partial response (RP); 48 (62%) disease stabilization (SD); 13 (17%) progression disease (PD) and 1 (1%) not evaluable. Accordingly, ORR (CR + PR) was 19.4%, and DCR (CR+PR+SD) was >82% (**Table 2**) summarized results for groups A and B respectively). The median response time in all patients (n = 78) was 6.5

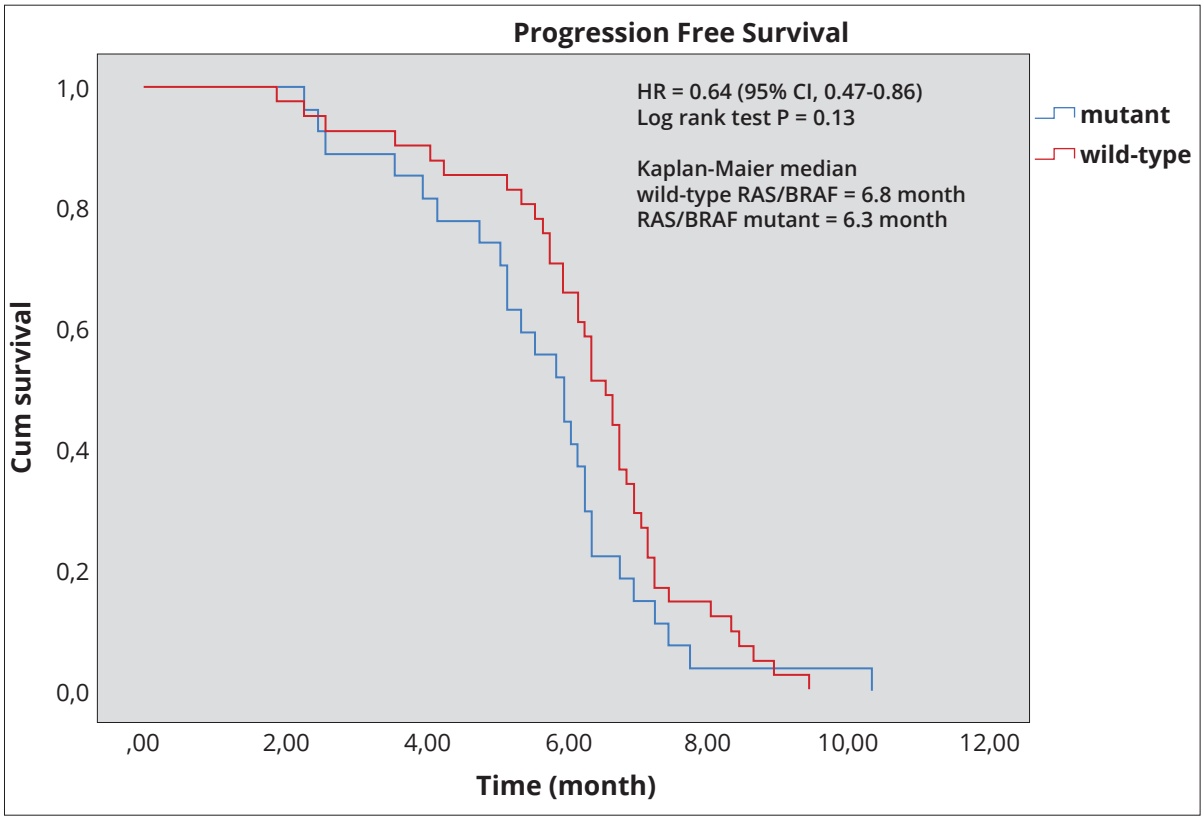

Figure 1. *Log rank test for PFS in wild-type RAS/BRAF (n. 49) and RAS/BRAF mutant (n. 29). PFS: progression-free survival.*

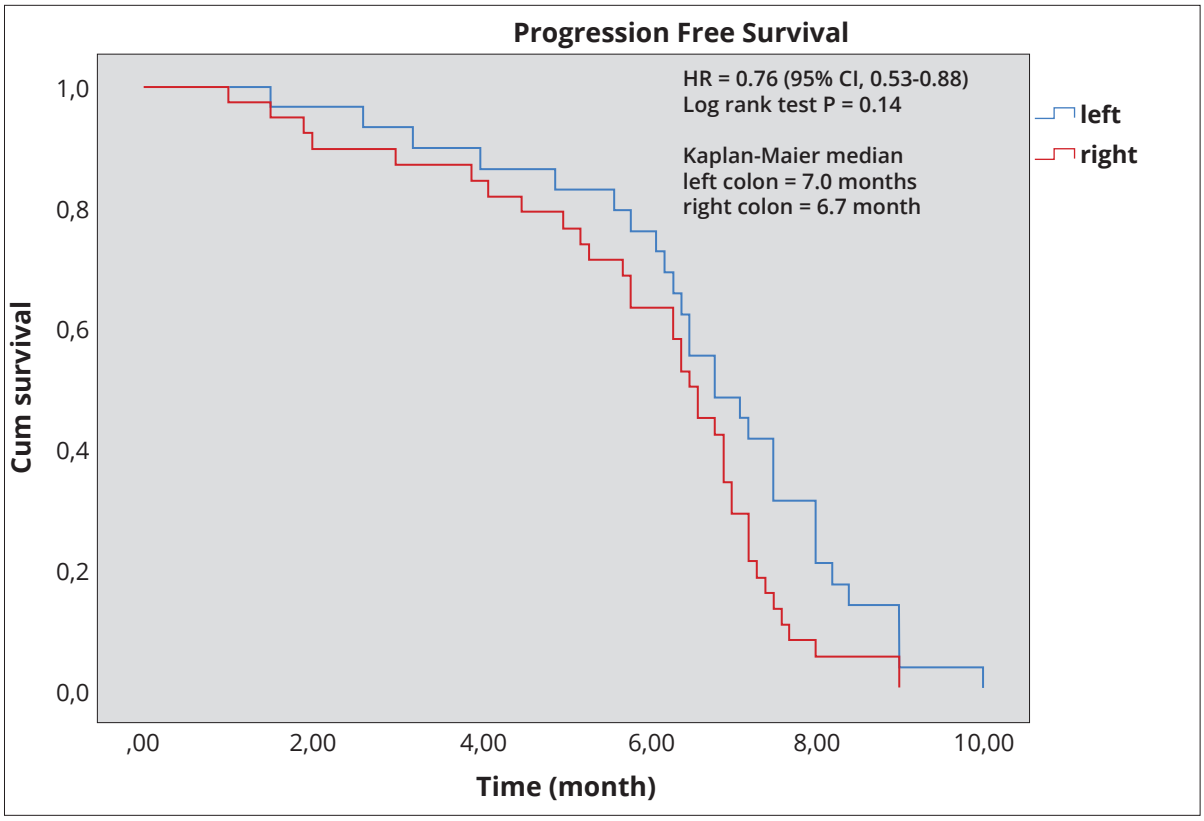

Figure 2. *Log rank test for PFS in primary tumor site in the left colon (n. 35) and primary tumor site in the right colon (n. 42). PFS: progression-free survival.*

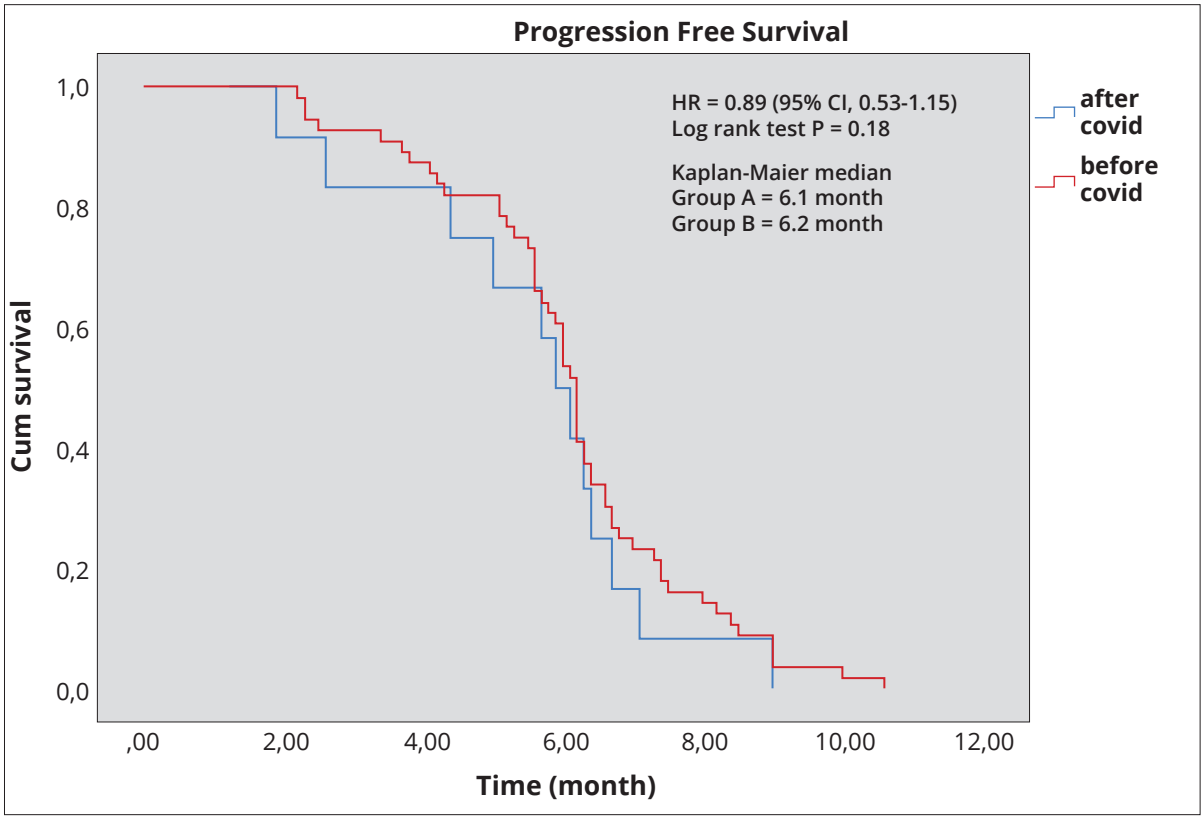

Figure 3. *Log rank test for PFS in patients treated before COVID-19 period (n. 56) and patients treated after COVID-19 period (n. 22). PFS: progression-free survival; Group A: cancer treatment before COVID-19; Group B: cancer treatment after COVID-19.*

Table 2. *Overall Response Rate divided in two groups (Group A and Group B).*

	GROUP A N = 56 (%)	GROUP B N = 22 (%)	*P* VALUE
Complete response	1 (2%)	-	
Partial response	9 (16%)	6 (27%)	0.05
Stable response	36 (64%)	12 (55%)	0.46
Progressive response	9 (16%)	4 (18%)	0.03
Not evaluable	1 (2%)	-	
Overall response rate (CR+PR)	10 (18%)	6 (27%)	0.12
Clinical benefit rate (CR+PR+SD)	46 (82%)	18 (82%)	0.46

Group A: cancer treatment before COVID-19; Group B: cancer treatment after COVID-19; ECOG: Eastern Cooperative Oncology Group; CR: complete response; PR: partial response; SD: stable response.

months (95% CI: 4.9-8.1) with a modest improved QoL. Bravais-Pearson index showed a positive correlation between PFS and CEA reduction, with a correlation coefficient (95% CI:0.31-0.76) value of 0.59, p = 0.009. We observed a mean CEA reduction >50% reflecting an increase in PFS.

Our analysis of the final cohort of patients showed a median PFS of 6.2 months (95% CI:5.1-7.2). According to RAS/BRAF status (WT vs mutant), PFS was 6.8 months versus 6.3 months respectively (**Figure 1**); also sideness (left vs right) did not affect PFS (7.0 months versus 6.7 months respectively) (**Figure 2**); we have recorded a PFS of 6.1 months in Group A versus 6.2 months in Group B with a HR of 0.89 (95%, CI 0.53-1.15), furthermore, in patients with low pre-treatment CEA levels and a low number of metastatic sites, we registered also greater PFS time. **Figure 3** reports the PFS analyses of patients, divided according to the treatment period before COVID-19 (group A) and after COVID-19 (group B). Our analyses showed no statistically significant differences in PFS between the two groups likely due to their small size.

Quality of Life

At baseline (treatment beginning), the EORTC QLQ-C30 questionnaires showed an overall slightly lower QoL outcome (the score for global health status was 57.7 in group A vs 56.3 in the group B). As for the symptom scales, patients reported sleeping disorders (24.3), fatigue (41.6) and nausea/vomiting (50.3) in two groups. Furthermore, the group B shows a worsening in the social area (47.0 group A vs 58.1 group B) and financial area (44.3 group A vs 45.7 group B). At follow-up, QoL had improved with a score of 61.3 for global health status (61.5 group A vs 60.7 group B). In the two groups we observe a reduction in pain symptoms (44.3 group A vs 45.2 group B), (**Table 3**). The reduction of financial impact appeared also relevant. In fact it was at baseline 44.3 in group A and 45.3

Table 3. *The Quality of Life score (n. 78) divided in two groups (Group A and Group B).*

	GROUP A N = 56		GROUP B N = 22		*P* VALUE
	BASELINE	FOLLOW UP	BASELINE	FOLLOW UP	
Global health status	57.7	61.5	56.3	60.7	0.05
Physical	46.1	47.4	46.9	47.0	0.35
Role	41.5	42.5	44.3	44.7	0.78
Cognitive	43.6	43.8	43.9	44.3	1.06
Emotional	51.4	50.6	52.7	51.8	0.58
Social	47.0	48.3	58.1	57.9	0.02
Fatigue	41.4	39.4	41.7	40.6	1.69
Pain	48.6	44.3	48.8	45.2	0.00
Nausea and vomiting	50.3	50.2	50.3	50.1	1.56
Dyspnoea	29.6	28.3	28.4	28.1	1.73
Loss of appetite	19.6	19.5	19.9	18.9	0.07
Insomnia	24.3	24.7	34.6	35.7	0.01
Constipation	18.6	18.8	18.9	18.4	1.83
Diarrhoea	18.6	18.9	17.5	18.2	0.59
Financial impact	44.3	45.7	45.3	48.1	0.06

in group B, respectively, and it became after three months 45. 7 in group A and 48.1 in group B, respectively.

Tolerability

No severe treatment-related hypersensitivity reactions were reported, and no patients died of treatment-related adverse events. The main hematological toxicities related to AFL and FOLFIRI were: neutropenia (all grades: 36%; G3-G4: 12%), febrile neutropenia (all grades: 8%), G2-G3 anemia (22%) G4 anemia (5 patients), and G3 thrombocytopenia (12%). Granulocyte colonies-stimulating factor (G-CSF), antibiotics, erythropoietin, oral steroids, and blood transfusions (3 patients) were used as expected in routine clinical practice. The most frequent major non-hematological toxicities were: asthenia (all grades: 26%); diarrhoea (all grades: 24%; G3-G4: 9%), treated with loperamide as needed; arterial hypertension; G3 hypertension (18%), treated with the dose-adjustment of the pre-existing antihypertensive therapies or with more than one drug; G4 hypertension (only 1 case); G3 proteinuria (6%); palmar-plantar erythro-dysesthesia (in 1-4%- of G2-G3 patients) (**Table 4**). No heart failure, left ventricular ejection fraction (LVEF) reduction, gastrointestinal perforations, or fistulas cases were reported. No significant differences were recorded in both incidence and severity of Ae in the two groups of patients.

Table 4. *Adverse events graded according CTCAE, Version 4.0 (n. 78).*

ADVERSE EVENTS	ALL GRADES	GRADE 3-4
Hematological		
Anemia	22%	6%
Neutropenia	38%	14%
Trombocitopenia	16%	12%
Febbrile neutropenia	13%	13%
Non-hematological		
Nausea	16%	12%
Vomiting	12%	8%
Hypertension	18%	12%
Fatigue	28%	28%
Hyperbilirubinemia	4%	2%
Hand foot syndrome	4%	4%
Peripheral neuropathy	0%	0%
Diarrhoea	48%	13%

CTCAE: Common Terminology Criteria for Adverse Events.

DISCUSSION

The outbreak of the COVID-19 pandemic has interfered with the normal practices of cancer patients' management in both providing and receiving care. This study was carried out before and after the COVID-19 pandemic. As a result of this analysis, AFL combined with FOLFIRI was proven effective and well-tolerated as second-line therapy for mCRC. Patients were previously treated with Oxaliplatin-based regimens as first-line chemotherapy and, in some cases, they also received anti-VEGF or anti-EGFR targeted agents. The pandemic has required new practices in patient management: to reduce the risk of exposition to the virus, preventive measures were introduced to limit access of cancer patients to the hospital. Our results were in line with the VELOUR trial experience (15-22). Although the retrospective design of this study, our results showed AFL effective and well-tolerated, with good disease control and a manageable toxicity profile, with a median PFS of 6.2 months (95% CI: 5.1-7.2). As a consequence, COVID-19 pandemic had probably no impact on PFS for FOLFIRI + Aflibercept treatment.

A significant impact on QoL was observed in most patients (23, 24), the result of this study showed no significant impact due to the onset of COVID-19 pandemic. In the present study, mutations of the RAS / BRAF genes were associated with a lower response rate with a median PFS of 6.3 months, compared to a median PFS of 6.8 months for the wild-type RAS/ BRAF subgroup. Similar trends were observed in the biomarker sub-analyses of the VELOUR study (16). The primary tumor site is an important independent prognostic factor in CRC due to the distinct biological characteristics of right-sided and left-sided tumors. Of interest, right colon cancer is associated with defective repair genes and increased numbers of KRAS / BRAF mutations (25). In our study, no clinically relevant differences were shown according to the localization of the tumor on the left or right side (7.0 months versus 6.7). Finally, a significant correlation was observed between lower pre-treatment CEA values, deceased post-treatment CEA values, increased PFS. Therefore, based on these results, the lack of RAS/BRAF mutations, the localization of the primary tumor, and the pre-treatment CEA levels may represent prognostic factors to achieve greater responses and prolongation of survival.

The results of this study suggest that AFL with FOLFIRI may have specific benefits in patients with the above-mentioned characteristics even during COV-

ID-19 pandemic. Moreover, patients treated with this combination did not experience QoL worsening. On the contrary, thanks to psyconcologist support, the 85% patients enrolled experienced either improvement or stability in QoL. The study confirms that the absence of RAS/BRAF gene mutations, the localization of the primary tumor on the left side, and low pre-treatment CEA levels might be prognostic biomarkers for the treatment with AFL plus FOLFIRI. In addition, a significant correlation between decreased CEA levels increased PFS, and clinical benefits were observed. AFL is an effective antiangiogenic therapy with a manageable tolerability profile that provides significant clinical benefits when combined with FOLFIRI in mCRC after Oxaliplatin with or without biological agents (26). The results obtained showed that AFL is well-tolerated by most patients. AFL does not alter QoL, and its efficacy in terms of survival is confirmed. The results show a good QoL for patients under treatment, without critical consequences in the management of the disease. The physical symptoms were well tolerated without any impact on QoL, however a greater impact was observed on the social area, affecting what were distracting and sociable activities.

CONCLUSIONS

During the recent COVID-19 pandemic, although treatment guidelines remained unchanged, patient management was modified. Nevertheless, the best oncological therapy was performed with a reasonable profile of complications and side effects of chemotherapy due to antiemetics, antiallergics, prophylaxis for immunodeficiencies (G-CSF). A specific patient management was crucial to limit the access of patients to the hospital and, consequently, to drastically reduce the risk of Covid infections in cancer patients (27). The psycho-oncological support, as established by national and international guidelines, is a tool that allows you to improve patients' mood and QoL. This study showed efficacy results for mCRC patients treated with AFL and FOLFIRI in common clinical practice, including patients previously treated with anti-EGFR antibody or bevacizumab during the COVID-19 pandemic (28). AFL plus FOLFIRI has a manageable safety profile, and the results regarding the efficacy and toxicity are consistent with previous studies (29, 30). Limitations of this analysis include the restricted number of patients enrolled as well as the non-randomized sampling. According to the retrospective nature of our analysis and the limited number of patients enrolled, we can suggest that our results can provide valid assumptions for future studies.

COMPLIANCE WITH ETHICAL STANDARDS

Fundings

This research received no external funding.

Conflict of interests

The authors have nothing to disclose, and all authors declare no conflict of interest.

Availability of data and materials

All the datasets on which the conclusions of this study rely were displayed in the manuscript.

Authors' contributions

All authors made a significant contribution to the work reported. GC as coordinating investigator, principal investigator and project manager: designed, initiated, managed and coordinated the research; AG, RD, RA, GR, SC and GL: contributed to the study conception and design; RD, AG and GC: performed material preparation and data collection. The final version of the article was approved by all authors for publication.

Ethical approval

Human studies and subjects

The study was conducted in full compliance with the provisions of the Declaration of Helsinki as well as with the Good Clinical Practice guidelines.

Animal studies

N/A.

Publications ethics

Plagiarism

The article provides a comprehensive review of the latest studies in the field, with accurate citations.

Data falsification and fabrication

The writing and contents of the article are entirely original and were developed entirely by the authors.

REFERENCES

1. World Health Organization. Coronavirus disease (COVID-19) Weekly Epidemiological Updates and Monthly Operational Updates. In: Situation reports. Available from: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports. Accessed: Apr 4, 2020.
2. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020;382(8):727-33. doi: 10.1056/NEJMoa2001017.
3. World Health Organization. Coronavirus disease 2019 (COVID-19) Situation Report – 100. Available from: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200429-sitrep-100-covid-19.pdf. Accessed: Mar 21, 2024.
4. Morris EJA, Goldacre R, Spata E, Mafham M, Finan PJ, Shelton J, et al. Impact of the COVID-19 pandemic on the detection and management of colorectal cancer in England: a population-based study. Lancet Gastroenterol Hepatol. 2021;6(3):199-208. doi: 10.1016/S2468-1253(21)00005-4.
5. Stintzing S, Fischer von Weikersthal L, Decker T, Vehling-Kaiser U, Jäger E, Heintges T, et al. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer-subgroup analysis of patients with KRAS: mutated tumours in the randomised German AIO study KRK-0306. Ann Oncol. 2012;23(7):1693-9. doi: 10.1093/annonc/mdr571.
6. Gong J, Cho M, Fakih M. RAS and BRAF in metastatic colorectal cancer management. J Gastrointest Oncol. 2016;7(5):687-704. doi: 10.21037/jgo.2016.06.12.
7. Afrăsânie VA, Marinca MV, Alexa-Stratulat T, Gafton B, Păduraru M, Adavidoaiei AM, et al. KRAS, NRAS, BRAF, HER2 and microsatellite instability in metastatic colorectal cancer - practical implications for the clinician. Radiol Oncol. 2019;53(3):265-74. doi: 10.2478/raon-2019-0033.
8. Golshani G, Zhang Y. Advances in immunotherapy for colorectal cancer: a review. Therap Adv Gastroenterol. 2020;13:1756284820917527. doi: 10.1177/1756284820917527.
9. Sun W. Angiogenesis in metastatic colorectal cancer and the benefits of targeted therapy. J Hematol Oncol. 2012;5:63. doi: 10.1186/1756-8722-5-63.
10. Giantonio BJ, Catalano PJ, Meropol NJ, O'Dwyer PJ, Mitchell EP, Alberts SR, et al. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. J Clin Oncol. 2007;25(12):1539-44. doi: 10.1200/JCO.2006.09.6305. Corrected and republished in: J Clin Oncol. 2023;41(21):3670-5.
11. Feliu J, Díez de Corcuera I, Manzano JL, Valladares-Ayerbes M, Alcaide J, García García T, et al. Effectiveness and safety of aflibercept for metastatic colorectal cancer: retrospective review within an early access program in Spain. Clin Transl Oncol. 2017;19(4):498-507. doi: 10.1007/s12094-016-1556-3.
12. Van Cutsem E, Tabernero J, Lakomy R, Prenen H, Prausová J, Macarulla T, et al. Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. J Clin Oncol. 2012;30(28):3499-506. doi: 10.1200/JCO.2012.42.8201.
13. Chong DQ, Manalo M, Imperial M, Teo P, Yong G, Ng M, et al. Safety and efficacy of aflibercept in combination with fluorouracil, leucovorin and irinotecan in the treatment of Asian patients with metastatic colorectal cancer. Asia Pac J Clin Oncol. 2016;12(3):275-83. doi: 10.1111/ajco.12496.
14. Lockhart AC, Rothenberg ML, Dupont J, Cooper W, Chevalier P, Sternas L, et al. Phase I study of intravenous vascular endothelial growth factor trap, aflibercept, in patients with advanced solid tumors. J Clin Oncol. 2010;28(2):207-14. doi: 10.1200/JCO.2009.22.9237.
15. Van Cutsem E, Joulain F, Hoff PM, Mitchell E, Ruff P, Lakomý R, et al. Aflibercept Plus FOLFIRI vs. Placebo Plus FOLFIRI in Second-Line Metastatic Colorectal Cancer: a Post Hoc Analysis of Survival from the Phase III VELOUR Study Subsequent to Exclusion of Patients who had Recurrence During or Within 6 Months of Completing Adjuvant Oxaliplatin-Based Therapy. Target Oncol. 2016;11(3):383-400. doi: 10.1007/s11523-015-0402-9.
16. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur

J Cancer. 2009;45(2):228-47. doi: 10.1016/j.ejca.2008.10.026.
17. Cicero G, Lo Re G, DE Luca R, Vernuccio F, Picone D, Midiri M, et al. Role of Densitometric Criteria in Evaluation of Effectiveness of Antiangiogenic Therapies in Metastatic Colorectal Cancer: An Italian Clinical Experience. Anticancer Res. 2017;37(9):5187-92. doi: 10.21873/anticanres.11941.
18. Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. J Clin Oncol. 2010;28(31):4697-705. doi: 10.1200/JCO.2009.27.4860.
19. Yusof MM, Abdullah NM, Sharial MM, Zaatar A. Safety and Management of Toxicity Related to Aflibercept in Combination with Fluorouracil, Leucovorin and Irinotecan in Malaysian Patients with Metastatic Colorectal Cancer. Asian Pac J Cancer Prev. 2016;17(3):973-8. doi: 10.7314/apjcp.2016.17.3.973.
20. Snyder CF, Blackford AL, Okuyama T, Akechi T, Yamashita H, Toyama T, et al. Using the EORTC-QLQ-C30 in clinical practice for patient management: identifying scores requiring a clinician's attention. Qual Life Res. 2013;22(10):2685-91. doi: 10.1007/s11136-013-0387-8.
21. Wirapati P, Pomella V, Vandenbosch B, Kerr P, Maiello E, Jeffery GM, et al. Velour trial biomarkers update: Impact of RAS, BRAF, and sidedness on aflibercept activity. *JCO*. 2017;35(15):3538. doi: 10.1200/JCO.2017.35.15_suppl.3538.
22. Riechelmann RP, Srimuninnimit V, Bordonaro R, Kavan P, Di Bartolomeo M, Maiello E, et al. Aflibercept Plus FOLFIRI for Second-line Treatment of Metastatic Colorectal Cancer: Observations from the Global Aflibercept Safety and Health-Related Quality-of-Life Program (ASQoP). Clin Colorectal Cancer. 2019;18(3):183-91.e3. doi: 10.1016/j.clcc.2019.05.003.
23. You XH, Wen C, Xia ZJ, Sun F, Li Y, Wang W, et al. Primary Tumor Sidedness Predicts Bevacizumab Benefit in Metastatic Colorectal Cancer Patients. Front Oncol. 2019;9:723. doi: 10.3389/fonc.2019.00723.
24. Loupakis F, Hurwitz HI, Saltz L, Arnold D, Grothey A, Nguyen QL, et al. Impact of primary tumour location on efficacy of bevacizumab plus chemotherapy in metastatic colorectal cancer. Br J Cancer. 2018;119(12):1451-5. doi: 10.1038/s41416-018-0304-6.
25. Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, et al. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol. 2004;22(1):23-30. doi: 10.1200/JCO.2004.09.046. Corrected and republished in: J Clin Oncol. 2023;41(19):3461-8.
26. Shen H, Yang J, Huang Q, Jiang MJ, Tan YN, Fu JF, et al. Different treatment strategies and molecular features between right-sided and left-sided colon cancers. World J Gastroenterol. 2015;21(21):6470-8. doi: 10.3748/wjg.v21.i21.6470.
27. Modest DP, Ricard I, Heinemann V, Hegewisch-Becker S, Schmiegel W, Porschen R, et al. Outcome according to KRAS-, NRAS- and BRAF-mutation as well as KRAS mutation variants: pooled analysis of five randomized trials in metastatic colorectal cancer by the AIO colorectal cancer study group. Ann Oncol. 2016;27(9):1746-53. doi: 10.1093/annonc/mdw261.
28. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol. 2010;11(8):753-62. doi: 10.1016/S1470-2045(10)70130-3.
29. Prager GW, Braemswig KH, Martel A, Unseld M, Heinze G, Brodowicz T, et al. Baseline carcinoembryonic antigen (CEA) serum levels predict bevacizumab-based treatment response in metastatic colorectal cancer. Cancer Sci. 2014;105(8):996-1001. doi: 10.1111/cas.12451.
30. Petrelli F, Tomasello G, Borgonovo K, Ghidini M, Turati L, Dallera P, et al. Prognostic Survival Associated With Left-Sided vs Right-Sided Colon Cancer: A Systematic Review and Meta-analysis. JAMA Oncol. 2017;3(2):211-9. doi: 10.1001/jamaoncol.2016.4227.

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REVIEW

THE EFFECTS OF VIRTUAL REALITY ON PAIN AND ANXIETY IN PEDIATRIC ONCOLOGY PATIENTS

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ABSTRACT: Pediatric patients undergoing cancer treatments often experience excessive pain and anxiety during medical procedures, especially those involving the insertion of needles. These feelings are usually associated with dangerous consequences such as attempts to escape and the avoidance of health care. Therefore, it is essential to improve the management of discomfort and fear to ensure appropriate care for the patients. In recent years, many studies and numerous randomized trials have been focusing on the effects of virtual reality (VR) during distressing procedures and rehabilitation sessions, and it has been reported that VR is a successful form of distraction from both pain and anxiety. This innovative form of non-pharmacological analgesic therapy has also been used together with opioids (standard care), such as morphine, while performing unpleasant therapies, successfully reducing the feelings of distress and fear compared to the patients treated with the standard medications.
This manuscript aims to analyze the most recent literature in VR for the management of pain and anxiety during cancer-related treatments in pediatric patients. The use of VR during medical procedures offers patients relaxing and pleasant scenarios. VR represents a promising strategy to alleviate the suffering and stress of pediatric patients, ensuring better patient management.

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Impact statement: The effectiveness of Virtual Reality (VR) has proven to be promising as a potential novel form of palliative pain and anxiety management for pediatric oncology patients.

Key words: *virtual reality; cancer pain; palliative care; opioids; analgesics; pediatric patients; oncology; rehabilitation; non-pharmacological.*

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INTRODUCTION

Cancer is a primary cause of death among children and teenagers. Globally, it has been estimated that almost 400,000 children and adolescents of 0-19 years develop cancer yearly (1).

Since pain and anxiety are significant symptoms experienced during cancer treatment, palliative care is considered an essential component of comprehensive oncology therapy. It has been demonstrated that patients receiving palliative treatment along

with standard cancer care, improved the management of their symptoms, their quality of life, and this latter effect has been showed to improve also for the caregivers and their families (2). According to the World Health Organization (WHO), pain in children is a public health concern of high significance in most parts of the world, especially in low-income countries. Numerous data suggest that these nations offer inadequate and frequently non-existent pain treatments to patients, who are subject to unnecessary agony (3).

The perception of children's pain has evolved through time, and now people value pain alleviation highly. Pain used to be extensively disregarded, and commonly ignored, and it was believed that children rapidly forgot about unpleasant experiences.

Since the 1970s, pain started to be the subject of many studies, resulting in the urgency for pediatric pain research (4).

At this time, the standard strategy for the management of pain and anxiety in cancer patients is pharmacological. The WHO guidelines to manage cancer pain usually recommend paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs), steroids and opioids (3). Opioids (morphine- like drugs) are used worldwide to alleviate painful symptoms. Most industrialized countries have widespread access to opioids in healthcare settings, although availability may be limited in developing nations.

Opioids are administered either parenterally or orally, and the dosage is often based on the body weight of the child (5).

However, these medications are well known for their significant side effects including nausea, constipation, and hallucination (6).

Therefore, to reduce these eventual risks during pain management, it is important to find efficacious nonpharmacological strategies.

Recently, virtual reality (VR) has been used to distract children during painful procedures, emerging as a novel non-pharmacological therapy for pain and anxiety (7). This innovative technology has been successfully appreciated by patients in the pediatric unit, as it appeals to various age groups, and sometimes it may be adapted to mobile phones, thus, providing a more economic option.

It has been shown that VR has the potential to keep patients distracted during painful procedures by keeping them active. For instance, using a VR headset, playing games or observing different scenarios during the procedure, will lead them to experience less pain and anxiety (8).

VR can be used during various medical procedures, helping the child to perceive the therapy as a safe environment (9).

Studies have shown that regional cerebral blood flow related to the processing of a painful event is decreased when a person is distracted (10). Similar to this, when the attention of the person is diverted by a task, there is less activation in the brain regions connected to pain, such as the thalamus, insula, and anterior cingulate cortex, which results in proportionately lower pain levels (11).

In this manuscript, we will review the most recent literature on the effects of VR on pain and anxiety for pediatric oncology patients.

VIRTUAL REALITY: HOW DOES IT WORK?

Virtual Reality can be best described as the simulation of a three-dimensional environment generated by a computer (10).

VR systems can be classified into three categories: Immersive VR, Semi-immersive VR, and Non-immersive VR.

Immersive VR relies on the full immersion of a person in a computer-generated world, instead of the actual one. This level of immersion is only achievable with a head-mounted display that excludes the view of the real world, and with headphones, that block the sounds of the surrounding environment (11).

Semi-immersive VR involves the utilization of a large screen for projecting the virtual environment (VE). Advanced interface devices, such as cybergloves, haptic feedback devices, or infrared cameras, are employed to facilitate user interaction with the VE. Notably, users can concurrently perceive the real world, resulting in a state of partial immersion and a heightened sense of presence (12).

Lastly, the non-immersive form is distinguished by a computer screen where the user can interact with the real world while also connected to the virtual one (11).

VR has proven to work as an efficient distraction tool from pain.

Studies have shown that regional cerebral blood flow related to processing a painful event is decreased when a person is distracted (13). Similar to this, when the attention of the person is diverted by a task, there is less activation in the brain regions connected to pain, such as the thalamus,

insula, and anterior cingulate cortex, which results in proportionately lower pain levels (14).
Various studies have been conducted in recent years to address the expanding field of Virtual Reality (VR) interventions, investigating their efficacy as palliative care for pain and anxiety management, along with their role in rehabilitation (VRR) (**Table 1**).

VR IN PAIN MANAGEMENT

Cancer therapy represents a very unpleasant experience for pediatric patients. Indeed, pediatric cancer patients often undergo treatments, such as chemotherapy, radiation therapy, surgery, and procedures involving the use of needles. These approaches can result in a status of anxiety and pain and make the management of these patients quite difficult (13).
Many studies have been using the following methods to measure pain intensity in the patients: Numerical Rating Scale (NRS), Visual Analogue Scale (VAS), Wong-Baker FACES pain scale and Faces Pain Scale-Revised (FPS-R) (14, 15, 16).
NRS is an 11-, 21-, or 101-point numeric rating scale. Patients are asked to express a numeric value within a specific numeric range to quantify their pain. Depending on the number of points that are present on the scale, the numeric range is from 0 to 10, or 0 to 20 or 0 to 100 where zero corresponds to "no pain" while the maximum range values represent "the worst pain"(15, 17). Instead, in VAS, patients indicate the intensity of their pain on a line whose extreme limits represent the absence of pain and the worst possible pain. The addition of a numerical scale or terms describing pain as " mild," "moderate," and "severe" in VAS defines the "Graphical Rating Scale" (GRS) (15). However, pain measurement methods involving the graphic representation of pain using drawn faces, such as Wong-Baker FACES and Faces Pain Scale-Revised, are highly preferred for younger children, compared with the usage of numerical scales (16). The Wong- Baker FACES is a scale that is based on the use of six drawings of faces with an expression ranging from absence of pain (smiling face) to maximum pain (crying face) with associated numerical scores from 0 to 5. The child is asked to indicate the face that is most representative to identify his or her level of pain (16). The Faces Pain Scale Revised also uses 6 drawn faces to represent pain, but unlike the Wong-Baker FACE, these drawings do not express too much emotion, since there are no significantly smiling or crying faces (16).
The measurement scales mentioned above are widely used to assess pain in the pediatric field (18). Since pain associated with painful procedures during cancer treatments negatively affects the emotional status and well-being of the child, it is crucial to develop approaches to reduce needle-induced anxiety and pain in pediatric patients (13, 19, 20). The use of VR is well known to improve the emotional and psychological status of cancer patients, acting as a distractor and pain reliever during painful procedures (11).
Several studies proved through various pain measurement scales, such as VAS, Wong-Baker faces and NRS, that VR exhibited a beneficial effect in reducing pain in pediatric oncology patients (21, 22, 23) and in distracting these patients during numerous medical procedures involving needles, including port access with a Huber needle, peripheral intravenous cannulation, and venipuncture (21, 24, 25). Indeed, VR can simultaneously engage several senses in patients. While using VR, patients focus their attention on pleasant stimuli determined by the virtual environment, thus distracting themselves from painful perceptions (26).
Immersive and non-immersive VR results in different outcomes in reducing pain in pediatric patients with solid or hematologic tumors (27). Although non-immersive VR is well accepted by children during procedures involving the use of needles, Nilsson *et al.* observed few statistically significant differences when comparing various quantitative pain assessments in pediatric oncology patients undergoing non-immersive type VR compared to control patients (28).
In contrast, the use of immersive VR exhibits a relevant beneficial effect on pain in pediatric cancer patients undergoing painful medical procedures (27). In detail, Atzori *et al.* proved that the use of immersive VR in venipuncture procedures led to a significant reduction in pain sensory perception and time spent by pediatric patients thinking about pain (25).
Examples of scenarios observed during immersive VR by pediatric cancer patients undergoing painful procedures include swimming with marine animals (Ocean Rift), discovering a forest through the eyes of an animal (In the Eyes of the Animal), throwing snowballs at animals or snowmen (SnowWorld) (21, 25); riding a rollercoaster that increases

Table 1. *Summary of Studies Conducted for Virtual Reality (VR) in Pain and Anxiety Management and Rehabilitation (VRR). A comprehensive overview of the aforementioned studies conducted in the field of research for Virtual Reality (VR) as palliative care for pain and anxiety management as well as rehabilitation (VRR). This table summarizes key findings from various studies exploring the effectiveness of VR in alleviating pain and anxiety, and enhancing rehabilitation outcomes for pediatric oncology patients. The studies cover diverse aspects. Each entry provides valuable insights into the evolving landscape of VR applications in the multifaceted care of pediatric patients undergoing cancer treatments.*

STUDY	PAIN OR ANXIETY MANAGEMENT OR REHABILITATION (VRR)	AUTHORS	METHODOLOGY	KEY FINDINGS
Effects of virtual reality on pain, fear and anxiety during blood draw in children aged 5-12 years old: A randomized controlled study	VR in Pain and Anxiety Management	Özalp *et al*. (2020)	CONSORT checklist, Child Fear Scale, Children's Anxiety Meter, Wong-Baker FACES pain rating scale	Virtual reality significantly reduces pain, fear, and anxiety during blood draw in children
A Pilot Randomized Controlled Trial of Virtual Reality Distraction to Reduce Procedural Pain During Subcutaneous Port Access in Children and Adolescents	VR in Pain Management	Hundert *et al*. (2021)	NRS, VAS, Wong-Baker FACES pain rating scale, FPS-R	VR exhibits a beneficial effect in reducing pain in pediatric oncology patients
Virtual Reality Intervention Targeting Pain and Anxiety Among Pediatric Cancer Patients Undergoing Peripheral Intravenous Cannulation: A Randomized Controlled Trial	VR in Pain Management	Wong *et al*. (2021)	NRS, VAS, Wong-Baker FACES pain rating scale, self-report scales of Anxiety	VR intervention significantly reduced pain and anxiety levels among pediatric cancer patients undergoing peripheral intravenous cannulation
Virtual Reality Analgesia During Venipuncture in Pediatric Patients With Onco Hematological Diseases	VR in Pain Management	Atzori *et al*. (2018)	VAS	The use of VR during venipuncture in pediatric patients with onco -hematological diseases was associated with an analgesic effect, contributing to reduced pain during the procedure
The use of Virtual Reality for needle related procedural pain and distress in children and adolescents in a pediatric oncology unit	VR in pain Management	Nilsson *et al*. (2009)	CAS, FAS, FLACC, oximeter	Non-immersive VR reduced pain and distress in children undergoing needle-related procedures
Effects of Virtual Reality on Pain During Venous Port Access in Pediatric Oncology Patients: A Randomized Controlled Study	VR in Pain Management	Semerci *et al*. (2021)	Wong–Baker FACES Pain Rating Scale	VR effectively reduces pain during venous port access in pediatric oncology patients
The effect of virtual reality on pain, fear, and anxiety during access of a port with huber needle in pediatric hematology-oncology patients: Randomized controlled trial	VR in Pain and Anxiety Management	Gerçeker *et al*. (2020)	Child Fear Scale, Children's Anxiety Meter, Wong-Baker Faces Pain Rating Scale	VR diminishes procedure-related pain, fear and anxiety in children aged 5-12 years old during blood draw
Effects of virtual reality therapy on perceived pain intensity, anxiety, catastrophizing and self-efficacy among adolescents with cancer.	VR in Pain and Anxiety Management	Sharifpour *et al*. (2021)	MPQ, PASS, PCS, PSEQ	VR was effective in reducing pain anxiety, pain intensity and pain catastrophizing. VR increases pain self-efficacy

STUDY	PAIN OR ANXIETY MANAGEMENT OR REHABILITATION (VRR)	AUTHORS	METHODOLOGY	KEY FINDINGS
Acupressure and anxiety in cancer patients	VR in Anxiety Management	Beikmoradi *et al.* (2015)	STAI	Acupressure is suggested as a complementary therapy to reduce anxiety in oncology patients
The Impact of an Interactive Computer Game on the Quality of Life of Children Undergoing Chemotherapy	VR in Anxiety Management	Fazelniya *et al.* (2017)	PedsQL	Computer games can be used to improve the quality of life of children undergoing chemotherapy
Is virtual reality ready for prime time in the medical space? A randomized control trial of pediatric virtual reality for acute procedural pain management	VR in Pain Management, VRR	Gold *et al.* (2018)	CAS, VAS, FAS, CASI, investigator-developed Child Presence Measure, Malaise Scale	VR has the potential to serve as a preventive intervention by making the blood draw experience less upsetting and possibly even painless
Unmet rehabilitation needs in 86% of Norwegian pediatric embryonal brain tumor survivors	VRR	Stensvold *et al.* (2020)	Individual Neuropsychological assessment (*e.g.* tests of intelligence, verbal and visual memory, attention, processing speed and executive functions), SIOP, Basal endocrinological tests, cardiac auscultation	Following treatment for pediatric medulloblastoma and central nervous system primitive neuroectodermal tumor, a significant number of survivors had unmet rehabilitative needs and significant late effects

**NRS = Numerical Rating Scale, VAS = Visual Analogue Scale, FPS-R = Faces Pain Scale-Revised, FAS = Facial Affective Scale, FLACC = Face Legs Activity Cry and Consolability Scale, MPQ = McGill Pain Questionnaire, PASS = Pain Anxiety Symptoms Scale, PCS = Pain Catastrophizing Scale, PSEQ = Pain Self-Efficacy Questionnaire, STAI = Spielberger's State-Trait Anxiety Inventory, PedsQL = Pediatric Quality of Life Inventory, CASI = Childhood Anxiety Sensitivity Index, SIOP = International Society of Paediatric Oncology Boston Ototoxicity Scale.*

and slows down the speed (29), and other videos from AAA VR Cinema v.1.6.1 application (30).

Some studies aimed to compare the effectiveness of immersive VR with non-immersive VR in relieving pain in pediatric oncology patients and found no significant difference in outcomes between the two types of VR (27). In a recent study conducted by Hundert *et al.*, values obtained from pain measurement using the NRS scale revealed a greater reduction in pain intensity in patients undergoing immersive VR than those undergoing non-immersive VR, during subcutaneous port access. Nevertheless, this difference was not statistically significant (23).

Thus, VR represents an effective distraction method to reduce pain in pediatric cancer patients. Based on registers for ongoing clinical trials, ISRCTN and clinicaltrials.gov, there seem to be several clinical trials to evaluate the effect of VR on pain in pediatric cancer patients (NCT05042479; NCT05275881; NCT02995434; NCT04092803; NCT03888690; NCT04138095; NCT04931745; NCT04853303) (27) (**Table 2**) and further studies are needed to clarify the mechanisms underlying the analgesic effect of VR (26).

VR IN ANXIETY MANAGEMENT

Anxiety has often been described as the "psychologic equivalent of physical pain" and in some cases, it can appear in the form of phobias (31).

Cancer patients may experience anxiety due to many different reasons, including the reaction to the diagnosis of the disease, critical pain, and long-term treatments (32). Anxiety has multiple negative effects on the therapies of oncology patients, slowing down the healing process (33).

For instance, chemotherapy's physical and psychological side effects cause patients to fear the chemotherapy and sometimes even reject or resist receiving anti-cancer treatment (34).

Table 2. *A Comprehensive List of Clinical Trials for Virtual Reality (VR) utilized as palliative care for pediatric oncology patients taken from clinicaltrials.gov as of February 2024. Based on the various filters applied ("virtual reality pain, anxiety pediatric oncology", childbirth-17, all sexes, not yet recruiting, recruiting, completed, enrolled by invitation, clinical trial), there are four clinical trials worldwide which seek or have sought to better understand the effects of VR as a method of distraction for pediatric oncology patients. There have been four completed clinical trials. These results do not include Scopus.gov clinical trial results, thus eliminating grant clinical trials. Source: clinicaltrials.gov.*

STUDY TITLE	NCT NUMBER	STATUS	AGE	CONDITIONS	INTERVENTIONS	LOCATION	LAST UPDATE POSTED
Virtual Reality on Pain, Fear, and Emotional Appearance During Phlebotomy in Pediatric Hematology and Oncology Patients	NCT05675358	Completed	4-12 years (Child)	Virtual Reality Pain, Acute Pediatric Cancer	Behavioral: Virtual Reality	Izmir, Turkey	2023–01-09
Impact of Virtual Reality on Peri-interventional Pain, Anxiety and Distress in a Pediatric Oncology Outpatient Clinic	NCT06235723	Completed	6-18 years (Child, Adult)	Pediatric Cancer Procedural Anxiety Procedural Pain	Device: Virtual Reality	Hanover, Lower Saxony, Germany	2024-02-01
The Effect of Biofeedback-Based Virtual Reality Game on Children	NCT05585840	Completed	6–12 years (Child)	Pediatric Cancer	Device: Biofeedback-based virtual reality game	Istanbul, Turkey	2022-10-19
Distracting Through Procedural Pain and Distress	NCT04892160	Completed	8-25 years (Child, Adult)	Chronic Illness Hematologic Malignancy Bone Marrow Transplant Infection Oncology Sickle Cell Disease	Other: Guided Imagery Other: Virtual Reality	Milwaukee, Wisconsin, United States	2021-05-19

Pharmacological and nonpharmacological techniques can both be used to treat anxiety in cancer patients (6).

However, the use of pharmacological medications (*e.g.*, benzodiazepine) to treat anxiety can have negative side effects, such as the development of tolerance, dependency, and drug interactions. Because of this, nonpharmacological methods for treating anxiety were seen to be safer (35).

Studies on VR technology have shown that feelings such as anxiety and fear can be decreased through immersion into a relaxing and engaging environment (20, 23, 36).

At this point, various VR applications can be used. The reaction of each child to VR may differ. Some kids prefer soothing, musical films while others enjoy moving, fascinating videos.

To assess the anxiety in children during medical procedures, many studies have used the "Children's Anxiety Meter" (CAM) (**Figure 1**).

CAM is displayed as a thermometer with a bulb at the bottom and horizontal lines spaced out along the top. To measure the anxiety state, children are asked to mark how they are feeling at that specific moment, and scores can be between 0 (very low anxiety) and 10 (very high anxiety) (7).

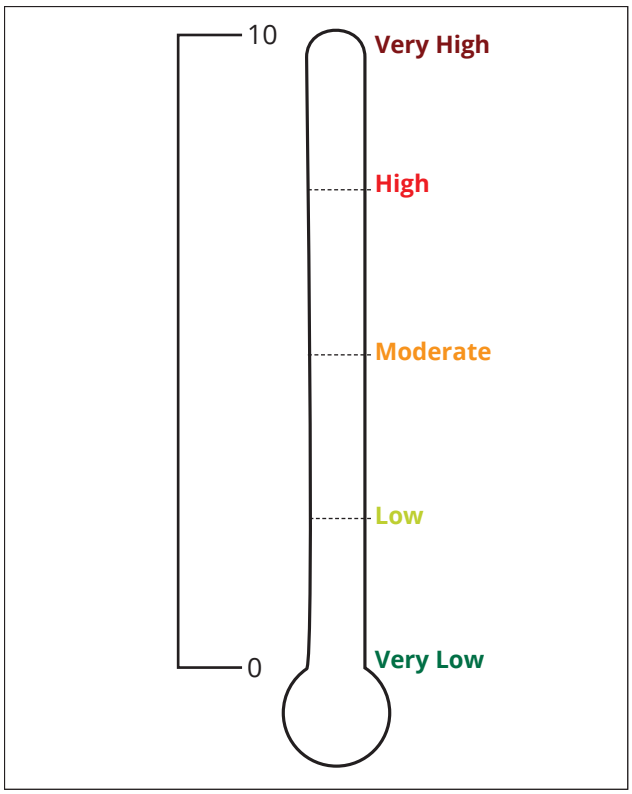

Figure 1. *Children's Anxiety Meter (CAM). This figure shows an example of CAM that can be used to assess anxiety in children. CAM is displayed as a thermometer and it is divided in multiple sections, each representing a specific anxiety level. Here, five sections have been considered: Very Low, Low, Moderate, High, Very High. Additionally, each anxiety level is color-coded to provide a visual reference and, to indicate a specific anxiety level, numeric values from 0 to 10 can be used.*

VR IN REHABILITATION (VRR)

Virtual reality rehabilitation (VRR) is an innovative approach that involves interactive computer- generated environments to simulate real-world experiences and promote physical and cognitive rehabilitation in a safe and engaging manner (36). Unlike virtual reality (VR) intervention, or therapy, which focuses primarily on distracting patients from pain and anxiety during medical procedures, VRR aims to improve patients' physical and cognitive abilities and promote recovery and rehabilitation (37). For pediatric oncology patients, who often experience a wide range of physical and cognitive challenges during and after treatment, including muscle weakness, balance impairments, and cognitive deficits, VRR offers a tailored approach that allows them to perform specific tasks designed to meet their unique needs and abilities (37).

Despite the studied benefits of traditional rehabilitation for cancer patients and survivors, a minority of cancer survivors are referred to rehabilitation programs (36). A 20-year follow- up study conducted by Stensvold *et al.* in Norway found that among the pediatric brain cancer survivors, 86% had an unmet rehabilitation need (38). This lack of access and information may be due to various factors, including the incapability of cancer care systems to deliver early detection of impairing symptoms, inadequate traditional training programs, transportation issues, and limited knowledge (39, 40, 41).

Virtual reality rehabilitation (VRR) offers a promising solution to these issues. VRR has been shown to improve adherence rates and training intensity due to its entertaining nature, which can lead to greater patient engagement and motivation (42). VRR also offers a safe and engaging environment for pediatric oncology patients to perform rehabilitation exercises and activities, which may help reduce anxiety, pain, and stress. Research studies have shown that VRR can be effective in improving physical function, reducing pain, and enhancing emotional well-being in pediatric oncology patients (37).

For example, a recent study conducted by Nuara *et al.* demonstrated that VRR was associated with significant improvements in upper extremity function and quality of life in pediatric cancer patients undergoing chemotherapy (41). Another study by Tanner *et al.* found that VRR was effective in reducing pain and anxiety in pediatric oncology patients undergoing bone marrow aspiration and biopsy procedures (37).

VRR offers a promising approach to improve outcomes in pediatric oncology patients. Despite the potential benefits, there is a need for greater awareness and access to VRR for patients. Health professionals and cancer care systems should consider incorporating VRR into rehabilitation programs to improve outcomes and quality of life for pediatric oncology patients.

VR SIDE EFFECTS AND LIMITATIONS

Despite the benefits of utilizing VR technology as non-pharmacological palliative care, there are potential side effects and limitations that must be considered for pediatric oncology patients.

One of the potential side effects of VR is motion sickness, which can cause nausea and discomfort in some patients (43). This can be especially problematic for younger patients who may not be able

to articulate their discomfort as well as adults. Therefore, it is recommended that children have a limit for screen time between 5 to 10 minutes to avoid "simulator sickness" (44). Prolonged use of VR headsets can also cause eye strain, headaches, and fatigue in some patients, which could exacerbate existing symptoms and negatively impact patient engagement (45). Additionally, the various types of VR and intervention scenarios presented to the children expose them to intense or frightening VR experiences which could potentially lead to negative behavioral changes, including increased aggression, nightmares, or anxiety (46). Some VR simulations expose the patient and family to a specific medical procedure, which increases the patient's procedural knowledge but at the same time may cause additional stress, anxiety and fear (47).

Physical limitations of VR technology exist despite offering a safe virtual environment. Although VR provides a promising tool to influence psychological and physiological functions, the headsets, or head-mounted displays (HMD) utilized may be too heavy or too large for pediatric patients (48). Therefore, alternative strategies, such as lighter and more comfortable headsets, or the use of other devices, such as smartphones, need to be explored to overcome the physical limitations of VR technology and enhance the patient's experience since active interaction, navigation and immersion are key characteristics of VR systems (49, 50).

Another limitation of VR technology is while there are some VR experiences specifically designed for pediatric oncology patients, there is still a limited amount of content available, which could limit the usefulness of the technology for some patients (51). Finally, it is important to note that VR technology is not a replacement for human interaction and emotional support that patients may need from healthcare providers, family, and friends (43). VR therapy can complement traditional care approaches, but it cannot replace them entirely. Children may still experience acute distress reactions based on the separation from parents, unfamiliar environments, medical equipment and negative experiences with previous medical procedures (47). Furthermore, preschool and elementary school-age children may have difficulty differentiating virtual experiences from real ones thus leading to potential false memories since they are still developing the ability to distinguish between reality and fantasy (44).

While VR technology can provide significant benefits to pediatric oncology patients, there are potential side effects and limitations that must be considered when using this technology. By carefully assessing patients and developing an individualized treatment plan, healthcare providers can help maximize the benefits of VR therapy while minimizing its potential risks.

CONCLUSION AND FUTURE DIRECTIONS

Although VR is based on the principle of distraction and has proven to be effective, there still remain drawbacks and limitations to its application in the medical field for palliative care. VR provides non-invasive real perceptual stimuli such as visual images, spatial sounds, tactile and olfactory feedback (48). Nonetheless, further research must be conducted to determine the safety of VR technology. Along with VR, Augmented Reality (AR) is also at the forefront these types of technologies in medical applications (52). AR differs from VR in which it utilizes elements of VR and superimposes them in the real-world environment (52). The preliminary evidence and use of these digital technologies has proven to be beneficial inn diagnostics, surgical procedures and rehabilitation. Further research must be performed to assess the short and long-term impact on clinical practices and patients' lives.

It is worth noting that not all studies have standardized criteria for what VR technology entails. VR technology encompasses studies that utilize VR video, VR games, and iPad videos (53), therefore it must be specified which VR type and scenario are used. This will allow researchers to better understand which VR type is more beneficial to reduce anxiety, pain and fear for a given pediatric patient.

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We have no conflicts of interest to disclose.

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The data underlying this article are available in the public domain, using various datasets primarily from Pubmed, GCO, WHO, Clinical Trials.gov, etc.

Authors' contributions

CN, SB, EA and AC: worked on the conception of the work; CN, SB, EA and AC: worked on drafting and revising it critically for important intellectual content; AC and AG: provided approval for publication of content; CN, SB, EA, AC and AG: agree to be accountable for all aspects of the work.

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Human studies and subjects

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Publications ethics

Plagiarism

The article provides a comprehensive review of the latest studies in the field, with accurate citations.

Data falsification and fabrication

The writing and contents of the article are entirely original and were developed entirely by the authors.

REFERENCES

1. World Health Organization. CureAll framework: WHO global initiative for childhood cancer. Increasing access, advancing quality, saving lives. World Health Organization 2021. Available from: https://www.who.int/publications/i/item/9789240025271. Accessed: Feb 15, 2024.
2. Snaman JM, Kaye EC, Lu JJ, Sykes A, Baker JN. Palliative Care Involvement Is Associated with Less Intensive End-of-Life Care in Adolescent and Young Adult Oncology Patients. J Palliat Med. 2017;20(5):509-16. doi: 10.1089/jpm.2016.0451.
3. World Health Organization. WHO Guidelines for the Pharmacological and Radiotherapeutic Management of Cancer Pain in Adults and Adolescents. Geneva: World Health Organization; 2018. Available from: https://www.ncbi.nlm.nih.gov/books/NBK537492/. Accessed: Feb 15, 2024.
4. Caes L, Boerner KE, Chambers CT, Campbell-Yeo M, Stinson J, Birnie KA, et al. A comprehensive categorical and bibliometric analysis of published research articles on pediatric pain from 1975 to 2010. Pain. 2016;157(2):302-13. doi: 10.1097/j.pain.0000000000000403.
5. Verghese ST, Hannallah RS. Acute pain management in children. J Pain Res. 2010;3:105-23. doi: 10.2147/jpr.s4554.
6. Ferrell BR, Coyle N, Paice J (Eds). *Oxford Textbook of Palliative Nursing. Oxford University Press, Oxford, 2015.*
7. Özalp Gerçeker G, Ayar D, Özdemir EZ, Bektaş M. Effects of virtual reality on pain, fear and anxiety during blood draw in children aged 5-12 years old: A randomised controlled study. J Clin Nurs. 2020;29(7-8):1151-61. doi: 10.1111/jocn.15173.
8. Bellieni CV, Cordelli DM, Raffaelli M, Ricci B, Morgese G, Buonocore G. Analgesic effect of watching TV during venipuncture. Arch Dis Child. 2006;91(12):1015-7. doi: 10.1136/adc.2006.097246.
9. Carmona-Torres JA, Cangas AJ, Langer AI. Applications of 3D simulation in Mental Health: Utilities and new developments. In: Mental Illnesses–Evaluation, Treatments and Implications. InTech, 2012: pp. 37-56.
10. Kardong-Edgren S, Farra SL, Alinier G, Young HM. A Call to Unify Definitions of Virtual Reality. Clinical Simulation in Nursing. 2019;31:28-34. doi: 10.1016/j.ecns.2019.02.006.
11. Chirico A, Lucidi F, De Laurentiis M, Milanese C, Napoli A, Giordano A. Virtual Reality in Health System: Beyond Entertainment. A Mini-Review on the Efficacy of VR During Cancer Treatment. J Cell Physiol. 2016;231(2):275-87. doi: 10.1002/jcp.25117.
12. Salatino A, Zavattaro C, Gammeri R, Cirillo E, Piatti ML, Pyasik M, et al. Virtual reality rehabilitation for unilateral spatial neglect: A systematic review of immersive, semi-immersive and non-immersive techniques. Neurosci Biobehav Rev. 2023;152:105248. doi: 10.1016/j.neubiorev.2023.105248.
13. Loeffen EAH, Mulder RL, Font-Gonzalez A, Leroy PLJM, Dick BD, Taddio A, et al. Reducing pain and distress related to needle procedures in children with cancer: A clinical practice guideline. Eur J Cancer. 2020;131:53-67. doi: 10.1016/j.ejca.2020.02.039.
14. Thong ISK, Jensen MP, Miró J, Tan G. The validity of pain intensity measures: what do

the NRS, VAS, VRS, and FPS-R measure? Scand J Pain. 2018;18(1):99-107. doi: 10.1515/sjpain-2018-0012.
15. Haefeli M, Elfering A. Pain assessment. Eur Spine J. 2006;15 Suppl 1(Suppl 1):S17-24. doi: 10.1007/s00586-005-1044-x.
16. Drendel AL, Kelly BT, Ali S. Pain assessment for children: overcoming challenges and optimizing care. Pediatr Emerg Care. 2011;27(8):773-81. doi: 10.1097/PEC.0b013e31822877f7.
17. Williamson A, Hoggart B. Pain: a review of three commonly used pain rating scales. J Clin Nurs. 2005;14(7):798-804. doi: 10.1111/j.1365-2702.2005.01121.x.
18. Gaglani A, Gross T. Pediatric Pain Management. Emerg Med Clin North Am. 2018;36(2):323-34. doi: 10.1016/j.emc.2017.12.002.
19. Kettwich SC, Sibbitt WL Jr, Brandt JR, Johnson CR, Wong CS, Bankhurst AD. Needle phobia and stress-reducing medical devices in pediatric and adult chemotherapy patients. J Pediatr Oncol Nurs. 2007;24(1):20-8. doi: 10.1177/1043454206296023.
20. Berman D, Duncan AM, Zeltzer LK. The evaluation and management of pain in the infant and young child with cancer. Br J Cancer Suppl. 1992;18:S84-91.
21. Gerçeker GÖ, Bektaş M, Aydınok Y, Ören H, Ellidokuz H, Olgun N. The effect of virtual reality on pain, fear, and anxiety during access of a port with huber needle in pediatric hematology-oncology patients: Randomized controlled trial. Eur J Oncol Nurs. 2021;50:101886. doi: 10.1016/j.ejon.2020.101886.
22. Cheng Z, Yu S, Zhang W, Liu X, Shen Y, Weng H. Virtual reality for pain and anxiety of pediatric oncology patients: A systematic review and meta-analysis. Asia Pac J Oncol Nurs. 2022;9(12):100152. doi: 10.1016/j.apjon.2022.100152.
23. Hundert AS, Birnie KA, Abla O, Positano K, Cassiani C, Lloyd S, et al. A Pilot Randomized Controlled Trial of Virtual Reality Distraction to Reduce Procedural Pain During Subcutaneous Port Access in Children and Adolescents With Cancer. Clin J Pain. 2021;38(3):189-96. doi: 10.1097/AJP.0000000000001017.
24. Wong CL, Li CK, Chan CWH, Choi KC, Chen J, Yeung MT, et al. Virtual Reality Intervention Targeting Pain and Anxiety Among Pediatric Cancer Patients Undergoing Peripheral Intravenous Cannulation: A Randomized Controlled Trial. Cancer Nurs. 2021;44(6):435-42. doi: 10.1097/NCC.0000000000000844.
25. Atzori B, Hoffman HG, Vagnoli L, Patterson DR, Alhalabi W, Messeri A, et al. Virtual Reality Analgesia During Venipuncture in Pediatric Patients With Onco-Hematological Diseases. Front Psychol. 2018;9:2508. doi: 10.3389/fpsyg.2018.02508.
26. Indovina P, Barone D, Gallo L, Chirico A, De Pietro G, Giordano A. Virtual Reality as a Distraction Intervention to Relieve Pain and Distress During Medical Procedures: A Comprehensive Literature Review. Clin J Pain. 2018;34(9):858-77. doi: 10.1097/AJP.0000000000000599.
27. Comparcini D, Simonetti V, Galli F, Saltarella I, Altamura C, Tomietto M, et al. Immersive and Non-Immersive Virtual Reality for Pain and Anxiety Management in Pediatric Patients with Hematological or Solid Cancer: A Systematic Review. Cancers (Basel). 2023;15(3):985. doi: 10.3390/cancers15030985.
28. Nilsson S, Finnström B, Kokinsky E, Enskär K. The use of Virtual Reality for needle-related procedural pain and distress in children and adolescents in a paediatric oncology unit. Eur J Oncol Nurs. 2009;13(2):102-9. doi: 10.1016/j.ejon.2009.01.003.
29. Semerci R, Akgün Kostak M, Eren T, Avci G. Effects of Virtual Reality on Pain During Venous Port Access in Pediatric Oncology Patients: A Randomized Controlled Study. J Pediatr Oncol Nurs. 2021;38(2):142-51. doi: 10.1177/1043454220975702.
30. Sharifpour S, Manshaee GR, Sajjadian I. Effects of virtual reality therapy on perceived pain intensity, anxiety, catastrophising and self-efficacy among adolescents with cancer. Counselling and Psychotherapy Research. 2020;21. doi: 10.1002/capr.12311.
31. Walker HK, Hall WD, Hurst JW, editors. Clinical Methods: The History, Physical, and Laboratory Examinations. 3rd edition. Boston: Butterworths; 1990. Available from: https://www.ncbi.nlm.nih.gov/books/NBK201/. Accessed: Feb 15, 2024.
32. Arrieta O, Angulo LP, Núñez-Valencia C, Dorantes-Gallareta Y, Macedo EO, Martínez-López D, et al. Association of depression and anxiety on quality of life, treatment adherence, and prognosis in patients with advanced non-small cell lung cancer. Ann Surg Oncol. 2013;20(6):1941-8. doi: 10.1245/s10434-012-2793-5.
33. Beikmoradi A, Najafi F, Roshanaei G, Pour Esmaeil Z, Khatibian M, Ahmadi A. Acupressure and anxiety in cancer patients. Iran Red Crescent Med J. 2015;17(3):e25919. doi: 10.5812/ircmj.25919.
34. Fazelniya Z, Najafi M, Moafi A, Talakoub S. The Impact of an Interactive Computer Game on the

Quality of Life of Children Undergoing Chemotherapy. Iran J Nurs Midwifery Res. 2017;22(6):431-5. doi: 10.4103/ijnmr.IJNMR_215_15.
35. Platt LM, Whitburn AI, Platt-Koch AG, Koch RL. Nonpharmacological Alternatives to Benzodiaze-pine Drugs for the Treatment of Anxiety in Out-patient Populations: A Literature Review. J Psy-chosoc Nurs Ment Health Serv. 2016;54(8):35-42. doi: 10.3928/02793695-20160725-07.
36. Melillo A, Chirico A, De Pietro G, Gallo L, Caggianese G, Barone D, et al. Virtual Reality Rehabilitation Systems for Cancer Survivors: A Narrative Review of the Literature. Cancers (Basel). 2022;14(13):3163. doi: 10.3390/cancers14133163.
37. Tanner L, Keppner K, Lesmeister D, Lyons K, Rock K, Sparrow J. Cancer Rehabilitation in the Pediatric and Adolescent/Young Adult Population. Semin Oncol Nurs. 2020;36(1):150984. doi: 10.1016/j.soncn.2019.150984.
38. Stensvold E, Stadskleiv K, Myklebust TÅ, Wesenberg F, Helseth E, Bechensteen AG, et al. Unmet rehabilitation needs in 86% of Norwegian paediatric embryonal brain tumour survivors. Acta Paediatr. 2020;109(9):1875-86. doi: 10.1111/apa.15188.
39. Cheville AL, Kornblith AB, Basford JR. An examination of the causes for the underutilization of rehabilitation services among people with advanced cancer. Am J Phys Med Rehabil. 2011;90(5 Suppl 1):S27-37. doi: 10.1097/PHM.0b013e31820be3be.
40. Pudkasam S, Polman R, Pitcher M, Fisher M, Chinlumprasert N, Stojanovska L, et al. Physical activity and breast cancer survivors: Importance of adherence, motivational interviewing and psychological health. Maturitas. 2018;116:66-72. doi: 10.1016/j.maturitas.2018.07.010.
41. Nuara A, Fabbri-Destro M, Scalona E, Lenzi SE, Rizzolatti G, Avanzini P. Telerehabilitation in response to constrained physical distance: an opportunity to rethink neurorehabilitative routines. J Neurol. 2022;269(2):627-38. doi: 10.1007/s00415-021-10397-w.
42. Rose T, Nam CS, Chen KB. Immersion of virtual reality for rehabilitation - Review. Appl Ergon. 2018;69:153-61. doi: 10.1016/j.apergo.2018.01.009.
43. Gold JI, Mahrer NE. Is Virtual Reality Ready for Prime Time in the Medical Space? A Randomized Control Trial of Pediatric Virtual Reality for Acute Procedural Pain Management. J Pediatr Psychol. 2018;43(3):266-75. doi: 10.1093/jpepsy/jsx129.
44. Stevens Aubrey J, Robb MB, Bailey J, Bailenson J. Virtual Reality 101: What You Need to Know About Kids and VR. Common Sense 2018. Available from: https://www.commonsensemedia.org/sites/default/files/research/report/csm_vr101_final_under5mb.pdf. Accessed: March 22, 2023.
45. Li A, Montaño Z, Chen VJ, Gold JI. Virtual reality and pain management: current trends and future directions. Pain Manag. 2011;1(2):147-57. doi: 10.2217/pmt.10.15.
46. Mahrer NE, Gold JI. The use of virtual reality for pain control: a review. Curr Pain Headache Rep. 2009;13(2):100-9. doi: 10.1007/s11916-009-0019-8.
47. Tennant M, Anderson N, Youssef GJ, McMillan L, Thorson R, Wheeler G, et al. Effects of immersive virtual reality exposure in preparing pediatric oncology patients for radiation therapy. Tech Innov Patient Support Radiat Oncol. 2021;19:18-25. doi: 10.1016/j.tipsro.2021.06.001.
48. Lambert V, Boylan P, Boran L, Hicks P, Kirubakaran R, Devane D, et al. Virtual reality distraction for acute pain in children. Cochrane Database Syst Rev. 2020;10(10):CD010686. doi: 10.1002/14651858.CD010686.pub2.
49. Buche H, Michel A, Blanc N. Use of virtual reality in oncology: from the state of the art to an integrative model.). Frontiers in Virtual Reality 3. 2022:104. doi: 10.3389/frvir.2022.894162.
50. Aguinas H, Henle C, Beaty Jr JC. Virtual Reality Technology: A New Tool for Personnel Selection. International Journal of Selection and Assessment. 2001;9(1-2):70-83. doi: 10.1111/1468-2389.00164.
51. Parsons TD, Rizzo AA. Affective outcomes of virtual reality exposure therapy for anxiety and specific phobias: a meta-analysis. J Behav Ther Exp Psychiatry. 2008;39(3):250-61. doi: 10.1016/j.jbtep.2007.07.007.
52. Yeung AWK, Tosevska A, Klager E, Eibensteiner F, Laxar D, Stoyanov J, et al. Virtual and Augmented Reality Applications in Medicine: Analysis of the Scientific Literature. J Med Internet Res. 2021;23(2):e25499. doi: 10.2196/25499.
53. Cheng Z, Yu S, Zhang W, Liu X, Shen Y, Weng H. Virtual reality for pain and anxiety of pediatric oncology patients: A systematic review and meta-analysis. Asia Pac J Oncol Nurs. 2022;9(12):100152. doi: 10.1016/j.apjon.2022.100152.

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REVIEW

CALORIE RESTRICTION AND PERIODIC FASTING FROM RODENT TO HUMAN: LOST IN TRANSLATION?

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ABSTRACT: In recent years, nutrition has attracted attention and interest from the scientific community, as it has emerged as a fundamental player in improving lifespan and healthspan by preventing non-communicable ageing-related diseases.
Preclinical studies have shown that caloric restriction and periodic fasting extend the lifespan in animal models, prevent tumorigenesis, delay the onset of age-related diseases, and enhance the efficacy of anticancer therapies. This review provides the current state of knowledge on the benefits of calorie restriction and periodic fasting on tumor development and ageing in a rodent model and summarizes the clinical progress with calorie restriction and periodic fasting in clinical trials. We also discuss the numerous caveats that might arise with the implementation of these dietary interventions in clinical practice.

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Impact statement: Dietary interventions have the potential to impact life expectancy and prevent age-related diseases like tumors and neurodegenerative disorders. However, the applicability of calorie restriction and fasting interventions from mouse models to human populations remains controversial and inconclusive. In this review, we examine the potential obstacles that may arise when attempting to apply dietary interventions from mouse models to human populations.

Key words: *calorie restriction; fasting, ageing; cancer; metabolism.*

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INTRODUCTION

The ageing of the world population and the increase in life expectancy have led to an increase in age-related diseases, including obesity, cardiovascular diseases (CVDs), type 2 diabetes, neurodegenerative diseases, and cancer (1). However, the health span (the period of life free of chronic disease) does not improve at the same rate as life expectancy. New approaches to therapy are being developed (World Health Organization, 2021) with the goal of minimizing the number of years lived with chronic diseases and the associated suffering, ultimately enhancing the overall well-being of the population and easing the growing burden of healthcare costs (2).
Although the advent of molecular genetics in model organisms has made it possible to identify and modulate the genes implicated in the ageing process, these genetic strategies are difficult to apply in clinical practice. To date, caloric restriction (CR) has proven to be the most effec-

tive, simplest and robust geroprotective intervention (3, 4).

In recent years, new dietary interventions, which involve alternating periods of fasting (complete abstinence from eating but not drinking) and normal diet, have been shown to have the same beneficial effects as CR in lifespan and healthspan extension (5-11).

CR consists of reducing daily calorie intake below energy demand without deprivation of essential micro- and macronutrients over a given period. In mice and rats, calorie intake is reduced by 10-50% compared to ad libitum food intake (12, 13), whereas it is reduced by 10-25% from the baseline level in humans (7, 14-16).

Periodic fasting (PF) involves several strategies that differ in the duration of fasting. The 5:2 diet involves two non-consecutive fasting days per week (*5*); alternate-day fasting (ADF) consists of fasting every other day (17); time-restricted eating (TRE) limits meal intake to a daily window of 6-8 hours followed by a fast of 14-16 hours (6-8). The fasting mimicking diet (FMD), on the other hand, is a 5-day plant-based low-calorie diet program alternated with a normal diet, repeated 2-3 times a year, which aims to reproduce the effects of fasting without complete food deprivation (9-11).

In this review, we will discuss the mechanisms regulated by CR and fasting, the effects of such dietary interventions on ageing and cancer, and the pitfalls that might be encountered in translating such dietary programs from the preclinical to clinical phase.

SYSTEMIC METABOLIC AND HORMONAL CHANGES INDUCED BY CR AND PF

Similar to CR, acute fasting triggers catabolic and hormonal responses in mice and humans. Drops in glucose levels due to nutritional restriction or abstinence reduce circulating insulin levels and promote glucagon secretion from the pancreas, which stimulates glucose release from the liver via glycogenolysis and lipolysis in adipocytes via lipase activation (18). Non-esterified fatty acids, released into the circulation upon triglyceride breakdown, undergo beta oxidation mainly in the liver mitochondria and peroxisomes, and are converted into acetyl-CoA, which is used to fuel the mitochondrial Krebs cycle and supply the cell's energy needs.

If the fasting period is prolonged, acetyl-CoA is converted in the liver to ketone bodies (KB; hydroxybutyrate, acetoacetate, and acetone), which become the main energy source for extrahepatic organs, such as the brain and heart (19). Conversely, KB can be catabolized to acetyl-CoA, which helps sustain the Krebs cycle and energy production (20, 21).

Simultaneously, acetyl-CoA and glucogenic amino acids are metabolized into glycolytic/gluconeogenetic or Krebs cycle intermediates that support glucagon-stimulated glucose synthesis (gluconeogenesis) via the cyclic AMP (cAMP)/protein kinase A (PKA) signaling pathway in the liver.

Nutrient depletion leads to an increase in ghrelin (hunger hormone) (22), branched-chain amino acids, adiponectin and FGF21 (fibroblast growth factor 21). The latter are involved in both the regulation of glucose levels and energy homeostasis (23). In contrast, the circulatory levels of various factors, such as insulin, IGF-1, glucose, gluconeogenic amino acids, triglycerides, and leptin (satiety hormone), decrease significantly over time.

These metabolic changes are accompanied by a decrease in inflammatory markers and oxidative stress markers, including C-reactive protein (CRP), a liver-derived acute-phase biomarker associated with the inflammatory response (18, 24, 25).

Furthermore, the thyroid gland function and the secretion of the thyroid hormone triiodothyronine (T3) decreases during fasting and caloric restriction and lead a reduction in the body's energy expenditure and a slowing of the metabolic rate in human (17, 26-28). In rodents, low thyroid function leads to a decrease in body temperature, which could contribute to metabolic changes related to CR and fasting (29).

CR and fasting affect intestinal microbiota populations, which depend on the diet's macronutrient composition and metabolome content (30). Clinical and preclinical studies have highlighted that CR and fasting enrich the microbiota with probiotic favorable strains (*e.g.*, *Lactobacillus* and *Bifidobacterium*), while reducing pro-inflammatory strains (*e.g.*, *Desulfovibrionaceae* and *Streptococcaceae*) (31-33).

However, returning to a normal diet restores the original microbiota composition and reverses the effects of CR and fasting (34).

The microbiota is a key factor in determining the positive outcome of CR and fasting, as germ-free mice do not benefit to the same extent from CR and fasting-mediated effects as conventional mice (*e.g.*, body weight and fat loss) (34, 35).

CELLULAR AND MOLECULAR MECHANISM UNDERLYING CR AND FASTING

Nutrient availability shapes the cellular metabolome, epigenome, transcriptome, and proteome by modulating several interconnected nutrient-sensing pathways and influencing various cellular processes.

Low glucose and insulin/IGF1 levels and amino acid restriction inhibit mechanistic target of rapamycin (mTOR). mTOR is a threonine-serine kinase that acts as the catalytic domain of two protein complexes: mTORC1 and mTORC2. Its inhibition leads to the arrest of protein synthesis, lipogenesis, ribosome and nucleotide biogenesis, and activation of autophagy.

mTORC1 senses and integrates different environmental stimuli, and coordinates cell growth and division based on environmental nutrient availability. mTORC2 is an effector of PI3K signaling, is sensitive only to growth factor stimulation, and is involved in cytoskeleton organization and cellular insulin sensitivity. Calorie restriction and fasting exert their beneficial effects through inhibition of mTORC1 and activation of mTORC2 (36-41) (**Figure 1**).

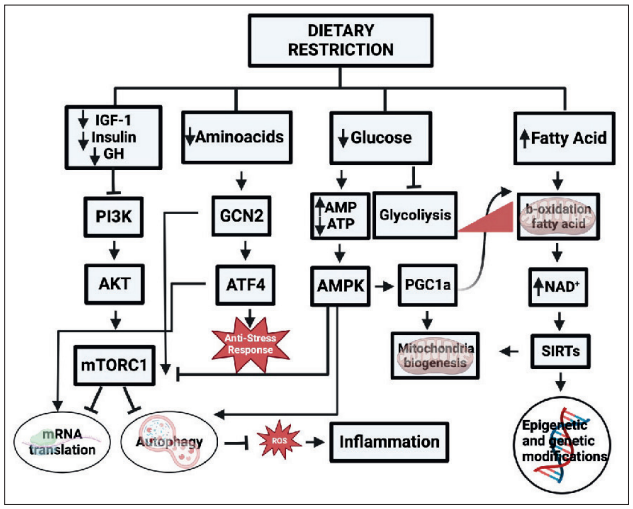

Figure 1. *Signaling pathways regulated by CR and PF. Reduced levels of most macronutrients (glucose and amino acids) lead to activation of autophagy, inhibition of mRNA translation, reduced inflammation, and anti-stress response through modulation of the IGF-1-PI3K-AKT-mTORC1 and GCN2-ATF4 signaling pathway. The high AMP/ATP ratio increases AMPK activity which results in mTORC1 inhibition, autophagy activation, and PGC1 α-mediated mitochondria biogenesis. The low glucose level, the increase in fatty acids and their beta oxidation promote the cellular transition from glycolysis to oxidative phosphorylation leading to an increase in the level of NAD+, the activation of sirtuins and consequently epigenetic and genetic remodelling. FGF21 = fibroblast growth factor 21, IGF-1 = insulin-like growth factor, mTORC1 = mechanistic target of rapamycin complex 1, NAD+= Nicotinamide adenine dinucleotide, AMP= Adenosine monophosphate, ATP= Adenosine triphosphate, SIRTS=sirtuins (created with BioRender.com).*

Under low-energy conditions, increasing the AMP/ATP ratio activates AMP-activated protein kinase (AMPK), a cellular energy sensor serine/threonine kinase involved in fatty acid oxidation, glucose uptake, and lipogenesis inhibition in various cell types (42).

AMPK activation increases NAD^+ levels by stimulating the expression and activity of nicotinamide phosphoribosyltransferase (NAMPT), a rate-limiting enzyme in NAD^+ synthesis, and indirectly by enhancing fatty acid oxidation and mitochondrial respiration (43, 44). Elevated NAD^+ levels increase sirtuin deacetylase activity, resulting in epigenetic remodeling, metabolic reprogramming, and transcriptional changes. The sirtuin family includes seven members (SIRT1-SIRT7) that differ in their subcellular localization and function (45, 46). Sirtuins also possess ADP ribosylation (SIRT4 and SIRT6) (47, 48), demalonylation and desuccinylation (SIRT5*)* (49, 50), and lysine decrotonylation (SIRT1 and SIRT2) activities, which could mediate the beneficial effects of CR and fasting (45).

AMPK and SIRT1 enhance mitochondrial respiration and mitochondrial fatty acid (FA) transport and utilization via peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), a master transcriptional regulator of mitochondrial biogenesis (51) (**Figure 1**).

To maintain homeostasis during fasting, cells initiate autophagy, which is a process that results in the degradation of damaged organelles and dysfunctional macromolecules via lysosomal pathways. This degradation provides energy and key metabolites for macromolecule synthesis to sustain nutrient-depleted cells (52-55).

Fasting and CR promote autophagy via AMPK-mediated control of mTOR and SIRTs and through the deacetylation of nuclear and cytoplasmic proteins due to the low availability of acetyl-CoA (56).

The effects of CR and fasting might also be mediated by the nutrient-sensitive GCN2 (general control non-derepressible 2) signaling pathway, which is involved in immune system homeostasis (57) and in the coordination of integrated stress responses and the inflammasome (58).

Upon uncharged tRNAs accumulation or ribosomal stalling (59-61), GCN2 phosphorylates eukaryotic translation initiation factor 2 (eIF2a) and inhibits mTORC1 and the translation of most mRNAs,

except for some selected proteins, such as ATF4 (62, 63) (**Figure 1**).

The transcription factor ATF4 promotes the transcription of genes involved in amino acid import, glutathione biosynthesis, and antioxidative stress response, including the energy balance hormone FGF21 (64). FGF21 is an endocrine hormone that improves metabolic health by increasing insulin sensitivity and energy expenditure by regulating lipid and glucose metabolism.

AMPK activation, low insulin and IGF-1 levels, and PI3K-AKT-mTOR inhibition increase the levels of reactive oxygen species (ROS) and induce a resistance response to oxidative stress by upregulating the expression of genes such as *NRF2*, a master regulator of several cytoprotective and detoxifying genes (20, 65) (**Figure 1**).

KB, produced during fasting, promote not only the switch of cellular metabolism from glycolysis to oxidative phosphorylation but also epigenetic remodeling and transcriptional reprogramming by inhibiting histone deacetylases (66). In addition, KB exerts an anti-inflammatory effect and improves lipid profile (low triglyceride and cholesterol levels combined with high HDL) by binding to G protein-coupled receptors (GPCRs) (67) (**Figure 1**).

CALORIE RESTRICTION, PERIODIC FASTING AND AGEING

Ageing is characterized by the progressive decay of biological functions due to: 1) mitochondrial dysfunction, 2) loss of proteostasis, 3) telomere shortening, 4) cellular senescence, 5) genomic instability, 6) stem cell exhaustion, 7) altered intercellular communication (inflammageing), 8) epigenetic alterations, and 9) deregulated nutrient detection (68, 69).

CR and PF promote longevity in mammals and prevent the onset of ageing hallmarks by regulating nutrient-sensing pathways, such as IGF1-AKT-mTOR, GCN2-ATF4- FGF21, AMPK, and sirtuin pathways (68, 70) (**Figure 2**).

Genetic mouse models bearing the growth hormone receptor (Ghr) deletion (Ames mice) or Pit1 inactivating mutation (Snell dwarf mice) (71) have a lifespan longer than that of their siblings (36, 37, 41) and show reduced mTORC1 function, elevated mTORC2 activity (71, 72), low circulating levels of IGF1, elevated insulin sensitivity, low AKT activity, and increased chaperone-mediated autophagy

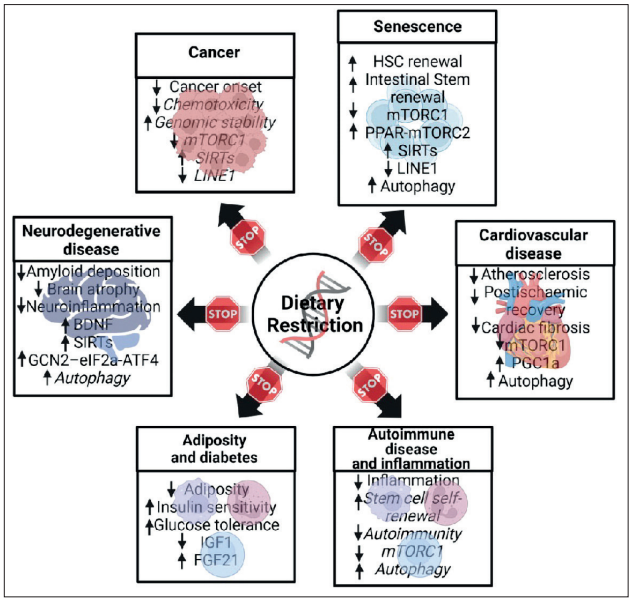

Figure 2. *The beneficial effects of calorie restriction and prolonged fasting on age-related disorders and diseases. CR and PF increase health and lifespan in rodents, non-human primates, and humans by preventing obesity and diabetes, cardiovascular diseases, cancer, senescence, autoimmune and inflammatory conditions (created with BioRender.com).*

compared to control mice (55). In these mouse models, mTORC1 inhibition and PPAR signaling pathway activation promote hematopoietic and intestinal stem cell renewal and regeneration, and reverse age-associated immune senescence and myelosuppression (**Figure 2**).

The GCN2-eIF2-ATF4 pathway could improve cellular health and preserve cellular and tissue homeostasis by modulating protein quality control mechanisms that remove abnormal proteins. This mechanism ensures cellular functional integrity by preventing progressive deterioration of physiological function with ageing (73).

FGF21 is a potent factor that mediates the beneficial effects of CR and PF on ageing and longevity in rodents. FGF21 treatment or transgenic overexpression extends the lifespan of mice and improves glucose tolerance and insulin sensitivity independently of calorie intake and mTORC1 activity (74-76).

Elevated levels of SIRTs increase longevity in normal mice, extend the lifespan of progeroid mice (77, 78), and improve the regenerative capacity of hematopoietic and intestinal stem cells (*79*). Epigenetic remodeling induced by SIRTs deacetylase activity enhances the anti-stress response, improves DNA double-strand repair through PARP activation (47, 80, 81), and represses LINE1 element transposition. These remodeling activities reduce

oxidative damage, increase DNA stability, prevent telomere shortening, inhibit cellular senescence, and decrease inflammation (82) (**Figure 2**).

Activation of PGC1α, mediated by SIRT1 and AMPK, increases mitochondrial biogenesis and respiration, reduces oxidative stress, and improves cellular function and health. Furthermore, SIRT1-dependent upregulation of brain-derived neurotrophic factor (BDNF) ameliorates cognitive function in mice (83).

CR- and PF-mediated modulation of nutrient-sensing signaling pathways leads to the activation of autophagy.

Autophagy suppresses inflammatory cytokine secretion by inhibiting ROS-mediated activation of the NLRP3 inflammasome. The p62-mediated autophagosomal degradation of Keap-1 releases the transcription factor NRF2 from its inhibitor and leads to the transcription of antioxidant genes, such as catalase and superoxide dismutase (SOD), which block ROS activity and enhance the cellular anti-stress response (84, 85). Autophagy prevents immune system exhaustion and senescence by attenuating inflammation and oxidative stress (**Figure 2**).

CR and PF protect against myocardial infarction and prevent the onset and progression of neurodegenerative diseases such as Alzheimer disease and Parkinson disease in mouse models via AMPK-mTOR-mediated activation of autophagy (86-88).

CR and PF elicit different ageing outcomes, even though they regulate several common pathways and mechanisms. While CR increases both the mean and maximum lifespan in rodents by up to 50%, PF only improves the median lifespan by 11% without affecting the maximum lifespan (9).

Although CR has proven to be a promising intervention to attenuate age-related decline in rodents, studies on non-human primates have produced controversial and debatable results. In a study conducted by the University of Wisconsin, 30% calorie restriction extended lifespan and healthspan in rhesus monkeys (89, 90), while a similar study at the National Institute on Aging (NIA) demonstrated that CR improves metabolic parameters without extending lifespan (91). However, it must be taken into consideration that in the NIA study, the average lifespan of monkeys was also very high (31.8 years); thus, this aspect may not have allowed the benefits of CR to be detected (92).

CR AND PF IN CANCER PREVENTION AND TREATMENT

Tumor cells contain genetic and epigenetic alterations that reprogram cellular metabolism towards enhanced glycolysis (Warburg effect) to meet the high energy demand to support their uncontrolled cellular proliferation. The high glycolytic and proliferative activity of tumor cells leads to acidification via lactate production and nutrient depletion in the tumor microenvironment.

Tumor cells release growth factors, cytokines, and chemokines and recruit immune cells and stromal cells to hijack their metabolism and transcriptional activity to constrain their antitumor capacity (93).

CR and PF prevent the onset of tumors by slowing tumor progression and improve the efficacy of anticancer therapy by remodeling the tumor micro-environment (TME) and promoting anti-tumor immune responses even in non-immunogenic tumors (93-103).

During CR and PF, glucose levels are reduced, thereby limiting the primary energy source of the tumor. Fasting leads to inhibition of the IGF1-PI3K-mTOR pathway, which is often altered in tumor cells. The metabolic switch from glycolysis to oxidative phosphorylation compromises the tumor growth, reduces tumor plasticity and causes increased vulnerability towards chemotherapies and molecularly targeted therapies (94, 96, 99, 104-110).

These metabolic and hormonal changes also affect the composition of the immune infiltrate and priming and activity of the immune system.

Low levels of IGF1 and inhibition of the stress-responsive enzyme heme oxygenase 1 (HO1) reduce immunosuppressive T cells and MDSCs in the TME. Fasting conditions increase fatty acid beta-oxidation, oxidative phosphorylation, short-chain fatty acid (SCFA) release, and reduced methionine levels in the TME. These changes in metabolism and metabolites reshape the epigenetic landscape of T cells leading to the activation of signaling pathways involved in stemness and secondary immune response (93, 96, 111-116).

In mouse models, CR and PF not only improve the antitumor response but also exert a protective effect against the adverse effects of chemotherapy and immunotherapy. CR and PF reverse chemotherapy-induced immunosuppression by promoting hematopoietic stem cell (HSC) self-renewal, while preventing immune-related cardiotoxicity of

checkpoint inhibitors by reducing T-cell infiltration into the heart and levels of inflammatory markers, such as the NLRP3 inflammasome and leukotrienes (9, 97, 98, 100, 117, 118).

To date, clinical studies evaluating the effects of CR and PF on cancer patients are few, controversial, and have been conducted on small cohorts of patients.

However, the reduced cancer rates of Okinawan people, who eat a low-calorie, antioxidant-rich diet of mostly vegetables, fruit, fish, and seafood (119, 120), might suggest that CR could have the same antineoplastic potential found in mouse models. Furthermore, recent clinical studies conducted on a limited number of cancer patients have shown that PF is safe and well tolerated, modulates the immune system and improves the quality of life by reducing the adverse effects of chemotherapy (98, 100-102, 121-123).

However, to confirm the benefits of CR and PF on tumor prevention and antitumor response in humans, new double-blind clinical trials in larger cohorts of patients with cancer are urgently needed.

TRANSLATABILITY OF CR AND PF EFFECTS FROM RODENTS TO HUMAN: LIMITS AND CONSIDERATIONS

Caloric restriction and periodic fasting have multiple effects on the health and lifespan of rodents; however, are these benefits transferable from mice to humans?

Although mice and humans share a high degree of genomic, anatomical, and physiological similarities, they differ in physiology, morphometric parameters, and evolutionary biology. Humans are approximately 3,000 times the size of mice, live 30 times longer than mice, and have a basal metabolic rate per gram of body weight (mass-specific metabolic rate) seven times lower than that of mice (**Figure 3**). A high mass-specific metabolic rate in mice implies high production of free radicals, an increase in oxidative damage, and enhancement of cellular senescence. This difference in metabolic rate could translate into conflicting effects on ageing and tumorigenesis in these two species (124-126) (**Figure 3**).

Mice and humans age at different rates, implying that the ageing mechanism is different between the two species. The decline in physiological functions associated with ageing depends on metabolic stability, that is, the cellular ability to maintain metabolic homeostasis in response to external stresses (127, 128).

Humans have higher metabolic stability and maximum potential lifespan than mice, which are susceptible to significant metabolic alterations in response to external stress (129).

In mice, one day of fasting causes profound changes in circulating levels of metabolites (glucose, amino acids, lipids, ketone bodies), hormones (thyroid, insulin, adiponectin), and inflammatory factors (C-reactive proteins, cytokines, and chemokines), and consequently, lowers body temperature and transiently lowers metabolism. One-day fasting also implies strong inhibition of endocrine hormone

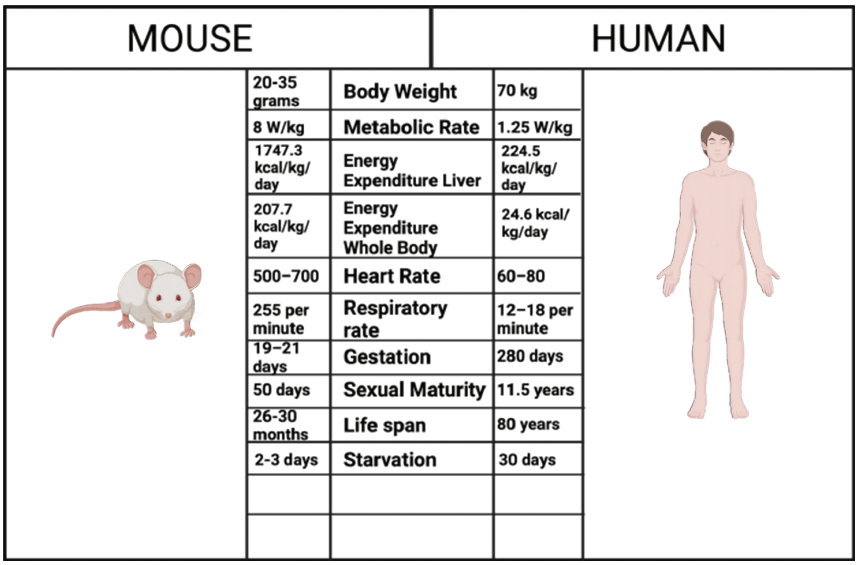

Figure 3. *Physiological properties for mouse (low metabolic stability) and human (strong metabolic stability) (created with BioRender.com).*

signaling pathways (130-134) and nutrient-sensing systems (135).

In some mouse strains, the glucose and thyroid hormone T3 levels are drastically reduced after 24 h of fasting, causing pathological and life-threatening conditions such as hypoglycemia, hypothyroidism, and hypothermia (132-134). In fact, 48 h of fasting can be lethal for some mouse strains because of their genetic and epigenetic features (2). In humans, 24-hour fasting has negligible to mild effects on glucose and T3 levels and must be extended for several days to achieve metabolic outcomes similar to those in mice (136, 137). The release of KB rises within a few hours in mice until it reaches millimolar concentrations after 24 h, while in humans, KB production increases after 24 hours (0.2-0.5 mM) and reaches millimolar concentrations after 48 h (134, 135). Therefore, the timescales of the physiological processes in rodents and humans are extremely different. Laboratory mice have an average lifespan of 2 years, whereas humans live on average up to 80 years. Thus, 24 h of fasting in mice corresponds to at least 5 days of fasting in humans (2) (**Figure 3**).

These fasting-induced metabolic changes lead to weight loss of at least 20% in mice over a short period of time. Caloric restriction of 20-30% reduces the body weight of mice by up to 20% within a few weeks, while fasting causes a 20% drop in body weight in 2-3 days. In humans, CR or PF cause weight loss of up to 10% continuously and gradually over a few months (9).

Therefore, the adaptation of cellular metabolism to food reduction or deprivation follows completely different dynamics in mice and humans. Human cellular metabolism is much more resistant to stress than murine cellular metabolism, and the human metabolic network and increased energy reserves confer greater metabolic stability during fasting, while minimizing systemic changes. Thus, it is possible that CR and PF may have only minimal effects on the metabolic stability of humans, but could have significant effects on the metabolic stability of mice, leading to an extension of lifespan (138).

The differences in metabolic rates between the two species could influence the pathogenesis of diseases, such as cancer susceptibility. If tumor genesis had been similar in mice and humans, we would expect to have a higher tumor incidence in human than in mice, given that humans live 30 times longer than mice. As the lifetime tumor incidence is similar between humans and mice, cancer development and progression follow different patterns in the two species (139, 140). In mice, genetic tumor alterations occur within a time window of 6 to 18 months and increase exponentially with age, whereas in humans, the multistep process of carcinogenesis takes several years to occur, begins at 40 years of age, and ceases at age 80 (141, 142). Approximately 30% of laboratory mice develop cancer during their lifetime, whereas in humans, this percentage is reached only after the age of 70 years (143).

CLINICAL TRIAL OUTCOMES OF THE CR AND PF EFFECTS ON HUMAN HEALTH

Given the large metabolic and physiological differences between the two species, it is uncertain whether such dietary interventions can have the same benefits as those found in rodents. So far, clinical studies aimed at evaluating the benefits of these dietary interventions in humans remain scarce and controversial. The main limitations of these human clinical studies are: 1) low adherence to diet; 2) the short duration of the study; 3) the limited number of participants; 4) enrolled participants are mostly overweight or obese (9, 144-146).

The compliance of obese and non-obese individuals to CR and PR interventions is low and represents the main challenge to overcome (15, 147). In the CALERIE-2 study, conducted on normal-weight healthy people to test the effect of CR on health and markers of longevity, adherence to 18% CR decreased significantly after 20 weeks, and the drop in body weight was significant in people who adhered to such dietary interventions for a long period (148). In the CALERIE clinical trial, participants who adhered to 18% CR for 6 months, followed by 10% CR for the remaining 18 months, demonstrated an average 8% reduction in body weight. Weight loss is far from the 20% observed in mice, and as such, the overall health impacts are less pronounced.

CALERIE clinical trials showed that participants who adhered to 11% CR over 2 years demonstrated a reduction in only some age-related cardiovascular and metabolic disease risk factors and improved only some longevity markers; therefore, it did not have the same impact on humans as rodents. Furthermore, this study found that CR reduced lean mass in young and older adults, muscle strength in older groups, and bone mass in some areas (149).

In the Minnesota Starvation Experiment, which was designed to assess the physiological effects of se-

vere and prolonged dietary restriction, participants following a 40% CR for 6 months lost 25% of their weight and developed severe socio-behavioral changes (*e.g.*, depression and chronic fatigue) (150). In an experiment conducted in Biosphere 2, eight participants subjected to a 20% CR for 2 years lost approximately 15% of their body weight and improved their metabolic profile. However, many participants experienced severe adverse events and psychological changes such as hunger, tiredness, mental confusion, licking of every dish rather obsessively, elaborate eating rituals, and depression (151).

Contrary to what has been observed in mice (152), prolonged CR or PF significantly reduced circulating levels of the neuroprotective factor BDNF in humans (153, 154), confirming that the mechanisms of adaptation to fasting in rodents and humans are vastly different.

It is necessary to assess whether the CR or PF metabolic and physiological benefits are extended and maintained after the end of the dietary intervention or reversed, or even worsened, when the participant returns to a normal diet.

At the 6-month follow-up of the Biosphera2 study, the participants' weight recovery was exclusively due to an increase in body fat reserves. Their lean mass did not change, while reduced energy expenditure due to low spontaneous physical activity could subsequently promote weight gain and obesity (151). The same phenomenon also occurred in famine victims and emaciated prisoners of World War II (155, 156), in patients with anorexia nervosa (157), cancer (158), and in subjects during the refeeding period of the Minnesota experiment (150, 159).

Therefore, such dietary interventions cannot be pursued for a long period of time in humans and are not recommended for lean people, minors, very elderly people, pregnant or breastfeeding women, anorexic people, those with low bone density, and patients affected by specific diseases, as they could cause psychological stress, depression, and a harmful impact on mental and physical health (160).

For instance, advanced-stage cancer patients already have high catabolism, are at risk of cachexia, and would not be able to tolerate such stringent regimens. Furthermore, in such patients, the lack of appetite and reduced absorption of nutrients should already activate the mechanisms regulated by CR and PF involved in enhancing the effectiveness of chemotherapies, targeted molecular therapies, and immunotherapies. In any case, a nutrient supplement is necessary for patients with cancer to avoid worsening their physical conditions. Correct nutrition during therapy and at the end of treatment is an aspect to be considered, which has only been receiving the right and necessary attention in recent years (161).

Furthermore, nutritional restriction or deprivation impairs immune function by suppressing the mTOR signaling pathway and makes mice more susceptible to viral infections (162, 163). Although the reduction in circulating leukocytes during fasting is much more marked in mice than in humans (*9, 96,* 100), such dietary interventions may not be suitable for immunosuppressed cancer patients, as they are more exposed to infections and experience serious complications that could lead to death (36, 164-166).

However, small clinical studies have shown that fasting for a few days is feasible and safe in patients with HER2 negative breast cancer, gynecological cancers, advanced stage cancers undergoing chemotherapy, suggesting that fasting can reduce toxicity and modulate the immune system (94, 98, 100-102, 122). Although these data are encouraging, it is not possible to draw conclusions on the effectiveness of these interventions because these studies were conducted on a limited number of cancer patients with various forms of cancer. The main objective of these studies was to evaluate the feasibility of the intervention and did not consider tumor stratification and different treatment strategies. Future studies are needed to determine whether immune system activation in cancer patients during fasting is indicative of an enhanced antitumor response or a stress condition associated with increased CRP. In fact, fasting-induced modulation of the immune system occurs even in healthy individuals and fades when oncologic patients return to their normal diet. Therefore, the activation of the immune system in oncologic patients may not be associated with a specific antitumor response, but could be a stress-induced effect.

Adopting an appropriate lifestyle, based on a balanced diet combined with physical exercise, plays a fundamental role in decreasing the onset and development of the most common tumors in Western countries. It has been shown that following a diet based on the Mediterranean model helps prevent the risk of tumors. In general, a diet rich in vegetables (fruit and vegetables), whole grains, and legumes and low in animal fats and meat constitutes a protective factor against the onset of tumors.

Overweight or obese people certainly tolerate these dietary interventions for longer periods and benefit from both weight loss and improvements in cardiometabolic markers (15, 153, 167). However, it is unclear whether the beneficial effects of these dietary interventions on cardiometabolic health are mediated by weight loss, the modulation of nutrient-sensing pathways, or dietary components. Several studies have shown that weight loss mainly depends on caloric intake. CR and PF cause different effects even if they act on the same signaling pathways and the composition of the diet influences cardiometabolic health and long-term metabolomic reprogramming in mice (147, 168-170). In patients with thyroiditis (inflammation of the thyroid due to an autoimmune pathology), a balanced, carbohydrate-free diet (bread, pasta, fruit, and rice) reduces autoimmune antibodies (antithyroid, antimicrosomal, and antiperoxidase antibodies), body weight, body mass index, and fat mass by inhibiting the translocation of carbohydrate-responsive element-binding protein (ChREBP) into the nucleus and consequently the transcription of genes involved in lipogenesis and autoantibody production (171).

Recent work has shown that isocaloric CR and PF (25% net calorie restriction), tested on lean subjects for 2 years, reduced body weight equally; however, PF causes greater loss of lean mass than CR, probably due to specific losses affecting skeletal muscle. Furthermore, the positive effects of PF on metabolic regulation or cardiovascular health are essentially due to reduced caloric intake and not to mechanisms regulated by fasting, as PF without energy restriction is less effective in reducing body fat mass and improving metabolic parameters or cardiovascular health (147). In addition, CR provides greater protection against tumor growth and lung metastasis compared to PF in the tumor xenograft mouse model, by enhancing the immune response (170).

Collectively, CR and PF could improve metabolic and cardiovascular health and have antineoplastic potential also in humans, however it is still early to draw conclusions in the absence of double-arm randomized clinical trials performed on a statistically significant sample (172).

FOOD DRUG INTERACTION: WARNING AND PRECAUTIONS

The composition of food can be a factor capable of interacting pharmacodynamically with chemotherapeutics. Food can influence some oral drugs that must be taken between meals, while others have only a mild effect on others. Foods rich in fats or proteins can significantly increase the absorption of the drug at the intestinal level and therefore its bioavailability, whereas foods with a high fiber content can alter the bioavailability of oral chemotherapy drugs owing to the link between the drugs and the fiber. Liquids accelerate passage through the stomach, thereby reducing the time interval between drug administration and the onset of its effects (173, 174). Grapefruit, cranberry, and cranberry juice must not be taken together with chemotherapy because they enhance the toxic effects of chemotherapy by increasing the concentration of the drug in the blood. Garlic sensitizes prostatic tumor cells to chemotherapy because biologically active garlic components interact with chemotherapy and suppress the growth of human prostate cancer cells (175).

Therefore, it is particularly important to respect the prescribed methods of administering oral chemotherapy drugs with respect to meals.

It should not be underestimated that cancer patients often consume complementary medicinal products, such as multivitamin or mineral salt supplements, to replenish nutritional deficiencies due to reduced food intake. Even the most popular nutritional supplements should be consumed after careful evaluation of their effectiveness.

Fish oil and its main components, omega 3 fatty acids, are commonly used as supplements for cancer patients. They can enhance the effects of chemotherapy while simultaneously reducing toxicity to healthy tissues and systemic inflammation. Instead, Vitamin D has poor antitumor activity and potential toxic effects; therefore, indiscriminate supplementation (*i.e.*, without an actual need) of vitamin D, alone or in combination with standard treatments, must be avoided (175).

Many cancer patients consume dietary supplements, vitamins, minerals, and herbal products along with cancer treatments because they perceive them to be anticancer and antitoxic agents.

Herbal remedies have clinical implications, with a potential effect on drug metabolism. In fact, herbal products are not subjected to rigorous scientific investigations to evaluate their effectiveness, tolerability, or quality control. Furthermore, they are rarely sold with a contraindication leaflet because they are not required by law; therefore, it is very important to pay attention to their consumption.

St. John's wort, most often used to treat depression, can modify hepatic metabolism and reduce chemotherapy efficacy. Ginseng, used by cancer patients for its alleged anti-neoplastic properties, increases the concentration of chemotherapeutic drugs in circulation and enhances their toxic effects. Green tea, known for its antioxidant and anti-inflammatory properties, exerts an antagonistic action with some chemotherapeutic drugs; therefore, the use of this drink in combination with therapy should be avoided. The consumption of Aloe Vera-based products reduces the absorption and effectiveness of the drugs as they have a laxative effect (175).
For these reasons, supplements and other substances with pharmacological effects should be taken under the supervision of a specialist in the sector because they can interact with chemotherapeutic drugs and affect their efficacy and toxicity.

CONCLUSION

In recent years, preclinical studies have shown that CR and PF prevent the development of diseases associated with ageing (cardiovascular, neurodegenerative, and cancer) and prolong life extension by improving metabolic and physiological profiles. Although these results are promising, it is too early to establish whether the broad health benefits of these dietary interventions could be translated from rodents to humans.
To date, few clinical studies have been conducted on small cohorts, in most cases including overweight or obese individuals, for short periods of time. Although these dietary interventions also appear promising in humans, new clinical trials need to be implemented to establish long-term benefits and safety in a large-scale population.

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Plagiarism

The article provides a comprehensive review of the latest studies in the field, with accurate citations.

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REFERENCES

1. Cardona C, Bishai D. The slowing pace of life expectancy gains since 1950. BMC Public Health. 2018;18(1):151. doi: 10.1186/s12889-018-5058-9.
2. World Health Organization. Decade of healthy ageing: baseline report. World Health Organization. (2021).
3. Green CL, Lamming DW, Fontana L. Molecular mechanisms of dietary restriction promoting health and longevity. Nat Rev Mol Cell Biol. 2022;23(1):56-73. doi: 10.1038/s41580-021-00411-4.
4. Fontana L. The scientific basis of caloric restriction leading to longer life. Curr Opin Gastroenterol. 2009;25(2):144-50. doi: 10.1097/MOG.0b013e32831ef1ba.
5. Scholtens EL, Krebs JD, Corley BT, Hall RM. Intermittent fasting 5:2 diet: What is the macronutrient and micronutrient intake and composition?

Clin Nutr. 2020;39(11):3354-60. doi: 10.1016/j.clnu.2020.02.022.
6. Hatori M, Vollmers C, Zarrinpar A, DiTacchio L, Bushong EA, Gill S, et al. Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. Cell Metab. 2012;15(6):848-60. doi: 10.1016/j.cmet.2012.04.019.
7. Gabel K, Kroeger CM, Trepanowski JF, Hoddy KK, Cienfuegos S, Kalam F, et al. Differential Effects of Alternate-Day Fasting Versus Daily Calorie Restriction on Insulin Resistance. Obesity (Silver Spring). 2019;27(9):1443-50. doi: 10.1002/oby.22564.
8. Regmi P, Heilbronn LK. Time-Restricted Eating: Benefits, Mechanisms, and Challenges in Translation. iScience. 2020;23(6):101161. doi: 10.1016/j.isci.2020.101161.
9. 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 Metab. 2015 Jul 7;22(1):86-99. doi: 10.1016/j.cmet.2015.05.012.
10. Choi IY, Piccio L, Childress P, Bollman B, Ghosh A, Brandhorst S, et al. A Diet Mimicking Fasting Promotes Regeneration and Reduces Autoimmunity and Multiple Sclerosis Symptoms. Cell Rep. 2016;15(10):2136-46. doi: 10.1016/j.celrep.2016.05.009.
11. 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. Sci Transl Med. 2017 Feb 15;9(377):eaai8700. doi: 10.1126/scitranslmed.aai8700.
12. Mitchell SJ, Madrigal-Matute J, Scheibye-Knudsen M, Fang E, Aon M, González-Reyes JA, et al. Effects of Sex, Strain, and Energy Intake on Hallmarks of Aging in Mice. Cell Metab. 2016;23(6):1093-112. doi: 10.1016/j.cmet.2016.05.027.
13. Acosta-Rodríguez VA, de Groot MHM, Rijo-Ferreira F, Green CB, Takahashi JS. Mice under Caloric Restriction Self-Impose a Temporal Restriction of Food Intake as Revealed by an Automated Feeder System. Cell Metab. 2017;26(1):267-77.e2. doi: 10.1016/j.cmet.2017.06.007.
14. Redman LM, Ravussin E. Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes. Antioxid Redox Signal. 2011;14(2):275-87. doi: 10.1089/ars.2010.3253.
15. Trepanowski JF, Kroeger CM, Barnosky A, Klempel MC, Bhutani S, Hoddy KK, et al. Effect of Alternate-Day Fasting on Weight Loss, Weight Maintenance, and Cardioprotection Among Metabolically Healthy Obese Adults: A Randomized Clinical Trial. JAMA Intern Med. 2017;177(7):930-8. doi: 10.1001/jamainternmed.2017.0936.
16. Most J, Tosti V, Redman LM, Fontana L. Calorie restriction in humans: An update. Ageing Res Rev. 2017;39:36-45. doi: 10.1016/j.arr.2016.08.005.
17. Stekovic S, Hofer SJ, Tripolt N, Aon MA, Royer P, Pein L, et al. Alternate Day Fasting Improves Physiological and Molecular Markers of Aging in Healthy, Non-obese Humans. Cell Metab. 2019;30(3):462-76.e6. doi: 10.1016/j.cmet.2019.07.016.
18. Steinhauser ML, Olenchock BA, O'Keefe J, Lun M, Pierce KA, Lee H, et al. The circulating metabolome of human starvation. JCI Insight. 2018;3(16):e121434. doi: 10.1172/jci.insight.121434.
19. Cahill GF Jr. Fuel metabolism in starvation. Annu Rev Nutr. 2006;26:1-22. doi: 10.1146/annurev.nutr.26.061505.111258.
20. Nencioni A, Caffa I, Cortellino S, Longo VD. Fasting and cancer: molecular mechanisms and clinical application. Nat Rev Cancer. 2018;18(11):707-19. doi: 10.1038/s41568-018-0061-0.
21. Guzmán M, Blázquez C. Ketone body synthesis in the brain: possible neuroprotective effects. Prostaglandins Leukot Essent Fatty Acids. 2004;70(3):287-92. doi: 10.1016/j.plefa.2003.05.001.
22. Müller TD, Nogueiras R, Andermann ML, Andrews ZB, Anker SD, Argente J, et al. Ghrelin. Mol Metab. 2015;4(6):437-60. doi: 10.1016/j.molmet.2015.03.005.
23. Geng L, Lam KSL, Xu A. The therapeutic potential of FGF21 in metabolic diseases: from bench to clinic. Nat Rev Endocrinol. 2020;16(11):654-67. doi: 10.1038/s41574-020-0386-0.
24. Collet TH, Sonoyama T, Henning E, Keogh JM, Ingram B, Kelway S, et al. A Metabolomic Signature of Acute Caloric Restriction. J Clin Endocrinol Metab. 2017;102(12):4486-95. doi: 10.1210/jc.2017-01020.
25. Anton SD, Moehl K, Donahoo WT, Marosi K, Lee SA, Mainous AG 3rd, et al. Flipping the Metabolic Switch: Understanding and Applying the Health Benefits of Fasting. Obesity (Silver Spring). 2018;26(2):254-68. doi: 10.1002/oby.22065.
26. Soare A, Cangemi R, Omodei D, Holloszy JO, Fontana L. Long-term calorie restriction, but not en-

durance exercise, lowers core body temperature in humans. Aging (Albany NY). 2011;3(4):374-9. doi: 10.18632/aging.100280.
27. Fontana L, Villareal DT, Das SK, Smith SR, Meydani SN, Pittas AG, et al. Effects of 2-year calorie restriction on circulating levels of IGF-1, IGF-binding proteins and cortisol in nonobese men and women: a randomized clinical trial. Aging Cell. 2016 Feb;15(1):22-7. doi: 10.1111/acel.12400.
28. Redman LM, Smith SR, Burton JH, Martin CK, Il'yasova D, Ravussin E. Metabolic Slowing and Reduced Oxidative Damage with Sustained Caloric Restriction Support the Rate of Living and Oxidative Damage Theories of Aging. Cell Metab. 2018;27(4):805-15.e4. doi: 10.1016/j.cmet.2018.02.019.
29. Guijas C, Montenegro-Burke JR, Cintron-Colon R, Domingo-Almenara X, Sanchez-Alavez M, Aguirre CA, et al. Metabolic adaptation to calorie restriction. Sci Signal. 2020;13(648):eabb2490. doi: 10.1126/scisignal.abb2490.
30. Durack J, Lynch SV. The gut microbiome: Relationships with disease and opportunities for therapy. J Exp Med. 2019;216(1):20-40. doi: 10.1084/jem.20180448.
31. Zheng X, Wang S, Jia W. Calorie restriction and its impact on gut microbial composition and global metabolism. Front Med. 2018;12(6):634-44. doi: 10.1007/s11684-018-0670-8.
32. Tanca A, Abbondio M, Palomba A, Fraumene C, Marongiu F, Serra M, et al. Caloric restriction promotes functional changes involving short-chain fatty acid biosynthesis in the rat gut microbiota. Sci Rep. 2018;8(1):14778. doi: 10.1038/s41598-018-33100-y.
33. Mesnage R, Grundler F, Schwiertz A, Le Maho Y, Wilhelmi de Toledo F. Changes in human gut microbiota composition are linked to the energy metabolic switch during 10 d of Buchinger fasting. J Nutr Sci. 2019;8:e36. doi: 10.1017/jns.2019.33.
34. Wang S, Huang M, You X, Zhao J, Chen L, Wang L, et al. Gut microbiota mediates the anti-obesity effect of calorie restriction in mice. Sci Rep. 2018;8(1):13037. doi: 10.1038/s41598-018-31353-1.
35. Grajeda-Iglesias C, Durand S, Daillère R, Iribarren K, Lemaitre F, Derosa L, et al. Oral administration of Akkermansia muciniphila elevates systemic antiaging and anticancer metabolites. Aging (Albany NY). 2021;13(5):6375-405. doi: 10.18632/aging.202739.
36. Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, et al. Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. Science. 2012;335(6076):1638-43. doi: 10.1126/science.1215135.
37. Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, et al. Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. Science. 2009;326(5949):140-4. doi: 10.1126/science.1177221. Erratum in: Science. 2011 Oct 7;334(6052):39.
38. Yu D, Tomasiewicz JL, Yang SE, Miller BR, Wakai MH, Sherman DS, et al. Calorie-Restriction-Induced Insulin Sensitivity Is Mediated by Adipose mTORC2 and Not Required for Lifespan Extension. Cell Rep. 2019;29(1):236-48.e3. doi: 10.1016/j.celrep.2019.08.084.
39. Chellappa K, Brinkman JA, Mukherjee S, Morrison M, Alotaibi MI, Carbajal KA, et al. Hypothalamic mTORC2 is essential for metabolic health and longevity. Aging Cell. 2019;18(5):e13014. doi: 10.1111/acel.13014.
40. Lamming DW, Mihaylova MM, Katajisto P, Baar EL, Yilmaz OH, Hutchins A, et al. Depletion of Rictor, an essential protein component of mTORC2, decreases male lifespan. Aging Cell. 2014;13(5):911-7. doi: 10.1111/acel.12256.
41. Wu JJ, Liu J, Chen EB, Wang JJ, Cao L, Narayan N, et al. Increased mammalian lifespan and a segmental and tissue-specific slowing of aging after genetic reduction of mTOR expression. Cell Rep. 2013;4(5):913-20. doi: 10.1016/j.celrep.2013.07.030.
42. Gonzalez JT, Dirks ML, Holwerda AM, Kouw IWK, van Loon LJC. Intermittent versus continuous enteral nutrition attenuates increases in insulin and leptin during short-term bed rest. Eur J Appl Physiol. 2020;120(9):2083-94. doi: 10.1007/s00421-020-04431-4.
43. Cantó C, Auwerx J. Caloric restriction, SIRT1 and longevity. Trends Endocrinol Metab. 2009;20(7):325-31. doi: 10.1016/j.tem.2009.03.008.
44. Fulco M, Cen Y, Zhao P, Hoffman EP, McBurney MW, Sauve AA, et al. Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. Dev Cell. 2008;14(5):661-73. doi: 10.1016/j.devcel.2008.02.004.
45. Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. Annu Rev

Pathol. 2010;5:253-95. doi: 10.1146/annurev.pathol.4.110807.092250.
46. Eldridge MJG, Pereira JM, Impens F, Hamon MA. Active nuclear import of the deacetylase Sirtuin-2 is controlled by its C-terminus and importins. Sci Rep. 2020;10(1):2034. doi: 10.1038/s41598-020-58397-6.
47. Haigis MC, Mostoslavsky R, Haigis KM, Fahie K, Christodoulou DC, Murphy AJ, et al. SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. Cell. 2006;126(5):941-54. doi: 10.1016/j.cell.2006.06.057.
48. Mao Z, Hine C, Tian X, Van Meter M, Au M, Vaidya A, et al. SIRT6 promotes DNA repair under stress by activating PARP1. Science. 2011;332(6036):1443-6. doi: 10.1126/science.1202723.
49. Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, et al. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. Science. 2011;334(6057):806-9. doi: 10.1126/science.1207861.
50. Peng C, Lu Z, Xie Z, Cheng Z, Chen Y, Tan M, et al. The first identification of lysine malonylation substrates and its regulatory enzyme. Mol Cell Proteomics. 2011;10(12):M111.012658. doi: 10.1074/mcp.M111.012658.
51. Lin J, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. Cell Metab. 2005;1(6):361-70. doi: 10.1016/j.cmet.2005.05.004.
52. Hansen M, Rubinsztein DC, Walker DW. Autophagy as a promoter of longevity: insights from model organisms. Nat Rev Mol Cell Biol. 2018;19(9):579-93. doi: 10.1038/s41580-018-0033-y. Erratum in: Nat Rev Mol Cell Biol.
53. Yang L, Licastro D, Cava E, Veronese N, Spelta F, Rizza W, et al. Long-Term Calorie Restriction Enhances Cellular Quality-Control Processes in Human Skeletal Muscle. Cell Rep. 2016;14(3):422-8. doi: 10.1016/j.celrep.2015.12.042.
54. Hetz C, Zhang K, Kaufman RJ. Mechanisms, regulation and functions of the unfolded protein response. Nat Rev Mol Cell Biol. 2020;21(8):421-38. doi: 10.1038/s41580-020-0250-z.
55. Endicott SJ, Boynton DN Jr, Beckmann LJ, Miller RA. Long-lived mice with reduced growth hormone signaling have a constitutive upregulation of hepatic chaperone-mediated autophagy. Autophagy. 2021;17(3):612-25. doi: 10.1080/15548627.2020.1725378.
56. Pietrocola F, Demont Y, Castoldi F, Enot D, Durand S, Semeraro M, et al. Metabolic effects of fasting on human and mouse blood in vivo. Autophagy. 2017;13(3):567-78. doi: 10.1080/15548627.2016.1271513.
57. Bunpo P, Cundiff JK, Reinert RB, Wek RC, Aldrich CJ, Anthony TG. The eIF2 kinase GCN2 is essential for the murine immune system to adapt to amino acid deprivation by asparaginase. J Nutr. 2010;140(11):2020-7. doi: 10.3945/jn.110.129197.
58. Ravindran R, Loebbermann J, Nakaya HI, Khan N, Ma H, Gama L, et al. The amino acid sensor GCN2 controls gut inflammation by inhibiting inflammasome activation. Nature. 2016;531(7595):523-7. doi: 10.1038/nature17186.
59. Wek SA, Zhu S, Wek RC. The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. Mol Cell Biol. 1995;15(8):4497-506. doi: 10.1128/MCB.15.8.4497.
60. Dong J, Qiu H, Garcia-Barrio M, Anderson J, Hinnebusch AG. Uncharged tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding domain. Mol Cell. 2000;6(2):269-79. doi: 10.1016/s1097-2765(00)00028-9.
61. Harding HP, Ordonez A, Allen F, Parts L, Inglis AJ, Williams RL, et al. The ribosomal P-stalk couples amino acid starvation to GCN2 activation in mammalian cells. Elife. 2019 Nov;8:e50149. doi: 10.7554/eLife.50149.
62. Dever TE, Feng L, Wek RC, Cigan AM, Donahue TF, Hinnebusch AG. Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. Cell. 1992;68(3):585-96. doi: 10.1016/0092-8674(92)90193-g.
63. Averous J, Lambert-Langlais S, Mesclon F, Carraro V, Parry L, Jousse C, et al. GCN2 contributes to mTORC1 inhibition by leucine deprivation through an ATF4 independent mechanism. Sci Rep. 2016 Jun 14;6:27698. doi: 10.1038/srep27698.
64. Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, et al. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell. 2000;6(5):1099-108. doi: 10.1016/s1097-2765(00)00108-8.
65. Fontana L, Partridge L. Promoting health and longevity through diet: from model organ-

isms to humans. Cell. 2015;161(1):106-18. doi: 10.1016/j.cell.2015.02.020.
66. Ruan HB, Crawford PA. Ketone bodies as epigenetic modifiers. Curr Opin Clin Nutr Metab Care. 2018;21(4):260-6. doi: 10.1097/MCO.0000000000000475.
67. Spigoni V, Cinquegrani G, Iannozzi NT, Frigeri G, Maggiolo G, Maggi M, et al. Activation of G protein-coupled receptors by ketone bodies: Clinical implication of the ketogenic diet in metabolic disorders. Front Endocrinol (Lausanne). 2022;13:972890. doi: 10.3389/fendo.2022.972890.
68. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013;153(6):1194-217. doi: 10.1016/j.cell.2013.05.039.
69. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: An expanding universe. Cell. 2023;186(2):243-78. doi: 10.1016/j.cell.2022.11.001.
70. Longo VD, Cortellino S. Fasting, dietary restriction, and immunosenescence. J Allergy Clin Immunol. 2020;146(5):1002-4. doi: 10.1016/j.jaci.2020.07.035.
71. Dominick G, Berryman DE, List EO, Kopchick JJ, Li X, Miller RA, et al. Regulation of mTOR activity in Snell dwarf and GH receptor gene-disrupted mice. Endocrinology. 2015;156(2):565-75. doi: 10.1210/en.2014-1690.
72. Garratt M, Nakagawa S, Simons MJP. Life-span Extension With Reduced Somatotrophic Signaling: Moderation of Aging Effect by Signal Type, Sex, and Experimental Cohort. J Gerontol A Biol Sci Med Sci. 2017;72(12):1620-6. doi: 10.1093/gerona/glx010.
73. Gallinetti J, Harputlugil E, Mitchell JR. Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. Biochem J. 2013;449(1):1-10. doi: 10.1042/BJ20121098.
74. Laeger T, Henagan TM, Albarado DC, Redman LM, Bray GA, Noland RC, et al. FGF21 is an endocrine signal of protein restriction. J Clin Invest. 2014;124(9):3913-22. doi: 10.1172/JCI74915.
75. Kharitonenkov A, Shiyanova TL, Koester A, Ford AM, Micanovic R, Galbreath EJ, et al. FGF-21 as a novel metabolic regulator. J Clin Invest. 2005;115(6):1627-35. doi: 10.1172/JCI23606.
76. Zhang Y, Xie Y, Berglund ED, Coate KC, He TT, Katafuchi T, et al. The starvation hormone, fibroblast growth factor-21, extends lifespan in mice. Elife. 2012;1:e00065. doi: 10.7554/eLife.00065.
77. North BJ, Rosenberg MA, Jeganathan KB, Hafner AV, Michan S, Dai J, et al. SIRT2 induces the checkpoint kinase BubR1 to increase lifespan. EMBO J. 2014;33(13):1438-53. doi: 10.15252/embj.201386907.
78. Sun S, Qin W, Tang X, Meng Y, Hu W, Zhang S, et al. Vascular endothelium-targeted Sirt7 gene therapy rejuvenates blood vessels and extends life span in a Hutchinson-Gilford progeria model. Sci Adv. 2020;6(8):eaay5556. doi: 10.1126/sciadv.aay5556.
79. Brown K, Xie S, Qiu X, Mohrin M, Shin J, Liu Y, et al. SIRT3 reverses aging-associated degeneration. Cell Rep. 2013;3(2):319-27. doi: 10.1016/j.celrep.2013.01.005.
80. Van Meter M, Simon M, Tombline G, May A, Morello TD, Hubbard BP, et al. JNK Phosphorylates SIRT6 to Stimulate DNA Double-Strand Break Repair in Response to Oxidative Stress by Recruiting PARP1 to DNA Breaks. Cell Rep. 2016;16(10):2641-50. doi: 10.1016/j.celrep.2016.08.006.
81. Chen J, Xie JJ, Jin MY, Gu YT, Wu CC, Guo WJ, et al. Sirt6 overexpression suppresses senescence and apoptosis of nucleus pulposus cells by inducing autophagy in a model of intervertebral disc degeneration. Cell Death Dis. 2018;9(2):56. doi: 10.1038/s41419-017-0085-5.
82. Simon M, Van Meter M, Ablaeva J, Ke Z, Gonzalez RS, Taguchi T, et al. LINE1 Derepression in Aged Wild-Type and SIRT6-Deficient Mice Drives Inflammation. Cell Metab. 2019 Apr 2;29(4):871-885.e5. doi: 10.1016/j.cmet.2019.02.014.
83. Pak HH, Haws SA, Green CL, Koller M, Lavarias MT, Richardson NE, et al. Fasting drives the metabolic, molecular and geroprotective effects of a calorie-restricted diet in mice. Nat Metab. 2021;3(10):1327-41. doi: 10.1038/s42255-021-00466-9.
84. Fan W, Tang Z, Chen D, Moughon D, Ding X, Chen S, et al. Keap1 facilitates p62-mediated ubiquitin aggregate clearance via autophagy. Autophagy. 2010;6(5):614-21. doi: 10.4161/auto.6.5.12189.
85. Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, et al. Extension of murine life span by overexpression of catalase targeted to mitochondria. Science. 2005;308(5730):1909-11. doi: 10.1126/science.1106653.
86. Mattson MP, Longo VD, Harvie M. Impact of intermittent fasting on health and disease pro-

cesses. Ageing Res Rev. 2017;39:46-58. doi: 10.1016/j.arr.2016.10.005.
87. Bokov AF, Garg N, Ikeno Y, Thakur S, Musi N, DeFronzo RA, et al. Does reduced IGF-1R signaling in Igf1r+/- mice alter aging? PLoS One. 2011;6(11):e26891. doi: 10.1371/journal.pone.0026891.
88. Blüher M, Kahn BB, Kahn CR. Extended longevity in mice lacking the insulin receptor in adipose tissue. Science. 2003;299(5606):572-4. doi: 10.1126/science.1078223.
89. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science. 2009;325(5937):201-4. doi: 10.1126/science.1173635.
90. Colman RJ, Beasley TM, Kemnitz JW, Johnson SC, Weindruch R, Anderson RM. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Nat Commun. 2014;5:3557. doi: 10.1038/ncomms4557.
91. Mattison JA, Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL, et al. Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. Nature. 2012;489(7415):318-21. doi: 10.1038/nature11432.
92. Stonebarger GA, Urbanski HF, Woltjer RL, Vaughan KL, Ingram DK, Schultz PL, et al. Amyloidosis increase is not attenuated by long-term calorie restriction or related to neuron density in the prefrontal cortex of extremely aged rhesus macaques. Geroscience. 2020;42(6):1733-49. doi: 10.1007/s11357-020-00259-0.
93. Cortellino S, Longo VD. Metabolites and Immune Response in Tumor Microenvironments. Cancers (Basel). 2023;15(15):3898. doi: 10.3390/cancers15153898.
94. Brandhorst S, Longo VD. Fasting and Caloric Restriction in Cancer Prevention and Treatment. Recent Results Cancer Res. 2016;207:241-66. doi: 10.1007/978-3-319-42118-6_12.
95. 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. doi: 10.1038/s41586-020-2502-7.
96. Cortellino S, Raveane A, Chiodoni C, Delfanti G, Pisati F, Spagnolo V, et al. Fasting renders immunotherapy effective against low-immunogenic breast cancer while reducing side effects. Cell Rep. 2022;40(8):111256. doi: 10.1016/j.celrep.2022.111256.
97. Cortellino S, Quagliariello V, Delfanti G, Blaževitš O, Chiodoni C, Maurea N, et al. Fasting mimicking diet in mice delays cancer growth and reduces immunotherapy-associated cardiovascular and systemic side effects. Nat Commun. 2023;14(1):5529. doi: 10.1038/s41467-023-41066-3.
98. de Groot S, Lugtenberg RT, Cohen D, Welters MJP, Ehsan I, Vreeswijk MPG, et al. Fasting mimicking diet as an adjunct to neoadjuvant chemotherapy for breast cancer in the multicentre randomized phase 2 DIRECT trial. Nat Commun. 2020;11(1):3083. doi: 10.1038/s41467-020-16138-3.
99. Salvadori G, Zanardi F, Iannelli F, Lobefaro R, Vernieri C, Longo VD. Fasting-mimicking diet blocks triple-negative breast cancer and cancer stem cell escape. Cell Metab. 2021;33(11):2247-59.e6. doi: 10.1016/j.cmet.2021.10.008.
100. Vernieri C, Fucà G, Ligorio F, Huber V, Vingiani A, Iannelli F, et al. Fasting-Mimicking Diet Is Safe and Reshapes Metabolism and Antitumor Immunity in Patients with Cancer. Cancer Discov. 2022;12(1):90-107. doi: 10.1158/2159-8290.CD-21-0030.
101. Ligorio F, Lobefaro R, Fucà G, Provenzano L, Zanenga L, Nasca V, et al. Adding fasting-mimicking diet to first-line carboplatin-based chemotherapy is associated with better overall survival in advanced triple-negative breast cancer patients: A subanalysis of the NCT03340935 trial. Int J Cancer. 2024;154(1):114-23. doi: 10.1002/ijc.34701.
102. Ligorio F, Provenzano L, Vernieri C. Fasting-mimicking diet: a metabolic approach for the treatment of breast cancer. Curr Opin Oncol. 2023;35(6):491-9. doi: 10.1097/CCO.0000000000000986.
103. Ajona D, Ortiz-Espinosa S, Lozano T, Exposito F, Calvo A, Valencia K, et al. Short-term starvation reduces IGF-1 levels to sensitize lung tumors to PD-1 immune checkpoint blockade. Nat Cancer. 2020;1(1):75-85. doi: 10.1038/s43018-019-0007-9.
104. 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. doi: 10.1016/j.ccell.2016.05.016.
105. de Cabo R, Mattson MP. Effects of Intermittent Fasting on Health, Aging, and Disease. N Engl J Med. 2019;381(26):2541-51. doi: 10.1056/NEJMra1905136. Erratum in: N Engl J Med. 2020 Jan

16;382(3):298. Erratum in: N Engl J Med. 2020 Mar 5;382(10):978.
106. Safdie F, Brandhorst S, Wei M, Wang W, Lee C, Hwang S, et al. Fasting enhances the response of glioma to chemo- and radiotherapy. PLoS One. 2012;7(9):e44603. doi: 10.1371/journal.pone.0044603.
107. 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. Sci Transl Med. 2012;4(124):124ra27. doi: 10.1126/scitranslmed.3003293.
108. Raffaghello L, Safdie F, Bianchi G, Dorff T, Fontana L, Longo VD. Fasting and differential chemotherapy protection in patients. Cell Cycle. 2010;9(22):4474-6. doi: 10.4161/cc.9.22.13954.
109. 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. doi: 10.1016/j.ccell.2019.03.007.
110. 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. Nat Commun. 2020;11(1):2332. doi: 10.1038/s41467-020-16243-3.
111. Zhang HM, Diaz V, Walsh ME, Zhang Y. Moderate lifelong overexpression of tuberous sclerosis complex 1 (TSC1) improves health and survival in mice. Sci Rep. 2017;7(1):834. doi: 10.1038/s41598-017-00970-7.
112. Di Biase S, Lee C, Brandhorst S, Manes B, Buono R, Cheng CW, et al. Fasting-Mimicking Diet Reduces HO-1 to Promote T Cell-Mediated Tumor Cytotoxicity. Cancer Cell. 2016 Jul 11;30(1):136-146. doi: 10.1016/j.ccell.2016.06.005.
113. Collins N, Han SJ, Enamorado M, Link VM, Huang B, Moseman EA, et al. The Bone Marrow Protects and Optimizes Immunological Memory during Dietary Restriction. Cell. 2019;178(5):1088-101.e15. doi: 10.1016/j.cell.2019.07.049.
114. Vodnala SK, Eil R, Kishton RJ, Sukumar M, Yamamoto TN, Ha NH, et al. T cell stemness and dysfunction in tumors are triggered by a common mechanism. Science. 2019;363(6434):eaau0135. doi: 10.1126/science.aau0135.
115. Jordan S, Tung N, Casanova-Acebes M, Chang C, Cantoni C, Zhang D, et al. Dietary Intake Regulates the Circulating Inflammatory Monocyte Pool. Cell. 2019;178(5):1102-14.e17. doi: 10.1016/j.cell.2019.07.050.
116. Lien EC, Westermark AM, Zhang Y, Yuan C, Li Z, Lau AN, et al. Low glycaemic diets alter lipid metabolism to influence tumour growth. Nature. 2021;599(7884):302-7. doi: 10.1038/s41586-021-04049-2.
117. 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. Proc Natl Acad Sci U S A. 2008;105(24):8215-20. doi: 10.1073/pnas.0708100105.
118. 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 Biol. 2017;15(3):e2001951. doi: 10.1371/journal.pbio.2001951. Erratum in: PLoS Biol.
119. Kagawa Y. Impact of Westernization on the nutrition of Japanese: changes in physique, cancer, longevity and centenarians. Prev Med. 1978;7(2):205-17. doi: 10.1016/0091-7435(78)90246-3.
120. Willcox BJ, Willcox DC, Todoriki H, Fujiyoshi A, Yano K, He Q, et al. Caloric restriction, the traditional Okinawan diet, and healthy aging: the diet of the world's longest-lived people and its potential impact on morbidity and life span. Ann N Y Acad Sci. 2007;1114:434-55. doi: 10.1196/annals.1396.037.
121. 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):476. doi: 10.1186/s12885-018-4353-2.
122. 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):988-1007. doi: 10.18632/aging.100114.
123. Zorn S, Ehret J, Schäuble R, Rautenberg B, Ihorst G, Bertz H, et al. Impact of modified short-term fasting and its combination with a fasting supportive diet during chemotherapy on the incidence and severity of chemotherapy-induced toxicities in cancer patients - a controlled cross-over pilot study. BMC Cancer. 2020;20(1):578. doi: 10.1186/s12885-020-07041-7.
124. Azzu V, Valencak TG. Energy Metabolism and Ageing in the Mouse: A Mini-Review. Gerontology. 2017;63(4):327-36. doi: 10.1159/000454924.

125. Perlman RL. Mouse models of human disease: An evolutionary perspective. Evol Med Public Health. 2016;2016(1):170-6. doi: 10.1093/emph/eow014.
126. Austad SN. Comparative aging and life histories in mammals. Exp Gerontol. 1997;32(1-2):23-38. doi: 10.1016/s0531-5565(96)00059-9.
127. Macìas-Núñez JF, Ribera Casado JM, de la Fuente del Rey M, Barja Quiroga G, Tresguerres JAF, Ariznavarreta C., et al. The Aging Kidney in Health and Disease. In: MachasNúñez, J.F., Cameron, J.S., Oreopoulos, D.G, Editors. Biology of the Aging Process and Its Clinical Consequences. Boston; Springer, 2008: pp.55-91. https://doi.org/10.1007/978-0-387-72659-5_4.
128. Demetrius L. Caloric restriction, metabolic rate, and entropy. J Gerontol A Biol Sci Med Sci. 2004;59(9):B902-15. doi: 10.1093/gerona/59.9.b902.
129. Demetrius L. Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. EMBO Rep. 2005;6 Spec No(Suppl 1):S39-44. doi: 10.1038/sj.embor.7400422.
130. Speakman JR, Mitchell SE. Caloric restriction. Mol Aspects Med. 2011;32(3):159-221. doi: 10.1016/j.mam.2011.07.001.
131. Mlekusch W, Tillian H, Lamprecht M, Trutnovsky H, Horejsi R, Reibnegger G. The effect of reduced physical activity on longevity of mice. Mech Ageing Dev. 1996;88(3):159-68. doi: 10.1016/0047-6374(96)01734-4.
132. Adibi SA. Interrelationships between level of amino acids in plasma and tissues during starvation. Am J Physiol. 1971;221(3):829-38. doi: 10.1152/ajplegacy.1971.221.3.829.
133. Cuendet GS, Loten EG, Cameron DP, Renold AE, Marliss EB. Hormone-substrate responses to total fasting in lean and obese mice. Am J Physiol. 1975;228(1):276-83. doi: 10.1152/ajplegacy.1975.228.1.276.
134. Wu SY. The effect of fasting on thyroidal T4-5′ monodeiodinating activity in mice. Acta Endocrinol (Copenh). 1990;122(2):175-80. doi: 10.1530/acta.0.1220175.
135. Barzilai N, Huffman DM, Muzumdar RH, Bartke A. The critical role of metabolic pathways in aging. Diabetes. 2012;61(6):1315-22. doi: 10.2337/db11-1300.
136. Aoki TT, Müller WA, Cahill GF Jr. Hormonal regulation of glutamine metabolism in fasting man. Adv Enzyme Regul. 1972;10:145-51. doi: 10.1016/0065-2571(72)90011-8.
137. Cahill GF Jr. Starvation in man. N Engl J Med. 1970;282(12):668-75. doi: 10.1056/NEJM197003192821209.
138. Weindruch R, Naylor PH, Goldstein AL, Walford RL. Influences of aging and dietary restriction on serum thymosin alpha 1 levels in mice. J Gerontol. 1988;43(2):B40-2. doi: 10.1093/geronj/43.2.b40.
139. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990;61(5):759-67. doi: 10.1016/0092-8674(90)90186-i.
140. Dragan YP, Pitot HC. Multistage hepatocarcinogenesis in the rat: insights into risk estimation. Prog Clin Biol Res. 1992;374:261-79.
141. Piantanelli L. Cancer and aging: from the kinetics of biological parameters to the kinetics of cancer incidence and mortality. Ann N Y Acad Sci. 1988;521:99-109. doi: 10.1111/j.1749-6632.1988.tb35268.x.
142. DePinho RA. The age of cancer. Nature. 2000 Nov 9;408(6809):248-54. doi: 10.1038/35041694.
143. Holliday R. Neoplastic transformation: the contrasting stability of human and mouse cells. Cancer Surv. 1996;28:103-15.
144. Lindl T, Voelkel M, Kolar R. Tierversuche in der biomedizinischen Forschung. Eine Bestandsaufnahme der klinischen Relevanz von genehmigten Tierversuchsvorhaben [Animal experiments in biomedical research. An evaluation of the clinical relevance of approved animal experimental projects]. ALTEX. 2005;22(3):143-51. German.
145. Perel P, Roberts I, Sena E, Wheble P, Briscoe C, Sandercock P, et al. Comparison of treatment effects between animal experiments and clinical trials: systematic review. BMJ. 2007;334(7586):197. doi: 10.1136/bmj.39048.407928.BE.
146. Contopoulos-Ioannidis DG, Ntzani E, Ioannidis JP. Translation of highly promising basic science research into clinical applications. Am J Med. 2003;114(6):477-84. doi: 10.1016/s0002-9343(03)00013-5.
147. Templeman I, Smith HA, Chowdhury E, Chen YC, Carroll H, Johnson-Bonson D, et al. A randomized controlled trial to isolate the effects of fasting and energy restriction on weight loss and metabolic health in lean adults. Sci Transl Med. 2021;13(598):eabd8034. doi: 10.1126/scitranslmed.abd8034.
148. Dorling JL, Das SK, Racette SB, Apolzan JW, Zhang D, Pieper CF, et al. Changes in body

weight, adherence, and appetite during 2 years of calorie restriction: the CALERIE 2 randomized clinical trial. Eur J Clin Nutr. 2020;74(8):1210-20. doi: 10.1038/s41430-020-0593-8.
149. Villareal DT, Fontana L, Das SK, Redman L, Smith SR, Saltzman E, et al. Effect of Two-Year Caloric Restriction on Bone Metabolism and Bone Mineral Density in Non-Obese Younger Adults: A Randomized Clinical Trial. J Bone Miner Res. 2016 Jan;31(1):40-51. doi: 10.1002/jbmr.2701.
150. Keys A, Brožek J, Henschel A, Mickelsen O, Taylor HL, Simonson E, et al. The Biology of Human Starvation: Volume I. University of Minnesota Press, Minneapolis, 1950. https://doi.org/10.5749/j.ctv9b2tqv.
151. Weyer C, Walford RL, Harper IT, Milner M, MacCallum T, Tataranni PA, et al. Energy metabolism after 2 y of energy restriction: the biosphere 2 experiment. Am J Clin Nutr. 2000;72(4):946-53. doi: 10.1093/ajcn/72.4.946.
152. Mattson MP, Arumugam TV. Hallmarks of Brain Aging: Adaptive and Pathological Modification by Metabolic States. Cell Metab. 2018;27(6):1176-99. doi: 10.1016/j.cmet.2018.05.011.
153. Schübel R, Nattenmüller J, Sookthai D, Nonnenmacher T, Graf ME, Riedl L, et al. Effects of intermittent and continuous calorie restriction on body weight and metabolism over 50 wk: a randomized controlled trial. Am J Clin Nutr. 2018;108(5):933-45. doi: 10.1093/ajcn/nqy196.
154. Catenacci VA, Pan Z, Ostendorf D, Brannon S, Gozansky WS, Mattson MP, et al. A randomized pilot study comparing zero-calorie alternate-day fasting to daily caloric restriction in adults with obesity. Obesity (Silver Spring). 2016;24(9):1874-83. doi: 10.1002/oby.21581.
155. DEBRAY C, ZARACOVITCH M, et al. Contribution à l'étude de la pathologie des déportés [Contribution to the study of the pathology of the deportees]. Sem Hop. 1946;22:863-70. French.
156. D. M. Martin E, Une experience scientifique d'alimentation controlee: la rationnement en Suisse et pendant la deuxieme guerre. (A scientific experiment of controlled food intake: food rationing in Switzerland during World War II.). A. M, Ed., Regulation de l'equilibre energetique chez 'homme. (Energy balance in man.). Paris: Masson, 1973:pp.185-193.
157. T. A. Mitchell PB, in Handbook of eating disorders., B. G. Beumont PJV, Casper RC, Ed. (Elsevier, Amsterdam 1987).
158. van Eys J. Nutrition and cancer: physiological interrelationships. Annu Rev Nutr. 1985;5:435-61. doi: 10.1146/annurev.nu.05.070185.002251.
159. Dulloo AG, Jacquet J, Girardier L. Poststarvation hyperphagia and body fat overshooting in humans: a role for feedback signals from lean and fat tissues. Am J Clin Nutr. 1997;65(3):717-23. doi: 10.1093/ajcn/65.3.717.
160. Wilhelmi de Toledo F, Grundler F, Bergouignan A, Drinda S, Michalsen A. Safety, health improvement and well-being during a 4 to 21-day fasting period in an observational study including 1422 subjects. PLoS One. 2019;14(1):e0209353. doi: 10.1371/journal.pone.0209353.
161. Da Prat V, Casirati A, Preziati G, Perrone L, Serra F, Caccialanza R, et al. The complex reality of malnutritionmanagement in oncology. Annals of Research in Oncology. 2023;3(2):59-62. doi: 10.48286/aro.2023.66.
162. Janssen H, Kahles F, Liu D, Downey J, Koekkoek LL, Roudko V, et al. Monocytes re-enter the bone marrow during fasting and alter the host response to infection. Immunity. 2023;56(4):783-96.e7. doi: 10.1016/j.immuni.2023.01.024.
163. Chi H. Regulation and function of mTOR signalling in T cell fate decisions. Nat Rev Immunol. 2012;12(5):325-38. doi: 10.1038/nri3198.
164. Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of immune responses by mTOR. Annu Rev Immunol. 2012;30:39-68. doi: 10.1146/annurev-immunol-020711-075024.
165. Schreiber KH, Arriola Apelo SI, Yu D, Brinkman JA, Velarde MC, Syed FA, et al. A novel rapamycin analog is highly selective for mTORC1 in vivo. Nat Commun. 2019;10(1):3194. doi: 10.1038/s41467-019-11174-0.
166. Arriola Apelo SI, Neuman JC, Baar EL, Syed FA, Cummings NE, Brar HK, et al. Alternative rapamycin treatment regimens mitigate the impact of rapamycin on glucose homeostasis and the immune system. Aging Cell. 2016;15(1):28-38. doi: 10.1111/acel.12405.
167. Harvie M, Howell A. Potential Benefits and Harms of Intermittent Energy Restriction and Intermittent Fasting Amongst Obese, Overweight and Normal Weight Subjects-A Narrative Review of Human and Animal Evidence. Behav Sci (Basel). 2017;7(1):4. doi: 10.3390/bs7010004.
168. Diaz-Ruiz A, Rhinesmith T, Pomatto-Watson LCD, Price NL, Eshaghi F, Ehrlich MR, et al. Diet composition influences the metabolic benefits of short cycles of very low caloric intake.

Nat Commun. 2021;12(1):6463. doi: 10.1038/s41467-021-26654-5.

169. Solon-Biet SM, McMahon AC, Ballard JW, Ruohonen K, Wu LE, Cogger VC, et al. The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. Cell Metab. 2014;19(3):418-30. doi: 10.1016/j.cmet.2014.02.009. Erratum in: Cell Metab. 2020 Mar 3;31(3):654.

170. Pomatto-Watson LCD, 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. Nat Commun. 2021;12(1):6201. doi: 10.1038/s41467-021-26431-4.

171. Esposito T, Lobaccaro JM, Esposito MG, Monda V, Messina A, Paolisso G, et al. Effects of low-carbohydrate diet therapy in overweight subjects with autoimmune thyroiditis: possible synergism with ChREBP. Drug Des Devel Ther. 2016;10:2939-46. doi: 10.2147/DDDT.S106440.

172. Pedrazzoli P,Rosti G, Caccialanza R. A novel and definitive approach to nutrition in prevention and treatment of cancer: a provocative call. Annals of Research in Oncology. 2022;2(3):209-210. doi: 10.48286/aro.2022.50.

173. Hathcock JN. Nutrient-drug interactions. Clin Geriatr Med. 1987;3(2):297-307.

174. Trovato A, Nuhlicek DN, Midtling JE. Drug-nutrient interactions. Am Fam Physician. 1991;44(5):1651-8.

175. Genser D. Food and drug interaction: consequences for the nutrition/health status. Ann Nutr Metab. 2008;52 Suppl 1:29-32. doi: 10.1159/000115345.