ISSN 2218-4333 (online)

World Journal of *Clinical Oncology*

World J Clin Oncol 2021 May 24; 12(5): 290-392

Published by Baishideng Publishing Group Inc

World Journal of Clinical Oncology

Contents

Monthly Volume 12 Number 5 May 24, 2021

World Journal of Clinical Oncology

Monthly Volume 12 Number 5 May 24, 2021

Contents

ABOUT COVER

Editorial Board Member of *World Journal of Clinical Oncology*, Qiang Huo, MD, MSc(Med), Doctor-In-Charge, Center for Translational Medicine, Zibo Central Hospital, No. 54 West Gongqingtuan Road, Zibo 255036, Shandong Province, China. qianghuo@mail.sdu.edu.cn

AIMS AND SCOPE

The primary aim of *World Journal of Clinical Oncology* (*WJCO*, *World J Clin Oncol*) is to provide scholars and readers from various fields of oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJCO mainly publishes articles reporting research results and findings obtained in the field of oncology and covering a wide range of topics including art of oncology, biology of neoplasia, breast cancer, cancer prevention and control, cancer-related complications, diagnosis in oncology, gastrointestinal cancer, genetic testing for cancer, gynecologic cancer, head and neck cancer, hematologic malignancy, lung cancer, melanoma, molecular oncology, neurooncology, palliative and supportive care, pediatric oncology, surgical oncology, translational oncology, and urologic oncology.

INDEXING/ABSTRACTING

The *WJCO* is now abstracted and indexed in PubMed, PubMed Central, Emerging Sources Citation Index (Web of Science), China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Clinical Oncology

ISSN
ISSN 2218-4333 (online)

LAUNCH DATE
November 10, 2010

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Hiten RH Patel, Stephen Safe

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2218-4333/editorialboard.htm

PUBLICATION DATE
May 24, 2021

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
https://www.wjgnet.com/bpg/gerinfo/240

PUBLICATION ETHICS
https://www.wjgnet.com/bpg/GerInfo/288

PUBLICATION MISCONDUCT
https://www.wjgnet.com/bpg/gerinfo/208

ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2021 May 24; 12(5): 290-308

DOI: 10.5306/wjco.v12.i5.290

ISSN 2218-4333 (online)

REVIEW

Cellular based treatment modalities for unresectable hepatocellular carcinoma

Konstantinos Damiris, Hamza Abbad, Nikolaos Pyrsopoulos

ORCID number: Konstantinos Damiris 0000-0001-9972-740X; Hamza Abbad 0000-0002-0655-9564; Nikolaos Pyrsopoulos 0000-0002-6950-8174.

Author contributions: Damiris K, Abbad H, and Pyrsopoulos N equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision/editing; all authors have read and approve the final manuscript.

Conflict-of-interest statement: The authors do not have any conflicts of interest relevant to this article.

Manuscript source: Invited manuscript

Konstantinos Damiris, Hamza Abbad, Department of Medicine, Rutgers New Jersey Medical School, Newark, NJ 07103, United States

Nikolaos Pyrsopoulos, Department of Medicine, Division of Gastroenterology and Hepatology, Rutgers New Jersey Medical School, Newark, NJ 07103, United States

Corresponding author: Nikolaos Pyrsopoulos, FAASLD, AGAF, FACG, MD, PhD, Professor, Department of Medicine, Division of Gastroenterology and Hepatology, Rutgers New Jersey Medical School, 185 South Orange Ave, Newark, NJ 07103, United States.
pyrsopni@njms.rutgers.edu

Abstract

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and is unfortunately associated with an overall poor prognosis and high mortality. Early and intermediate stages of HCC allow for treatment with surgical resection, ablation and even liver transplantation, however disease progression warrants conventional systemic therapy. For years treatment options were limited to molecular-targeting medications, of which sorafenib remains the standard of care. The recent development and success of immune checkpoint inhibitors has proven to be a breakthrough in the treatment of HCC, but there is an urgent need for the development of further novel therapeutic treatments that prolong overall survival and minimize recurrence. Current investigation is focused on adoptive cell therapy including chimeric antigen receptor-T cells (CAR-T cells), T cell receptor (TCR) engineered T cells, dendritic cells, natural killer cells, and tumor infiltrating lymphocyte cells, which have shown remarkable success in the treatment of hematological and solid tumor malignancies. In this review we briefly introduce readers to the currently approved systemic treatment options and present clinical and experimental evidence of HCC immunotherapeutic treatments that will hopefully one day allow for revolutionary change in the treatment modalities used for unresectable HCC. We also provide an up-to-date compilation of ongoing clinical trials investigating CAR-T cells, TCR engineered T cells, cancer vaccines and oncolytic viruses, while discussing strategies that can help overcome commonly faced challenges when utilizing cellular based treatments.

Key Words: Hepatocellular carcinoma; Immunotherapy; Immune cells; Adoptive T cell therapy; Chimeric antigen receptor-T cell; Clinical trials

Specialty type: Oncology

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B, B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Received: March 12, 2021
Peer-review started: March 12, 2021
First decision: April 6, 2021
Revised: April 19, 2021
Accepted: April 28, 2021
Article in press: April 28, 2021
Published online: May 24, 2021

P-Reviewer: Li ZM, Pongcharoen S, Zhang L
S-Editor: Gao CC
L-Editor: A
P-Editor: Yuan YY

Core Tip: Over the recent years investigation for safe and effective treatments for unresectable hepatocellular carcinoma (HCC) has shifted focus from various chemotherapeutic agents to immune based therapy. Although far from being finalized, immune cell-based therapy has shown efficacy in a variety of clinical trials, indicating possible future utilization alone or in combination for the prevention and treatment of HCC.

Citation: Damiris K, Abbad H, Pyrsopoulos N. Cellular based treatment modalities for unresectable hepatocellular carcinoma. *World J Clin Oncol* 2021; 12(5): 290-308
URL: https://www.wjgnet.com/2218-4333/full/v12/i5/290.htm
DOI: https://dx.doi.org/10.5306/wjco.v12.i5.290

INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant primary tumor of the liver, accounting for approximately ninety percent of total liver tumor cases worldwide[1]. HCC commonly occurs in patients with preexisting liver cirrhosis, chronic hepatitis B virus (HBV), chronic hepatitis C virus (HCV), heavy alcohol consumption, aflatoxin exposure and metabolic associated fatty liver disease associated with diabetes and metabolic syndrome[2]. According to the Global Cancer Observatory (GLOBOCAN) in 2020, HCC is considered the 6th most common cancer worldwide, with the highest incidence in eastern Asia and Africa[3]. It has been estimated that by 2025, there will be more than one million individuals diagnosed with liver cancer annually[4]. Worldwide incidence of HCC has shown a greater predominance among men in comparison to women, with most diagnoses made in patients over the age of 60 years old[5]. HCC risk factors in the United States have varied significantly over the last two decades, with a majority of cases currently related to chronic HCV infection[6].

Management of HCC varies depending on the staging status of the tumor according to the standardized Barcelona-Clinic Liver Cancer (BCLC) system endorsed by both the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases[7]. Locally diagnosed tumors (BCLC stages 0-A), which are small in size (≤ 3 cm), early in progression, and limited to the liver parenchyma are amendable to surgical resection, radiofrequency ablation or ideally liver transplantation[8], with a median survival rate of 60 mo[9]. Advanced HCC on the other hand is defined by a significant increase in tumor size, vascular invasion and/or metastatic disease that is not amenable to loco-regional therapy (BCLC Stage C-D)[10], warranting treatment with systemic therapy[11]. In cases necessitating liver transplantation, the Milan criteria has been adopted by The United Network for Organ Sharing in order to determine eligibility[12], however this approach is limited by organ availability[13].

For over a decade, sorafenib has been considered the first line systemic treatment for advanced HCC. More recently, other systemic treatments such as the tyrosine kinase inhibitors (lenvatinib, regorafenib, and cabozantinib), immune checkpoint inhibitors (ICIs) (atezolizumab, ipilimumab, nivolumab, pembrolizumab) and the monoclonal antibody ramucirumab have been approved as first- and second-line treatment options[14]. With only modest established improvement in overall survival (OS) and an assortment of adverse effects, there has been a recent push for the development of immunobiological treatments, which first demonstrated efficacy in the treatment of hematological cancers. Treatments include chimeric antigen receptor-T cells (CAR-T cells), T cell receptor (TCR) engineered T cells, dendritic cells (DC), natural killer (NK) cells, and tumor infiltrating lymphocyte (TIL) cellular therapies (Figure 1). In this article we will review the novel range of cellular based treatments for HCC non-amenable to loco-regional therapy and introduce readers to various preclinical and clinical trials investigating their efficacy.

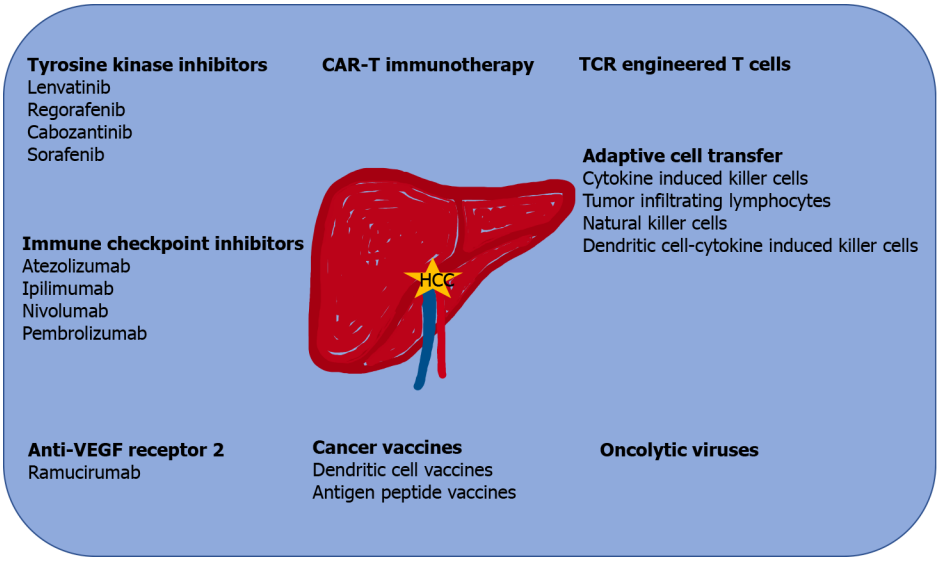


Figure 1 Treatment modalities available for unresectable (advanced) hepatocellular carcinoma, which is non-amendable to loco-regional therapies. CAR: Chimeric antigen receptor; HCC: Hepatocellular carcinoma; TCR: T cell receptor; VEGF: Vascular endothelial growth factor.

SYSTEMIC THERAPIES: APPROVED FIRST LINE THERAPIES

Sorafenib

Sorafenib is a multi-kinase inhibitor that inhibits cellular proliferation and angiogenesis through its effects on various receptor tyrosine kinases including vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and c-kit [15]. Its efficacy was demonstrated in two large phase III randomized controlled trials; SHARP conducted in the United States and Europe, and a similar study conducted in the Asia- Pacific focusing on patients with unresectable and advanced HCC[16]. In the SHARP trial, median OS was increased to 10.7 mo compared to 7.9 mo in placebo[17]. Similar findings were demonstrated in the Asia-Pacific trial; median OS increased in the treatment group (6.5 mo *vs* 4.2 mo) and time-to-progression (TTP) was also significantly greater (2.8 mo *vs* 1.4 mo)[18]. These findings led to sorafenib being approved by the Food and Drug Administration (FDA) in 2007, making it the standard of care and first line treatment option of advanced HCC[15,19].

Lenvatinib

Lenvatinib is another inhibitor of multiple receptor tyrosine kinases that has recently gained much attention[20]. In the randomized, open-label, non-inferiority phase III trial (REFLECT), lenvatinib was compared to sorafenib in patients who were deemed to have non-resectable HCC. Findings indicated an improved median OS of 13.6 mo with lenvatinib, compared to 12.3 mo with sorafenib. Lenvatinib also demonstrated an increase in median progression-free survival (PFS) and overall response rate (ORR) [21]. This allowed for FDA approval in 2018 as a fist line agent for treatment of unresectable HCC[22].

SYSTEMIC THERAPIES: APPROVED SECOND LINE THERAPIES

Ramucirumab

The phase III randomized controlled trial REACH (NCT01140347) investigated ramucirumab, a monoclonal antibody against VEGF receptor 2. While the study failed to demonstrate prolonged OS in the entire cohort, patients with elevated serum alpha-fetoprotein (AFP) (> 400 ng/mL) had significantly longer median OS when compared to placebo (7.8 mo *vs* 4.2 mo, P = 0.006)[23]. A subsequent phase III study, REACH-2 (NCT02435433) reported a significantly prolonged median OS (8.5 mo *vs* 7.3 mo) in sorafenib-experienced patients receiving ramucirumab *vs* placebo with AFP of 400 ng/mL or greater[24]. These findings showed promise as an effective treatment option in those with elevated AFP, leading to FDA approval in May 2019.

Regorafenib

Regorafenib is a multikinase inhibitor that inhibits various molecules including VEGF receptor 2/3, PDGF receptor and fibroblast growth factor receptor 1[25]. In the randomized, double-blind, phase III clinical trial RESORCE (NCT01774344) patients who failed sorafenib therapy were assigned to receive regorafenib *vs* placebo. This study determined that regorafenib significantly improved OS in individuals who experienced radiological progression of HCC during sorafenib treatment (10.6 mo *vs* 7.8 mo, $P < 0.0001$)[26]. Based on the promising findings of the RESORCE trial and a tolerable safety profile, the FDA approved regorafenib as a second line treatment option in those who have HCC progression while on sorafenib or who are not eligible for alternative therapy[25].

Cabozantinib

A double-blind, phase III clinical trial CELESTIAL (NCT01908426) was conducted testing cabozantinib, a multikinase inhibitor, in patients with unresectable HCC who had progressed with sorafenib. The study demonstrated a significant prolongation of OS when compared to placebo (10.2 mo *vs* 8.0 mo, $P = 0.005$) coupled with a longer median PFS when compared to placebo[27]. Based on the findings of CELESTIAL, the FDA approved cabozantinib as a second line treatment option for HCC in those who have undergone previous therapy with sorafenib.

IMMUNOTHERAPY: ICIS

Limited efficacy and undesirable side effects of traditional therapies (*i.e.*, sorafenib) have led to further investigation of immunotherapeutic agents over the years. A recent and emerging field of treatment includes cancer immunotherapies using ICIs that target programmed cell death protein-1 and its ligand (PD-1/PD-L1) and cytotoxic T-lymphocyte-associated protein- 4 (CTLA-4), which has significantly altered treatment of various types of cancer including HCC[28].

Immune checkpoint molecules play essential roles in regulating cancer cell interactions with the host immune system; and the development of drugs that suppress these interactions used by tumor cells to evade host immunity has shown promising results. PD-1, a member of CD28, is expressed on activated immune cells including T cells, B cells, and myeloid cells; and its interaction with ligand PD-L1 negatively regulates the immune system through suppressive signals that induce peripheral tolerance[29]. In HCC tumor cells, PD-L1 is aberrantly expressed, allowing for such an escape from host immunity[30]. CTLA-4 is another member of the CD28 family, and it is induced on T cells by antigen activation leading to subsequent binding with CD80 and CD86 with more affinity compared to CD28, negatively regulating costimulatory T cell signals and allowing for peripheral tolerance[31]. Ultimately these immunotherapeutic drugs strategically suppress key checkpoints that may be used by tumor cells to evade the host immune system, hindering a hallmark for tumor progression.

Atezolizumab

Atezolizumab is a fully humanized monoclonal antibody targeting PD-L1 that has recently changed the treatment landscape of unresectable HCC. A phase-Ib study evaluating synergistic atezolizumab and bevacizumab (anti-VEGF antibody) in patients with previously untreated and unresectable HCC not only demonstrated an acceptable safety profile, but promising antitumor activity with ORR 36%, disease control rate (DCR) 71%, and a median PFS of 7.3 mo when compared to monotherapy with nivolumab or pembrolizumab[32]. These results led to IMbrave150 (NCT 03434379), an open-label, randomized phase III study assessing atezolizumab (anti-PDL1) in combination with bevacizumab (anti-VEGF antibody) compared to sorafenib in patients with untreated locally advanced or metastatic HCC. OS at 12 mo was significantly longer in the combination group when compared to sorafenib (67.2% *vs* 54.6%) and PFS was significantly longer in atezolizumab + bevacizumab *vs* sorafenib (6.8 mo *vs* 4.3 mo, hazard ratio for disease progression or death [0.59; 95% confidence interval (CI): 0.47-0.76, $P < 0.001$], leading to FDA approval in May 2020 for the treatment of unresectable or metastatic HCC who have not received prior systemic therapy[33]. Currently a phase III, multicenter study of atezolizumab plus bevacizumab *vs* active surveillance as adjuvant therapy in HCC at high risk of recurrence after surgical resection or ablation is recruiting (IMbrave050; NCT04102098)[34]. In addition, a phase III study evaluating the safety and efficacy of atezolizumab plus

levantinib or sorafenib *vs* levantinib or sorafenib alone in locally advanced or metastatic and/or unresectable HCC following prior treatment with combination atezolizumab and bevacizumab was recently posted in February 2021 (IMbrave251; NCT04770896).

Ipilimumab

Ipilimumab is a monoclonal antibody targeting CTLA-4, which was recently granted accelerated FDA approval in May 2020, when used in combination with nivolumab (anti-PD-1) for HCC that has previously been treated with sorafenib. Approval was based on a single arm of the phase I/II clinical trial CheckMate 040 (NCT01658878), in which patients were treated with nivolumab 3 mg/kg plus ipilimumab 3 mg/kg every 3 wk for 4 doses. In addition to a substantial reduction in tumor burden, the overall ORR was 32%, with median OS 22.8 mo while having manageable safety profiles[35]. A phase III trial of ipilimumab in combination with nivolumab *vs* sorafenib or levantinib as first line treatment in patients with advanced HCC is currently recruiting participants (CheckMate 9DW; NCT04039607). A variety of clinical trials evaluating ipilimumab in combination with nivolumab during various treatment phases are ongoing and include; prior to liver resection (PRIME-HCC; NCT03682276), as neoadjuvant therapy (NCT03510871), and in combination with trans-arterial chemoembolization (CheckMate 74W; NCT04340193).

Nivolumab

Nivolumab is an anti-PD-1 monoclonal antibody that has shown promising results, leading to FDA approval for second-line treatment of advanced HCC. The phase I/II clinical trial CheckMate 040 (NCT01658878) investigated nivolumab in sorafenib-naïve and sorafenib-experienced patients with intermediate-advanced HCC and Child-Pugh Stage A. During the dose-expansion phase, ORR was 20%, while DCR was 64% with a median progression free survival of 4.1 mo[36]. The subsequent phase III study CheckMate 459 (NCT2576509) evaluating nivolumab *vs* sorafenib as first line treatments for unresectable HCC failed to demonstrate statistical significance for the primary endpoint of OS[37]. Currently a phase III trial, CheckMate-9DX (NCT 03383458), is actively evaluating recurrence free survival (RFS) in those with HCC at high risk of recurrence after curative hepatic resection or ablation.

Pembrolizumab

Pembrolizumab is a humanized anti-PD-1 monoclonal antibody that has been investigated across a variety of malignancies[38]. The phase II open-label clinical trial KEYNOTE-224 (NCT02702414) studied the safety and efficacy of pembrolizumab in patient with advanced HCC after having failed treatment with sorafenib. Favorable findings included an ORR of 17%, DCR of 61%, along with PFS of 4.8 mo, median OS of 12.9 mo and TTP of 4.9 mo[39]. This promising data led to FDA approval in November 2018 as a second line treatment after sorafenib. A subsequent phase III double-blind, randomized control trial, KEYNOTE-240 (NCT02702401) compared pembrolizumab to placebo, demonstrated longer OS and PFS; however, these findings were not statistically significant and failed to meet primary endpoints[40]. There are currently two-phase III trials that are ongoing at the time of this publication; KEYNOTE-394 (NCT03062358) , evaluating pembrolizumab in Asian patients with systemically treated advanced HCC and KEYNOTE-937 (NCT03867084) as an adjuvant therapy in HCC after curative treatment[29].

Ultimately, cancer uses a variety of unique mechanisms to evade host immune response and to develop drug resistance in HCC. This has sparked much interest in combining treatment modalities, particularly combination ICIs and checkpoint inhibitors with other chemotherapeutics. HIMALAYA (NCT03298451) is an open-label, multi-center, phase III study assessing durvalumab (anti-PDL1) plus tremelimumab (anti-CTLA4) in patients with advanced HCC. Scientists and physicians alike are eagerly awaiting results of other ongoing phase III trials such as LEAP-002 (NCT03713593) evaluating pembrolizumab and levantinib in combination and COSMIC-312 (NCT03755791) investigating atezolizumab and cabozantinib synergistically.

BEYOND CHECKPOINT INHIBITORS

Cell based immunotherapy

In recent years cancer therapy has begun shifting focus from the aforementioned conventional therapies, to the use of immune cell therapies, which utilize the host immune system to target and treat cancer. One such avenue of therapy includes gene modified T cell therapy, particularly TCR engineered T cells and CAR-T cells, which have shown promise in various malignancies and continue to be tested in clinical trials targeting HCC. Below we will discuss the concept of gene modified T cell therapies, and other modalities of adoptive cell transfer while reporting the results of recent and ongoing clinical trials where applicable.

When engineering T-cell based immune cell therapies targeting HCC, identification of tumor-associated antigens (TAAs) that can allow for appropriately mediated immune response is critical. AFP is a glycoprotein composed of 591 amino acids, identified as the first oncofetal biomarker for patients with HCC allowing for quantitative estimation of tumor burden and response to therapy[41]. Elevated levels of AFP have been found in approximately 70% of patients diagnosed with HCC, however elevations can also be found in other pathological conditions including cirrhosis, various hepatic disorders, germ cell tumors, lung cancer, gastric cancer, and pancreatic cancer[42]. Pre-clinical models have demonstrated the potential of AFP in the development of cellular immunotherapies[43,44]. Glypican-3 (GPC-3) belongs to the transmembrane heparan sulfate proteoglycan family, that regulates cellular division and growth[45]. GPC-3 expression is elevated in HCC, and recent studies have demonstrated that elevated levels correlate with an overall worse prognosis[46]. Therefore GPC-3 has been investigated in pre-clinical studies as a target for adoptive cell immunotherapy[47,48]. Melanoma antigen gene proteins (MAGE) was first identified in melanoma patients and has been found to be almost exclusively expressed in a variety of cancer tissues. Aberrant expression of MAGE has been demonstrated to significantly correlate with clinical characteristics of HCC, however functions of the multitude of MAGE proteins have yet to be thoroughly understood [49]. New York esophageal squamous cell carcinoma (NY-ESO-1) is a member of the cancer testis antigen family expressed in a variety of cancer cells including HCC[50]. NY-ESO-1 has been deemed as the most promising cancer testis antigen for the development of cancer immunotherapy with a multitude of studies demonstrating promising results across a variety of malignancies[51]. When examining HCC caused by viral infection (HBV or HCV) there is prospect in targeting viral antigens as a strategy to etiologically treat HCC. Targets include HBV S or L protein (envelope proteins), which have shown promise by eliminating HBV positive hepatocytes when targeted by antigen specific T cells[52]. Targeting of hepatitis B surface antigen has also shown efficacy in mouse models utilizing CAR-T cell therapy as well[53]. Other tumor antigens that are of particular interest include epithelial cell adhesion molecules (EpCAM)[54], mucin 1 glycoprotein (MUC1)[55] and human telomerase reverse transcriptase (hTERT)[56].

CAR-T and TCR engineered T cells: The basics

Gene modified T cell therapy is a method used to deliver T cells that are genetically engineered to produce TCRs that recognize specific tumor associated antigens and their epitopes. There are currently two methods of developing gene modified T cells, CAR-T cells and TCR transgenic T-cells (engineered T-cells), which have both shown efficacy and promise in a variety of solid and hematological malignancies[57]. CAR is composed of three basic elements: (1) the extracellular antigen recognition domain, (2) the transmembrane domain and (3) the intracellular signaling domain. The extracellular portion is a single chain antigen recognition receptor composed of heavy and light chains of a monoclonal antibody specific to the tumor surface antigen, allowing for no restrictions of target antigen recognition by major histocompatibility complex (MHC) molecules. The intracellular portion is formed by combining co-stimulatory molecules to the intracellular portion of TCRs[58]. The basic process of CAR-T generation includes: (1) T cell isolation from peripheral blood mononuclear cells using immunoselective beads and activation with anti-CD3 and Il-2, (2) genetic modification of CAR-T, (3) expansion of T cells *in vitro*, (4) evaluation ensuring CAR expression and T cell viability, and (5) CAR-T infusion back into the patient[59]. TCR transgenic T cells possess a TCR on the surface consisting of two distinct protein chains; alpha and beta, which bind to the MHC of antigen presenting cells, allowing for a highly specific interaction which is not limited to membrane bound antigens[60,61]. As previously described the selection and validation of target antigens is of utmost importance when

designing both TCR-T cells and CAR-T cells that are efficacious and minimize on target/off-tumor side effects[62].

CAR-T immunotherapy

CAR-T cell therapy has been tested both preclinically and clinically when targeting HCC. Below we will discuss findings of completed and ongoing clinical studies that seek to demonstrate safety and efficacy of cellular therapy. Shi *et al*[63] recently published results from two prospective phase I trials involving GPC-CAR-T cells (NCT02395250 and NCT03146234) in patients with advanced GPC3 positive relapsed or refractory HCC following chemotherapeutic induced lymphodepletion. A total of 13 patients received CAR-T cell infusion, with two partial responses and one patient with sustained stable disease after 44.2 mo. OS rates at 3 years, 1 year, and 6 mo were 10.5%, 42% and 50.3% respectively with median OS duration of 278 d according to the Kaplan-Meier method. Unfortunately, toxic effects were noted with major concern as one patient developed cytokine release syndrome (CRS) leading to death from multi-organ failure[63]. A recently completed, open-label, phase I-II study by Dai *et al*[64] demonstrated efficacy of CD133-CAR-T cell therapy in patients with biopsy proven HCC (BCLC stage C) not amenable to curative treatment. One patient demonstrated a partial response, while 66.7% of patients had stable disease after infusion with an overall PFS of 6.8 mo and median OS of 12 mo (NCT02541370). Currently an open-label, single center, phase I-II study is underway investigating CAR-T/TCR-T cell immunotherapy targeting a variety of different malignancies including hepatoma (NCT03638206). A list of currently ongoing clinical trials investigating CAR-T cells for the treatment of HCC is listed in (Table 1).

Improving CAR-T delivery and efficacy

Proven clinical efficacy of CAR-T across a variety of hematological malignancies has led to the investigation of its use in solid tumors. However, treatment of solid tumors poses many challenges to clinical investigators, one of which includes access to the tumor site. Many cases of HCC occur on the background of a fibrosed and cirrhotic liver, and the presence of such fibrotic extracellular matrix (ECM) poses a barrier for CAR-T cell penetration. In order to overcome such a challenge, CAR-T cells co-expressing heparinase, which degrades ECM heparan sulfate proteoglycans were developed. These newly engineered CAR-T cells improved cellular ability to degrade the ECM and displayed antitumor activity, suggesting a feasible strategy for the development of CAR-T cells targeting stroma-rich solid tumors[65]. Similar strategies are being investigated with the use of matrix metalloproteinase 8, which can allow CAR-T cells to overcome physical barriers[66]. Local administration of immunotherapy directly into tumors has shown promising results for organ selective delivery. In a study conducted by Katz *et al*[67], not only were CAR-T cells detected in biopsies of liver metastasis, but effective CAR-T cellular response was noted within the tumor following delivery *via* hepatic artery infusion. Such results have paved light into the investigation of direct tumor inoculation *via* a variety of local administration techniques including hepatic artery infusion in a multitude of clinical trials. Novel delivery platforms including nanoparticles, scaffolds and other biomaterials are also being investigated as a safe and effective way to deliver immunotherapy to tumor cells while avoiding off-target adverse effects[68]. Recent findings from the 2020 study conducted by Ma *et al*[69], demonstrated superior targeting and anti-tumor capabilities of CAR-T cell membrane coated nanoparticles both *in vivo* and *in vitro*.

In addition to tumor location being inaccessible, the tumor microenvironment has been found to be detrimental to CAR-T cells in regard to their survival and proliferation. The consumption of glucose by malignant cells renders the surrounding environment hypoxic, acidic, and low in nutrients leading to oxidative stress; leaving glycolytic T cells unable to adequately function, ultimately hindering the immune response[70,71]. In order to help eliminate reactive oxygen species (ROS) accumulation, CAR-T cells co expressing catalase were designed, and were subsequently able to survive and function in such unfavorable environments[72]. However, oxidative stress with release of compounds such as ROS and hydrogen peroxide, not only prevents recruitment and adequate function/persistence of immune cells, it also allows for recruitment of immune suppressor cells that lead to CAR-T cell exhaustion [73]. Strategies to help overcome such exhaustion include the use of ICIs that target PD-1, PD-L1, CTLA-4 in conjunction to CAR-T cells administration[74], and by genetically deleting T-cell PD-1 protein with the use of CRISPR-CAS9 gene editing [75]. There is optimism that these findings coupled with continued advance-ments in biomedical engineering will allow for new delivery and tumor microenvironment altering capabilities that can help amplify immunotherapeutic response in the

Table 1 Ongoing clinical trials investigating chimeric antigen receptor-T cell therapy in the treatment of hepatocellular carcinoma according to ClinicalTrials.gov

Product	Phase	Estimated completion	Study/identifier
GPC3- CAR-T	I	November 2021	NCT04121273
CD147- CAR-T	I	May 2022	NCT03993743
GPC3- CAR-T	I	June 2022	NCT03980288
CAR-CLD18	N/A	December 2023	NCT03302403
GPC3- CAR-T	I	May 2024	NCT03884751
GPC3/TGF-CAR-T	I	August 2024	NCT03198546
GPC3- CAR-T	I	October 2036	NCT02905188

CAR: Chimeric antigen receptor; CD: Cluster of differentiation; GPC3: Glypican-3; TGF: Transforming growth factor.

treatment of HCC.

Improving CAR-T safety

When utilizing cellular immunotherapy with CAR-T cells, a number of important safety concerns are to be noted including CRS, central nervous system toxicity (CNS) and the "on target-off tumor" effect. CRS is characterized by flu like symptoms including fever, fatigue and headache, which can progress to hypotension and organ dysfunction secondary to uncontrolled systemic inflammatory response associated with elevated serum cytokines; particularly interleukin (IL)-6, IL-10, tumor necrosis factor α (TNF-α) and interferon (IFN)-γ[76]. Symptoms of CNS toxicity can range from headache and dizziness, to memory loss and delirium, and can be found along with CRS or after the resolution of CRS. Although the exact etiology remains unclear at this time, it is speculated that various cytokines may alter the blood brain barrier[77], in conjunction with an increase in protein, white blood cells, IFN-γ, TNF-α, and IL-6 within cerebrospinal fluid[78].

These adverse reactions stem from CAR-T cells recognizing TAAs, which are not only heterogenous amongst tumors, but can also be present in normal tissues, elevating the risk of off target toxicity[71]. Such toxic effects have been reported following administration of anti-ERBB2 CAR-T cells for the treatment of metastatic malignancy, which led to CRS with subsequent respiratory distress and death due to low levels of ectopic ERBB2 in lung epithelium[79]. In order to help overcome such toxicities, a variety of strategies are being investigated. One such strategy includes the controlled removal of CAR-T cells with the use of suicide genes. There has been promise with the use of a drug inducible caspase 9 system (pro-apoptotic molecule that activates downstream pro-apoptotic caspase 3 when chemically induced) leading to apoptosis of CAR-T cells on demand[80] and with a system utilizing a truncated epidermal growth factor receptor, which allows for elimination following administration of a receptor specific antibody[81]. More recently, Amatya *et al*[82] developed CAR-T cells with a caspase 9 suicide gene for the treatment of multiple myeloma. Following administration of a dimerizing agent, CAR-T cells underwent apoptosis and were no longer active.

As previously mentioned, identification of a suitable TAA is imperative in the development of efficacious CAR-T cells, however highly specific TAAs are rarely identified, leading to investigation of dual- targeted CAR-T cells. Chen *et al*[83] developed dual-target CAR-T cells (targeting GPC3 and Asialoglycoprotein receptor 1) for the treatment of HCC. Not only did these cells demonstrate superior anticancer activity coupled with higher cytokine secretion when compared to single target CAR-T cells, but their specificity toward cellular targets may reduce the risk of on target- off tumor toxicity[83]. Recent developments have shown efficacy of a novel, split anti-GPC3 CAR-T, into two components with the use of SpyCatcher (extracellular 116 amino acid sequence linked to the intracellular domain) and SpyTag (13 amino acid peptide linked to single chain variable fragment). Such dual component delivery of CAR-T cells not only suppressed tumor growth, but displayed decreased cytokine release, representing a safer alternative when compared with conventional CAR-T cells[84]. Other perspectives include formation of a split, universal and programmable CAR system that allows for enhancement of specificity, safety and programmability of

CAR delivery systems[85].

TCR engineered T cells

TCR engineered T cells are modified T cells that are able to precisely recognize tumor surface antigen peptides and MHC, rendering a response only effective when tumor cells express both antigen epitopes and MHC molecules[57]. TCR engineered T cells specific to NY-ESO-1 have demonstrated significant antitumor effects in the treatment of multiple myeloma[86] and synovial sarcomas[87]. The presence of NY-ESO-1 over-expression in HCC has made it an investigated target when developing engineered T cells in one phase II study (NCT01967823), and two phase I studies (NCT02869217) and (NCT03159585), for which we eagerly await results. In addition, TCR engineered T cells targeting hepatitis B viral antigens in HBV related HCC have been of interest in both preclinical and clinical models[88,89]. Recently in November 2020, Chen *et al*[90] presented an abstract at the 2020 Society for Immunotherapy of Cancer, demonstrating safety and efficacy of HBV specific TCR T-cells in recurrent HBV related HCC patients post liver transplant (NCT02719782).

There are currently five ongoing trials investigating TCR engineered cells targeting various TAAs in HCC (Table 2). All trials are investigating efficacy in advanced, unresectable HCC, with the exception of (NCT03899415), which is evaluating the safety and clinical benefit of TCR engineered T cells in HBV positive HCC patients post hepatectomy or radiofrequency ablation.

Cytokine induced killer cells

Cytokine induced killer (CIK) cell therapy is a form of adaptive cell transfer that has been investigated as adjuvant therapy in patients with HCC. CIK cells are isolated from the patient's peripheral mononuclear cells, at which point they are grown *ex vivo* with the addition of cytokines such as recombinant human IFN-γ, anti-CD3 monoclonal antibody, and recombinant human IL-2 prior to transfusion back into the patient; allowing for recognition and subsequent death of tumor cells[91]. In a multicenter, randomized phase III trial conducted in Korea, 230 patients were assigned to receive adjuvant CIK or no adjuvant therapy (control) following treatment with surgical resection, radiofrequency ablation or percutaneous ethanol injection (NCT00699816). Median RFS was significantly prolonged in the immunotherapy group (44 mo *vs* 30 mo, P = 0.01), coupled with lower hazard ratios for all cause death (P = 0.08) and cancer-related death (P = 0.02)[92]. A 5-year follow-up study demonstrated continued efficacy of autologous CIK immunotherapy without any repeated injections (NCT01890291). After an average 68.5 mo follow up, RFS was 44.8% in the immunotherapy group and 33.1% in the control group, coupled with a significantly lower risk of all-cause death (hazard ratio 0.33, 95%CI: 0.15-0.76, P = 0.006)[93]. Similar results were demonstrated in a meta-analysis and systemic review conducted by Cai *et al*[94] examining adjuvant CIK after invasive treatments for HCC. Pooled analysis demonstrated a significant improvement of RFS, PFS and OS in patients receiving autologous CIK immunotherapy. Although many studies have been conducted examining the safety and efficacy of CIK as an adjuvant treatment for HCC with mixed results; research is limited when examining CIK therapy as a sole treatment for unresectable HCC.

TILs

TIL therapy is an immunotherapeutic technique that involves isolation and subsequent cell culture followed by autologous administration of tumor specific T cells present in infiltrated tumors. Success of adoptive cell therapy using TIL is based on the diverse antigenic specificity displayed by TIL cells toward the tumor and the lytic capabilities possessed allowing for eradication of malignancy[95]. TILs play an essential role in cancer progression[96] and even as a predictive biomarker of response to neoadjuvant chemotherapy in a variety of malignancies[97]. After the recognition of tumor associated antigens[98], various types of TILs infiltrate to the tumor site; including NK cells, NK T cells, mucosal-associated invariant T cells, and gamma delta T cells[99]. CD4+ T helper cells on the other hand further differentiate in the periphery into a variety of subsets (*i.e.*, Th1, Th2, Th9, Th17, Th22, regulatory T cells, and T follicular helper cells)[100]. CD8+ and NK cells contribute to a direct cytotoxic antitumor effect induced by a cascade of activating and inactivating receptors[99].

In a study conducted by Chew *et al*[101], examining immune gene expression profiles of resected tumor, HCC patient survival was positively correlated to higher expression of inflammatory immune genes. In conjunction, there was an increased presence of NK cells and T cells promoting apoptosis and reducing proliferation in the

Table 2 Ongoing clinical trials investigating T cell receptor engineered T cells in the treatment of hepatocellular carcinoma according to ClinicalTrials.gov

Product/target	Phase	Estimated completion	Study/identifier
C-TCR055/AFP	I	April 2021	NCT03971747
C-TCR055/AFP	I	November 2021	NCT04368182
HBV-TCR T cell/HBV Ag	I	June 2024	NCT03899415
IMA202-101/MAGE A1	I	June 2024	NCT03441100
AFP T cells/AFP	I	June 2026	NCT03132792

AFP: Alpha fetoprotein; HBV: Hepatitis B virus; Ag: Antigen; MAGE: Melanoma antigen gene protein.

tumors of patients with longer survival, suggesting that TILs can be used as a specific immunotherapy for treatment of HCC[101]. In a hallmark randomized trial, patients who had undergone curative resection for HCC were assigned to receive adoptive immunotherapy *vs* no adjuvant therapy. Administration of lymphocyte infusion led to a reduced frequency of recurrence by 18%, significantly longer time to first recurrence (P = 0.008), longer RFS (P = 0.01) and disease- specific survival (P = 0.04) when compared to controls[102]. A phase I trial (NCT01462903) examined the use of autologous TILs in HCC patients post resection. Results demonstrated that 80% of patients remained disease free after 14-mo follow-up, while displaying a favorable side effect profile, further suggesting TIL therapy as a safe treatment method for HCC[103]. Currently an active clinical trial is investigating the safety and efficacy of autologous TILs in patients with high-risk recurrent HCC (NCT04538313). Research is limited on the use of TIL immunotherapy for unresectable HCC, and we hope that further studies can be initiated using TILs alone or in combination with other therapies such as chemotherapy or ICIs.

NK cells

NK cells play an active and critical role in the innate and adaptive immune defense against viral infections and hepatocellular malignancy[104], forming as much as 50% of innate immunity cell infiltrate within the liver[105]. Several clinical studies have demonstrated the safety and efficacy of allogenic NK cell adoptive immunotherapy in the treatment of various malignancies[106,107] including HCC[108]. In a study conducted by Lin *et al*[108], patients were enrolled to receive cryoablation alone *vs* allogenic NK cell therapy for the treatment of unresectable HCC. Results demonstrated synergistic effects of combination therapy leading to enhanced immune function, reduction in AFP, and improved quality of life in addition to an increased median PFS (9.1 mo *vs* 7.6 mo, P = 0.01), higher response rate (60% *vs* 46.1%, P < 0.05) and DCR (85.7% *vs* 69.2%, P < 0.01) when compared to cryoablation alone. Similar results were demonstrated in a study conducted by Alnaggar *et al*[109], which investigated allogenic NK cell immunotherapy in combination with irreversible electroporation for Stage IV unresectable HCC. Efficacy of treatment was synergistic, with statistically significant lower AFP levels at 1 and 3 mo after treatment (P < 0.01), coupled with a higher median OS (10.1 mo *vs* 8.9 mo, P = 0.0078) when comparing combo therapies *vs* electroporation alone. Currently a phase I/II study investigating allogenic NK cell therapy in patients with advanced HCC (NCT04162158) is underway, as well as a phase II/III study examining the effects of autologous NK therapy when coupled with transcatheter arterial chemoembolization (TACE) for the treatment of advanced HCC (NCT04011033). Combination therapy of "off the shelf" FT500 NK cells with ICIs is also being investigated in subjects with advanced solid tumors including HCC (NCT03841110).

The advent of genetic modification techniques has led to the use of CAR technology to develop CAR-NK cells to improve specificity and efficacy of NK cell cytotoxi-city [110]. Yu *et al*[111] developed a GPC3-specific NK cell for the treatment of HCC, which demonstrated *in vitro* cytotoxicity and cytokine production in addition to potent anti-tumor activities in HCC xenografts. Similar treatment benefits were noted with the development of c-MET specific CAR-NK cells, which demonstrated specific cytotoxicity against malignant hepatic cells *in vitro*[112]. While there are many ongoing clinical trials investigating CAR-NK immunotherapy for the treatment of hematological malignancies; investigation of treatment efficacy in solid tumors remains

scarce.

DC-CIK cells

DC are antigen presenting cells that bridge the gap between innate and adaptive immunity, allowing for activation of naïve T cells that promote tumor-specific T cell immune response and adaptive immunity[113]. The use of *in vitro* generated DC combined with CIK cells has shown to be an effective and promising immunotherapy technique when used alone or in combination with other treatment options for a variety of malignancies[114]. In a study conducted by Zhou *et al*[115], advanced HCC patients were assigned to receive sorafenib alone *vs* a combination of sorafenib and DC-CIK immunotherapy. Results not only demonstrated a significant reduction in AFP levels when using combination therapy, but a significant increase in clinical benefit rate (41.9% *vs* 88.6%, $P < 0.05$) and prolonged median survival time (13.8 m *vs* 18.6 m, $P < 0.05$) while maintaining safety. Examination of combined therapy of TACE with DC-CIK therapy *vs* TACE alone has shown statistically prolonged OS time in HCC patients with HBV[116]. A meta-analysis of 22 studies, conducted in 2019 by Cao *et al*[117], has shown a prolonged survival and reduced recurrence rate of HCC when combining conventional clinical treatment and immunotherapy with DC and/or CIKs. There are currently studies recruiting participants for treatment of various solid tumors (including HCC) with DC-CIK combination immunotherapy (NCT04214717, NCT04476641, NCT03190811).

HCC vaccines

Current advancements in medicine and immunobiology have demonstrated promising treatment strategies of cancer vaccines and oncolytic immunotherapy in patients with advanced stage cancer, leading to a variety of studies under clinical investigation [118]. Modalities include DC vaccinations, vaccines that target the antigens on tumor cells which in return activate cellular and humoral immunity causing a phagocytic activity towards the tumor cells, and oncolytic viruses that can attack and destroy tumor cells. These vaccines have both prophylactic and therapeutic aspects that can aid in the treatment of advanced cases of HCC. An up-to-date list of cancer vaccines and oncolytic virus therapies under current clinical investigation is provided (Table 3).

Antigen peptide vaccines

Targeting these unique antigens can contribute to a significant approach in the development of vaccines in cases of HCC. Foremost antigens include AFP, GPC3, NY-ESO-1, hTERT, and hepatocellular and melanoma antigen gene-A (*MAGE-A*) as previously mentioned[119]. GPC3 peptide, a heparan sulfate glycoprotein and member of the GPC proteoglycan group, functions through attaching to the cell membrane by a glycophosphatidylinositol anchor, further regulating several growth factors through the Wnts, hedgehog, and the BMP signaling pathways[120]. This peptide can be an ideal target for HCC vaccine development, as GPC3 is detected in the vast majority of HCC cases[121]. In a phase I trial conducted on 33 patients to ensure the safety of the GPC3-derived peptide vaccine for advanced HCC, results indicated a well-tolerated vaccine with significant immune response, and an improvement in the median OS rate, 12.2 mo (95%CI: 6.5-18.0) in patients with high GPC3-specific cytotoxic T-lymphocytes frequencies, as compared with 8.5 mo (95%CI: 3.7-13.1) in those with low GPC3-specific cytotoxic lymphocyte frequencies ($P = 0.033$)[122]. However, it was not determined if these tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide-specific cytotoxic lymphocytes, prompting the initiation of a phase II trial (UMIN000005093) in patients with advanced HCC having failed sorafenib, which demonstrated induction of GPC3 peptide specific cytotoxic lymphocytes capable of infiltrating tumors[123].

DC vaccines

DCs have a potent effect on the T cell responses in HCC and other malignancies by acting as antigen presenting cells towards cytotoxic T lymphocytes[124]. Prior to administration of these vaccines to subjects, allogenic DCs are loaded with tumor antigens *ex vivo*, and then stimulated with cytokines such as granulocyte-macrophage colony-stimulating factor for further expansion[125]. In a recent 2016 study, DC vaccines loaded with autologous tumor cell lysate (Hepa1-6 cells) were studied using an orthotopic murine model of HCC. Treatment using this combination not only inhibited progression of murine HCC, but results were promising with a 90% survival rate compared to survival rate lower than 5% in untreated mice, suggesting alteration of the immunosuppressive tumor microenvironment as measured by lymphocyte and

Table 3 Ongoing clinical trials investigating cancer vaccines and oncolytic viruses in the treatment of hepatocellular carcinoma according to ClinicalTrials.gov

Product	Phase	Estimated completion	Study/identifier
OV telomelysin (OBP-301)	I	April 2021	NCT02293850
DC vaccine	II	April 2022	NCT04317248
DC vaccine + pneumococcal 13	I	May 2022	NCT03942328
OV M1-c6v1	I	October 2022	NCT04665362
DC vaccine	I	June 2023	NCT04147078
DSP-7888 PV + nivolumab or pembrolizumab	I/II	February 2024	NCT03311334
DNAJB1-PRKACA fusion kinase vaccine	I	March 2024	NCT04248569
TAEK-VAC-HerBy vaccine	I/II	December 2024	NCT04246671

DC: Dendritic cell; OV: Oncolytic virus; PV: Peptide vaccine.

cytokine analysis[126]. Although DCs seem to be an attractive treatment modality, further research is required to establish better *in vitro* handling of DCs, better methods of cellular delivery as well as further adaptation to the in-vivo environment following cellular administration.

Oncolytic virus therapy

The use of exogenously administered viruses has paved way as a new and promising modality of targeting cancer cells and promoting neoplastic destruction following replication and subsequent lysis of tumor cells. Such actions allow for the release of antigens after lysis, which triggers antitumor activity[29]. One such virus is Pexa-Vec (pexastimogene devacirepvec, JX-594), a modified pox virus expressing granulocyte-macrophage stimulating factor that activates both innate and adaptive immune responses, remaining as the leading oncolytic virus of interest when combating HCC [127,128]. A randomized phase II trial conducted in patients with advanced HCC, demonstrated dose-related significant improvement in OS at high doses (14.1 mo) compared to low-dose treatment (6.7 mo), coupled with tumor regression involving one complete response[129]. Current interest has sparked trials with combination of common therapies. We eagerly await the results of Pexa-Vec with nivolumab (NCT03071094), and Pexa-Vec with sorafenib (PHOCUS; NCT02562755) for treatment of patients with advanced HCC.

CONCLUSION

Treatment of HCC has made significant strides over the past decade since the approval of sorafenib as the first line, standard of care, molecular based treatment in 2007. More recently over the past few years four novel drugs: lenvatinib, ramucirumab, regorafenib, cabozantinib, have proven to be both safe and efficacious leading to approval for clinical use as an alternative or in addition to sorafenib. Clinical trials investigating the check point inhibitors atezolizumab, ipilimumab, nivolumab, and pembrolizumab have proven efficacy and identified new treatment modalities for HCC. As discussed in the paper adoptive immunotherapy including CAR-T cells, TCR engineered T cells, CIK cells, TIL cells, NK cells, DCs, and various vaccines have proven to be a promising therapeutic strategy for the treatment of HCC, however there are still many obstacles that need to be overcome prior to clinical application. Such challenges include identification of the ideal targeted antigen, overcoming the heterogenous and immunosuppressive environment of HCC, ensuring adequate delivery and persistence of immune cells, avoiding toxicity, and continued development of safe and cost-effective measures for cellular based treatment production and subsequent delivery to patients. The differences displayed between hepatic tumors amongst individuals across various stages of liver disease can possibly explain the difference in both clinical effects and adverse outcomes of immunotherapies studied. Therefore, we believe that the future of HCC treatment lies in the prospect of combined treatment modalities to exhibit synergistic effects, which have already entered various clinical trials as described

within this paper. While most completed and ongoing studies focus on individuals with adequate underlying hepatic function (Child-Pugh Class A), it is imperative to expand studies to other patient subgroups including those with early-stage disease and those undergoing palliative treatment. We believe that continued investigation over the coming years will overcome current barriers and lead to the development of novel and multifactorial immunotherapeutic strategies allowing for great advances in the treatment of HCC.

REFERENCES

1 **Kim E**, Viatour P. Hepatocellular carcinoma: old friends and new tricks. *Exp Mol Med* 2020; **52**: 1898-1907 [PMID: 33268834 DOI: 10.1038/s12276-020-00527-1]

2 **Janevska D**, Chaloska-Ivanova V, Janevski V. Hepatocellular Carcinoma: Risk Factors, Diagnosis and Treatment. *Open Access Maced J Med Sci* 2015; **3**: 732-736 [PMID: 27275318 DOI: 10.3889/oamjms.2015.111]

3 **Sung H**, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021 [PMID: 33538338 DOI: 10.3322/caac.21660]

4 **Bray F**, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; **68**: 394-424 [PMID: 30207593 DOI: 10.3322/caac.21492]

5 **Yang JD**, Altekruse SF, Nguyen MH, Gores GJ, Roberts LR. Impact of country of birth on age at the time of diagnosis of hepatocellular carcinoma in the United States. *Cancer* 2017; **123**: 81-89 [PMID: 27571320 DOI: 10.1002/cncr.30246]

6 **Yang M**, Parikh ND, Liu H, Wu E, Rao H, Feng B, Lin A, Wei L, Lok AS. Incidence and risk factors of hepatocellular carcinoma in patients with hepatitis C in China and the United States. *Sci Rep* 2020; **10**: 20922 [PMID: 33262356 DOI: 10.1038/s41598-020-77515-y]

7 **Maida M**, Orlando E, Cammà C, Cabibbo G. Staging systems of hepatocellular carcinoma: a review of literature. *World J Gastroenterol* 2014; **20**: 4141-4150 [PMID: 24764652 DOI: 10.3748/wjg.v20.i15.4141]

8 **Dhir M**, Melin AA, Douaiher J, Lin C, Zhen WK, Hussain SM, Geschwind JF, Doyle MB, Abou-Alfa GK, Are C. A Review and Update of Treatment Options and Controversies in the Management of Hepatocellular Carcinoma. *Ann Surg* 2016; **263**: 1112-1125 [PMID: 26813914 DOI: 10.1097/SLA.0000000000001556]

9 **Llovet JM**, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, Gores G. Hepatocellular carcinoma. *Nat Rev Dis Primers* 2016; **2**: 16018 [PMID: 27158749 DOI: 10.1038/nrdp.2016.18]

10 **Kinoshita A**, Onoda H, Fushiya N, Koike K, Nishino H, Tajiri H. Staging systems for hepatocellular carcinoma: Current status and future perspectives. *World J Hepatol* 2015; **7**: 406-424 [PMID: 25848467 DOI: 10.4254/wjh.v7.i3.406]

11 **Gbolahan OB**, Schacht MA, Beckley EW, LaRoche TP, O'Neil BH, Pyko M. Locoregional and systemic therapy for hepatocellular carcinoma. *J Gastrointest Oncol* 2017; **8**: 215-228 [PMID: 28480062 DOI: 10.21037/jgo.2017.03.13]

12 **Václav T**. Surgical treatment of hepatocellular carcinoma. *Klin Onkol* 2020; **33**: 30-33 [PMID: 33213163 DOI: 10.14735/amko20203S30]

13 **Jadlowiec CC**, Taner T. Liver transplantation: Current status and challenges. *World J Gastroenterol* 2016; **22**: 4438-4445 [PMID: 27182155 DOI: 10.3748/wjg.v22.i18.4438]

14 **Rimassa L**, Pressiani T, Merle P. Systemic Treatment Options in Hepatocellular Carcinoma. *Liver Cancer* 2019; **8**: 427-446 [PMID: 31799201 DOI: 10.1159/000499765]

15 **Sim HW**, Knox J. Hepatocellular carcinoma in the era of immunotherapy. *Curr Probl Cancer* 2018; **42**: 40-48 [PMID: 29150141 DOI: 10.1016/j.currproblcancer.2017.10.007]

16 **Kudo M**. Lenvatinib May Drastically Change the Treatment Landscape of Hepatocellular Carcinoma. *Liver Cancer* 2018; **7**: 1-19 [PMID: 29662829 DOI: 10.1159/000487148]

17 **Llovet JM**, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378-390 [PMID: 18650514 DOI: 10.1056/NEJMoa0708857]

18 **Cheng AL**, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25-34 [PMID: 19095497 DOI: 10.1016/S1470-2045(08)70285-7]

19 **Alqahtani A**, Khan Z, Alloghbi A, Said Ahmed TS, Ashraf M, Hammouda DM. Hepatocellular Carcinoma: Molecular Mechanisms and Targeted Therapies. *Medicina (Kaunas)* 2019; **55** [PMID: 31450841 DOI: 10.3390/medicina55090526]

20 **Al-Salama ZT**, Syed YY, Scott LJ. Lenvatinib: A Review in Hepatocellular Carcinoma. *Drugs*

2019; **79**: 665-674 [PMID: 30993651 DOI: 10.1007/s40265-019-01116-x]

21 **Kudo M**, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, Baron A, Park JW, Han G, Jassem J, Blanc JF, Vogel A, Komov D, Evans TRJ, Lopez C, Dutcus C, Guo M, Saito K, Kraljevic S, Tamai T, Ren M, Cheng AL. Lenvatinib *vs* sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet* 2018; **391**: 1163-1173 [PMID: 29433850 DOI: 10.1016/S0140-6736(18)30207-1]

22 **Personeni N**, Pressiani T, Rimassa L. Lenvatinib for the treatment of unresectable hepatocellular carcinoma: evidence to date. *J Hepatocell Carcinoma* 2019; **6**: 31-39 [PMID: 30775342 DOI: 10.2147/JHC.S168953]

23 **Zhu AX**, Park JO, Ryoo BY, Yen CJ, Poon R, Pastorelli D, Blanc JF, Chung HC, Baron AD, Pfiffer TE, Okusaka T, Kubackova K, Trojan J, Sastre J, Chau I, Chang SC, Abada PB, Yang L, Schwartz JD, Kudo M; REACH Trial Investigators. Ramucirumab *vs* placebo as second-line treatment in patients with advanced hepatocellular carcinoma following first-line therapy with sorafenib (REACH): a randomised, double-blind, multicentre, phase 3 trial. *Lancet Oncol* 2015; **16**: 859-870 [PMID: 26095784 DOI: 10.1016/S1470-2045(15)00050-9]

24 **Zhu AX**, Kang YK, Yen CJ, Finn RS, Galle PR, Llovet JM, Assenat E, Brandi G, Lim HY, Pracht M, Rau KM, Merle P, Motomura K, Ohno I, Daniele B, Shin D, Gerken G, Abada P, Hsu Y, Kudo M. REACH-2: A randomized, double-blind, placebo-controlled phase 3 study of ramucirumab *vs* placebo as second-line treatment in patients with advanced hepatocellular carcinoma (HCC) and elevated baseline alpha-fetoprotein (AFP) following first-line sorafenib. *J Clin Oncol* 2018; **36**: 4003 [DOI: 10.1200/JCO.2018.36.15_suppl.4003]

25 **Cerrito L**, Ponziani FR, Garcovich M, Tortora A, Annicchiarico BE, Pompili M, Siciliano M, Gasbarrini A. Regorafenib: a promising treatment for hepatocellular carcinoma. *Expert Opin Pharmacother* 2018; **19**: 1941-1948 [PMID: 30345837 DOI: 10.1080/14656566.2018.1534956]

26 **Bruix J**, Qin S, Merle P, Granito A, Huang YH, Bodoky G, Pracht M, Yokosuka O, Rosmorduc O, Breder V, Gerolami R, Masi G, Ross PJ, Song T, Bronowicki JP, Ollivier-Hourmand I, Kudo M, Cheng AL, Llovet JM, Finn RS, LeBerre MA, Baumhauer A, Meinhardt G, Han G; RESORCE Investigators. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017; **389**: 56-66 [PMID: 27932229 DOI: 10.1016/S0140-6736(16)32453-9]

27 **Abou-Alfa GK**, Meyer T, Cheng AL, El-Khoueiry AB, Rimassa L, Ryoo BY, Cicin I, Merle P, Chen Y, Park JW, Blanc JF, Bolondi L, Klümpen HJ, Chan SL, Zagonel V, Pressiani T, Ryu MH, Venook AP, Hessel C, Borgman-Hagey AE, Schwab G, Kelley RK. Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma. *N Engl J Med* 2018; **379**: 54-63 [PMID: 29972759 DOI: 10.1056/NEJMoa1717002]

28 **Hargadon KM**, Johnson CE, Williams CJ. Immune checkpoint blockade therapy for cancer: An overview of FDA-approved immune checkpoint inhibitors. *Int Immunopharmacol* 2018; **62**: 29-39 [PMID: 29990692 DOI: 10.1016/j.intimp.2018.06.001]

29 **Nakano S**, Eso Y, Okada H, Takai A, Takahashi K, Seno H. Recent Advances in Immunotherapy for Hepatocellular Carcinoma. *Cancers (Basel)* 2020; **12** [PMID: 32218257 DOI: 10.3390/cancers12040775]

30 **Gao Q**, Wang XY, Qiu SJ, Yamato I, Sho M, Nakajima Y, Zhou J, Li BZ, Shi YH, Xiao YS, Xu Y, Fan J. Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. *Clin Cancer Res* 2009; **15**: 971-979 [PMID: 19188168 DOI: 10.1158/1078-0432.CCR-08-1608]

31 **Fritz JM**, Lenardo MJ. Development of immune checkpoint therapy for cancer. *J Exp Med* 2019; **216**: 1244-1254 [PMID: 31068379 DOI: 10.1084/jem.20182395]

32 **Lee MS**, Ryoo BY, Hsu CH, Numata K, Stein S, Verret W, Hack SP, Spahn J, Liu B, Abdullah H, Wang Y, He AR, Lee KH; GO30140 investigators. Atezolizumab with or without bevacizumab in unresectable hepatocellular carcinoma (GO30140): an open-label, multicentre, phase 1b study. *Lancet Oncol* 2020; **21**: 808-820 [PMID: 32502443 DOI: 10.1016/S1470-2045(20)30156-X]

33 **Finn RS**, Qin S, Ikeda M, Galle PR, Ducreux M, Kim TY, Kudo M, Breder V, Merle P, Kaseb AO, Li D, Verret W, Xu DZ, Hernandez S, Liu J, Huang C, Mulla S, Wang Y, Lim HY, Zhu AX, Cheng AL; IMbrave150 Investigators. Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. *N Engl J Med* 2020; **382**: 1894-1905 [PMID: 32402160 DOI: 10.1056/NEJMoa1915745]

34 **Hack SP**, Spahn J, Chen M, Cheng AL, Kaseb A, Kudo M, Lee HC, Yopp A, Chow P, Qin S. IMbrave 050: a Phase III trial of atezolizumab plus bevacizumab in high-risk hepatocellular carcinoma after curative resection or ablation. *Future Oncol* 2020; **16**: 975-989 [PMID: 32352320 DOI: 10.2217/fon-2020-0162]

35 **Yau T**, Kang YK, Kim TY, El-Khoueiry AB, Santoro A, Sangro B, Melero I, Kudo M, Hou MM, Matilla A, Tovoli F, Knox JJ, Ruth He A, El-Rayes BF, Acosta-Rivera M, Lim HY, Neely J, Shen Y, Wisniewski T, Anderson J, Hsu C. Efficacy and Safety of Nivolumab Plus Ipilimumab in Patients With Advanced Hepatocellular Carcinoma Previously Treated With Sorafenib: The CheckMate 040 Randomized Clinical Trial. *JAMA Oncol* 2020 [PMID: 33001135 DOI: 10.1001/jamaoncol.2020.4564]

36 **El-Khoueiry AB**, Sangro B, Yau T, Crocenzi TS, Kudo M, Hsu C, Kim TY, Choo SP, Trojan J, Welling TH Rd, Meyer T, Kang YK, Yeo W, Chopra A, Anderson J, Dela Cruz C, Lang L, Neely J, Tang H, Dastani HB, Melero I. Nivolumab in patients with advanced hepatocellular carcinoma

(CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet* 2017; **389**: 2492-2502 [PMID: 28434648 DOI: 10.1016/S0140-6736(17)31046-2]

37 **Yau T**, Park J, Finn R, Cheng AL, Mathurin P, Edeline J, Kudo M, Han KH, Harding J, Merle P. CheckMate 459: A randomized, multi-center phase III study of nivolumab (NIVO) *vs* sorafenib (SOR) as first-line (1L) treatment in patients (pts) with advanced hepatocellular carcinoma (aHCC). *Ann Oncol* 2019; **30**: v874-v875 [DOI: 10.1093/annonc/mdz394.029]

38 **Kwok G**, Yau TC, Chiu JW, Tse E, Kwong YL. Pembrolizumab (Keytruda). *Hum Vaccin Immunother* 2016; **12**: 2777-2789 [PMID: 27398650 DOI: 10.1080/21645515.2016.1199310]

39 **Zhu AX**, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, Verslype C, Zagonel V, Fartoux L, Vogel A, Sarker D, Verset G, Chan SL, Knox J, Daniele B, Webber AL, Ebbinghaus SW, Ma J, Siegel AB, Cheng AL, Kudo M; KEYNOTE-224 investigators. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. *Lancet Oncol* 2018; **19**: 940-952 [PMID: 29875066 DOI: 10.1016/S1470-2045(18)30351-6]

40 **Finn RS**, Ryoo BY, Merle P, Kudo M, Bouattour M, Lim HY, Breder V, Edeline J, Chao Y, Ogasawara S, Yau T, Garrido M, Chan SL, Knox J, Daniele B, Ebbinghaus SW, Chen E, Siegel AB, Zhu AX, Cheng AL; KEYNOTE-240 investigators. Pembrolizumab As Second-Line Therapy in Patients With Advanced Hepatocellular Carcinoma in KEYNOTE-240: A Randomized, Double-Blind, Phase III Trial. *J Clin Oncol* 2020; **38**: 193-202 [PMID: 31790344 DOI: 10.1200/JCO.19.01307]

41 **Özdemir F**, Baskiran A. The Importance of AFP in Liver Transplantation for HCC. *J Gastrointest Cancer* 2020; **51**: 1127-1132 [PMID: 32845425 DOI: 10.1007/s12029-020-00486-w]

42 **Mizejewski GJ**. Levels of alpha-fetoprotein during pregnancy and early infancy in normal and disease states. *Obstet Gynecol Surv* 2003; **58**: 804-826 [PMID: 14668662 DOI: 10.1097/01.OGX.0000099770.97668.18]

43 **Zhu W**, Peng Y, Wang L, Hong Y, Jiang X, Li Q, Liu H, Huang L, Wu J, Celis E, Merchen T, Kruse E, He Y. Identification of α-fetoprotein-specific T-cell receptors for hepatocellular carcinoma immunotherapy. *Hepatology* 2018; **68**: 574-589 [PMID: 29443377 DOI: 10.1002/hep.29844]

44 **Liu H**, Xu Y, Xiang J, Long L, Green S, Yang Z, Zimdahl B, Lu J, Cheng N, Horan LH, Liu B, Yan S, Wang P, Diaz J, Jin L, Nakano Y, Morales JF, Zhang P, Liu LX, Staley BK, Priceman SJ, Brown CE, Forman SJ, Chan VW, Liu C. Targeting Alpha-Fetoprotein (AFP)-MHC Complex with CAR T-Cell Therapy for Liver Cancer. *Clin Cancer Res* 2017; **23**: 478-488 [PMID: 27535982 DOI: 10.1158/1078-0432.CCR-16-1203]

45 **Montalbano M**, Georgiadis J, Masterson AL, McGuire JT, Prajapati J, Shirafkan A, Rastellini C, Cicalese L. Biology and function of glypican-3 as a candidate for early cancerous transformation of hepatocytes in hepatocellular carcinoma (Review). *Oncol Rep* 2017; **37**: 1291-1300 [PMID: 28098909 DOI: 10.3892/or.2017.5387]

46 **Kaseb AO**, Hassan M, Lacin S, Abdel-Wahab R, Amin HM, Shalaby A, Wolff RA, Yao J, Rashid A, Vennapusa B, Feng J, Ohtomo T. Evaluating clinical and prognostic implications of Glypican-3 in hepatocellular carcinoma. *Oncotarget* 2016; **7**: 69916-69926 [PMID: 27655712 DOI: 10.18632/oncotarget.12066]

47 **Zhou F**, Shang W, Yu X, Tian J. Glypican-3: A promising biomarker for hepatocellular carcinoma diagnosis and treatment. *Med Res Rev* 2018; **38**: 741-767 [PMID: 28621802 DOI: 10.1002/med.21455]

48 **Jiang Z**, Jiang X, Chen S, Lai Y, Wei X, Li B, Lin S, Wang S, Wu Q, Liang Q, Liu Q, Peng M, Yu F, Weng J, Du X, Pei D, Liu P, Yao Y, Xue P, Li P. Anti-GPC3-CAR T Cells Suppress the Growth of Tumor Cells in Patient-Derived Xenografts of Hepatocellular Carcinoma. *Front Immunol* 2016; **7**: 690 [PMID: 28123387 DOI: 10.3389/fimmu.2016.00690]

49 **Li R**, Gong J, Xiao C, Zhu S, Hu Z, Liang J, Li X, Yan X, Zhang X, Li D, Liu W, Chong Y, Jie Y. A comprehensive analysis of the MAGE family as prognostic and diagnostic markers for hepatocellular carcinoma. *Genomics* 2020; **112**: 5101-5114 [PMID: 32941982 DOI: 10.1016/j.ygeno.2020.09.026]

50 **Gordeeva O**. Cancer-testis antigens: Unique cancer stem cell biomarkers and targets for cancer therapy. *Semin Cancer Biol* 2018; **53**: 75-89 [PMID: 30171980 DOI: 10.1016/j.semcancer.2018.08.006]

51 **Thomas R**, Al-Khadairi G, Roelands J, Hendrickx W, Dermime S, Bedognetti D, Decock J. NY-ESO-1 Based Immunotherapy of Cancer: Current Perspectives. *Front Immunol* 2018; **9**: 947 [PMID: 29770138 DOI: 10.3389/fimmu.2018.00947]

52 **Bohne F**, Chmielewski M, Ebert G, Wiegmann K, Kürschner T, Schulze A, Urban S, Krönke M, Abken H, Protzer U. T cells redirected against hepatitis B virus surface proteins eliminate infected hepatocytes. *Gastroenterology* 2008; **134**: 239-247 [PMID: 18166356 DOI: 10.1053/j.gastro.2007.11.002]

53 **Kruse RL**, Shum T, Tashiro H, Barzi M, Yi Z, Whitten-Bauer C, Legras X, Bissig-Choisat B, Garaigorta U, Gottschalk S, Bissig KD. HBsAg-redirected T cells exhibit antiviral activity in HBV-infected human liver chimeric mice. *Cytotherapy* 2018; **20**: 697-705 [PMID: 29631939 DOI: 10.1016/j.jcyt.2018.02.002]

54 **Krause J**, von Felden J, Casar C, Fründt TW, Galaski J, Schmidt C, Jung C, Ittrich H, Weidemann SA, Krech T, Heumann A, Li J, Fischer L, Sauter G, Lohse AW, Wege H, Schulze K. Hepatocellular carcinoma: Intratumoral EpCAM-positive cancer stem cell heterogeneity identifies high-risk tumor

subtype. *BMC Cancer* 2020; **20**: 1130 [PMID: 33225916 DOI: 10.1186/s12885-020-07580-z]
55 **Ma Y**, Wang Z, Gong R, Li L, Wu H, Jin H. Specific cytotoxicity of MUC1 chimeric antigen receptor-engineered Jurkat T cells against hepatocellular carcinoma. *Acad J Second Mil Med Univ* 2014; **5**: 1177-1182 [DOI: 10.3724/SP.J.1008.2014.01177]
56 **Mizukoshi E**, Nakamoto Y, Marukawa Y, Arai K, Yamashita T, Tsuji H, Kuzushima K, Takiguchi M, Kaneko S. Cytotoxic T cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma. *Hepatology* 2006; **43**: 1284-1294 [PMID: 16729333 DOI: 10.1002/hep.21203]
57 **Mizukoshi E**, Kaneko S. Immune cell therapy for hepatocellular carcinoma. *J Hematol Oncol* 2019; **12**: 52 [PMID: 31142330 DOI: 10.1186/s13045-019-0742-5]
58 **Benmebarek MR**, Karches CH, Cadilha BL, Lesch S, Endres S, Kobold S. Killing Mechanisms of Chimeric Antigen Receptor (CAR) T Cells. *Int J Mol Sci* 2019; **20** [PMID: 30875739 DOI: 10.3390/ijms20061283]
59 **Chen Y**, E CY, Gong ZW, Liu S, Wang ZX, Yang YS, Zhang XW. Chimeric antigen receptor-engineered T-cell therapy for liver cancer. *Hepatobiliary Pancreat Dis Int* 2018; **17**: 301-309 [PMID: 29861325 DOI: 10.1016/j.hbpd.2018.05.005]
60 **Ping Y**, Liu C, Zhang Y. T-cell receptor-engineered T cells for cancer treatment: current status and future directions. *Protein Cell* 2018; **9**: 254-266 [PMID: 28108950 DOI: 10.1007/s13238-016-0367-1]
61 **Rochigneux P**, Chanez B, De Rauglaudre B, Mitry E, Chabannon C, Gilabert M. Adoptive Cell Therapy in Hepatocellular Carcinoma: Biological Rationale and First Results in Early Phase Clinical Trials. *Cancers (Basel)* 2021; **13** [PMID: 33450845 DOI: 10.3390/cancers13020271]
62 **Kershaw MH**, Westwood JA, Darcy PK. Gene-engineered T cells for cancer therapy. *Nat Rev Cancer* 2013; **13**: 525-541 [PMID: 23880905 DOI: 10.1038/nrc3565]
63 **Shi D**, Shi Y, Kaseb AO, Qi X, Zhang Y, Chi J, Lu Q, Gao H, Jiang H, Wang H, Yuan D, Ma H, Li Z, Zhai B. Chimeric Antigen Receptor-Glypican-3 T-Cell Therapy for Advanced Hepatocellular Carcinoma: Results of Phase I Trials. *Clin Cancer Res* 2020; **26**: 3979-3989 [PMID: 32371538 DOI: 10.1158/1078-0432.CCR-19-3259]
64 **Dai H**, Tong C, Shi D, Chen M, Guo Y, Chen D, Han X, Wang H, Wang Y, Shen P. Efficacy and biomarker analysis of CD133-directed CAR T cells in advanced hepatocellular carcinoma: a single-arm, open-label, phase II trial. *Oncoimmunology* 2020; **9**: 1846926 [PMID: 33312759 DOI: 10.1080/2162402X.2020.1846926]
65 **Caruana I**, Savoldo B, Hoyos V, Weber G, Liu H, Kim ES, Ittmann MM, Marchetti D, Dotti G. Heparanase promotes tumor infiltration and antitumor activity of CAR-redirected T lymphocytes. *Nat Med* 2015; **21**: 524-529 [PMID: 25849134 DOI: 10.1038/nm.3833]
66 **Mardomi A**, Abediankenari S. Matrix Metalloproteinase 8: Could it Benefit the CAR-T Cell Therapy of Solid Tumors? *Cancer Microenviron* 2018; **11**: 93-96 [PMID: 29589335 DOI: 10.1007/s12307-018-0208-2]
67 **Katz SC**, Burga RA, McCormack E, Wang LJ, Mooring W, Point GR, Khare PD, Thorn M, Ma Q, Stainken BF, Assanah EO, Davies R, Espat NJ, Junghans RP. Phase I Hepatic Immunotherapy for Metastases Study of Intra-Arterial Chimeric Antigen Receptor-Modified T-cell Therapy for CEA+ Liver Metastases. *Clin Cancer Res* 2015; **21**: 3149-3159 [PMID: 25850950 DOI: 10.1158/1078-0432.CCR-14-1421]
68 **Riley RS**, June CH, Langer R, Mitchell MJ. Delivery technologies for cancer immunotherapy. *Nat Rev Drug Discov* 2019; **18**: 175-196 [PMID: 30622344 DOI: 10.1038/s41573-018-0006-z]
69 **Ma W**, Zhu D, Li J, Chen X, Xie W, Jiang X, Wu L, Wang G, Xiao Y, Liu Z, Wang F, Li A, Shao D, Dong W, Liu W, Yuan Y. Coating biomimetic nanoparticles with chimeric antigen receptor T cell-membrane provides high specificity for hepatocellular carcinoma photothermal therapy treatment. *Theranostics* 2020; **10**: 1281-1295 [PMID: 31938065 DOI: 10.7150/thno.40291]
70 **Franchina DG**, Dostert C, Brenner D. Reactive Oxygen Species: Involvement in T Cell Signaling and Metabolism. *Trends Immunol* 2018; **39**: 489-502 [PMID: 29452982 DOI: 10.1016/j.it.2018.01.005]
71 **Dal Bo M**, De Mattia E, Baboci L, Mezzalira S, Cecchin E, Assaraf YG, Toffoli G. New insights into the pharmacological, immunological, and CAR-T-cell approaches in the treatment of hepatocellular carcinoma. *Drug Resist Updat* 2020; **51**: 100702 [PMID: 32371296 DOI: 10.1016/j.drup.2020.100702]
72 **Ligtenberg MA**, Mougiakakos D, Mukhopadhyay M, Witt K, Lladser A, Chmielewski M, Riet T, Abken H, Kiessling R. Coexpressed Catalase Protects Chimeric Antigen Receptor-Redirected T Cells as well as Bystander Cells from Oxidative Stress-Induced Loss of Antitumor Activity. *J Immunol* 2016; **196**: 759-766 [PMID: 26673145 DOI: 10.4049/jimmunol.1401710]
73 **Wu AA**, Drake V, Huang HS, Chiu S, Zheng L. Reprogramming the tumor microenvironment: tumor-induced immunosuppressive factors paralyze T cells. *Oncoimmunology* 2015; **4**: e1016700 [PMID: 26140242 DOI: 10.1080/2162402x.2015.1016700]
74 **Gargett T**, Yu W, Dotti G, Yvon ES, Christo SN, Hayball JD, Lewis ID, Brenner MK, Brown MP. GD2-specific CAR T Cells Undergo Potent Activation and Deletion Following Antigen Encounter but can be Protected From Activation-induced Cell Death by PD-1 Blockade. *Mol Ther* 2016; **24**: 1135-1149 [PMID: 27019998 DOI: 10.1038/mt.2016.63]
75 **Rupp LJ**, Schumann K, Roybal KT, Gate RE, Ye CJ, Lim WA, Marson A. CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. *Sci Rep*

2017; **7**: 737 [PMID: 28389661 DOI: 10.1038/s41598-017-00462-8]

76 **Shimabukuro-Vornhagen A**, Gödel P, Subklewe M, Stemmler HJ, Schlößer HA, Schlaak M, Kochanek M, Böll B, von Bergwelt-Baildon MS. Cytokine release syndrome. *J Immunother Cancer* 2018; **6**: 56 [PMID: 29907163 DOI: 10.1186/s40425-018-0343-9]

77 **Yáñez L**, Sánchez-Escamilla M, Perales MA. CAR T Cell Toxicity: Current Management and Future Directions. *Hemasphere* 2019; **3**: e186 [PMID: 31723825 DOI: 10.1097/HS9.0000000000000186]

78 **Gust J**, Taraseviciute A, Turtle CJ. Neurotoxicity Associated with CD19-Targeted CAR-T Cell Therapies. *CNS Drugs* 2018; **32**: 1091-1101 [PMID: 30387077 DOI: 10.1007/s40263-018-0582-9]

79 **Morgan RA**, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 2010; **18**: 843-851 [PMID: 20179677 DOI: 10.1038/mt.2010.24]

80 **Gargett T**, Brown MP. The inducible caspase-9 suicide gene system as a "safety switch" to limit on-target, off-tumor toxicities of chimeric antigen receptor T cells. *Front Pharmacol* 2014; **5**: 235 [PMID: 25389405 DOI: 10.3389/fphar.2014.00235]

81 **Paszkiewicz PJ**, Fräßle SP, Srivastava S, Sommermeyer D, Hudecek M, Drexler I, Sadelain M, Liu L, Jensen MC, Riddell SR, Busch DH. Targeted antibody-mediated depletion of murine CD19 CAR T cells permanently reverses B cell aplasia. *J Clin Invest* 2016; **126**: 4262-4272 [PMID: 27760047 DOI: 10.1172/JCI84813]

82 **Amatya C**, Pegues MA, Lam N, Vanasse D, Geldres C, Choi S, Hewitt SM, Feldman SA, Kochenderfer JN. Development of CAR T Cells Expressing a Suicide Gene Plus a Chimeric Antigen Receptor Targeting Signaling Lymphocytic-Activation Molecule F7. *Mol Ther* 2021; **29**: 702-717 [PMID: 33129371 DOI: 10.1016/j.ymthe.2020.10.008]

83 **Chen C**, Li K, Jiang H, Song F, Gao H, Pan X, Shi B, Bi Y, Wang H, Li Z. Development of T cells carrying two complementary chimeric antigen receptors against glypican-3 and asialoglycoprotein receptor 1 for the treatment of hepatocellular carcinoma. *Cancer Immunol Immunother* 2017; **66**: 475-489 [PMID: 28035433 DOI: 10.1007/s00262-016-1949-8]

84 **Liu X**, Wen J, Yi H, Hou X, Yin Y, Ye G, Wu X, Jiang X. Split chimeric antigen receptor-modified T cells targeting glypican-3 suppress hepatocellular carcinoma growth with reduced cytokine release. *Ther Adv Med Oncol* 2020; **12**: 1758835920910347 [PMID: 32215059 DOI: 10.1177/1758835920910347]

85 **Cho JH**, Collins JJ, Wong WW. Universal Chimeric Antigen Receptors for Multiplexed and Logical Control of T Cell Responses. *Cell* 2018; **173**: 1426-1438.e11 [PMID: 29706540 DOI: 10.1016/j.cell.2018.03.038]

86 **Rapoport AP**, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, Badros AZ, Garfall A, Weiss B, Finklestein J, Kulikovskaya I, Sinha SK, Kronsberg S, Gupta M, Bond S, Melchiori L, Brewer JE, Bennett AD, Gerry AB, Pumphrey NJ, Williams D, Tayton-Martin HK, Ribeiro L, Holdich T, Yanovich S, Hardy N, Yared J, Kerr N, Philip S, Westphal S, Siegel DL, Levine BL, Jakobsen BK, Kalos M, June CH. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med* 2015; **21**: 914-921 [PMID: 26193344 DOI: 10.1038/nm.3910]

87 **D'Angelo SP**, Melchiori L, Merchant MS, Bernstein D, Glod J, Kaplan R, Grupp S, Tap WD, Chagin K, Binder GK, Basu S, Lowther DE, Wang R, Bath N, Tipping A, Betts G, Ramachandran I, Navenot JM, Zhang H, Wells DK, Van Winkle E, Kari G, Trivedi T, Holdich T, Pandite L, Amado R, Mackall CL. Antitumor Activity Associated with Prolonged Persistence of Adoptively Transferred NY-ESO-1 ^{c259}T Cells in Synovial Sarcoma. *Cancer Discov* 2018; **8**: 944-957 [PMID: 29891538 DOI: 10.1158/2159-8290.CD-17-1417]

88 **Gehring AJ**, Xue SA, Ho ZZ, Teoh D, Ruedl C, Chia A, Koh S, Lim SG, Maini MK, Stauss H, Bertoletti A. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. *J Hepatol* 2011; **55**: 103-110 [PMID: 21145860 DOI: 10.1016/j.jhep.2010.10.025]

89 **Tan AT**, Yang N, Lee Krishnamoorthy T, Oei V, Chua A, Zhao X, Tan HS, Chia A, Le Bert N, Low D, Tan HK, Kumar R, Irani FG, Ho ZZ, Zhang Q, Guccione E, Wai LE, Koh S, Hwang W, Chow WC, Bertoletti A. Use of Expression Profiles of HBV-DNA Integrated Into Genomes of Hepatocellular Carcinoma Cells to Select T Cells for Immunotherapy. *Gastroenterology* 2019; **156**: 1862-1876.e9 [PMID: 30711630 DOI: 10.1053/j.gastro.2019.01.251]

90 **Chen W**, Cheng J, Zheng X, Yang F, Fam R, Koh S, Wai L-E, Wang T, Bertoletti A, Zhang Q. 273 Phase i study of LioCyx-M, autologous hepatitis B virus (HBV)-specific T cell receptor (TCR) T-cells, in recurrent HBV-related hepatocellular carcinoma (HCC) post-liver transplantation. *J Immunother Cancer* 2020; **8**: A167-A167 [DOI: 10.1136/jitc-2020-SITC2020.0273]

91 **Zhang R**, Zhang Z, Liu Z, Wei D, Wu X, Bian H, Chen Z. Adoptive cell transfer therapy for hepatocellular carcinoma. *Front Med* 2019; **13**: 3-11 [PMID: 30659408 DOI: 10.1007/s11684-019-0684-x]

92 **Lee JH**, Lee JH, Lim YS, Yeon JE, Song TJ, Yu SJ, Gwak GY, Kim KM, Kim YJ, Lee JW, Yoon JH. Adjuvant immunotherapy with autologous cytokine-induced killer cells for hepatocellular carcinoma. *Gastroenterology* 2015; **148**: 1383-91.e6 [PMID: 25747273 DOI: 10.1053/j.gastro.2015.02.055]

93 **Lee JH**, Lee JH, Lim YS, Yeon JE, Song TJ, Yu SJ, Gwak GY, Kim KM, Kim YJ, Lee JW, Yoon

JH. Sustained efficacy of adjuvant immunotherapy with cytokine-induced killer cells for hepatocellular carcinoma: an extended 5-year follow-up. *Cancer Immunol Immunother* 2019; **68**: 23-32 [PMID: 30232520 DOI: 10.1007/s00262-018-2247-4]

94 **Cai XR**, Li X, Lin JX, Wang TT, Dong M, Chen ZH, Jia CC, Hong YF, Lin Q, Wu XY. Autologous transplantation of cytokine-induced killer cells as an adjuvant therapy for hepatocellular carcinoma in Asia: an update meta-analysis and systematic review. *Oncotarget* 2017; **8**: 31318-31328 [PMID: 28412743 DOI: 10.18632/oncotarget.15454]

95 **Forget MA**, Tavera RJ, Haymaker C, Ramachandran R, Malu S, Zhang M, Wardell S, Fulbright OJ, Toth CL, Gonzalez AM, Thorsen ST, Flores E, Wahl A, Peng W, Amaria RN, Hwu P, Bernatchez C. A Novel Method to Generate and Expand Clinical-Grade, Genetically Modified, Tumor-Infiltrating Lymphocytes. *Front Immunol* 2017; **8**: 908 [PMID: 28824634 DOI: 10.3389/fimmu.2017.00908]

96 **Gomez Perdiguero E**, Geissmann F. Cancer immunology. Identifying the infiltrators. *Science* 2014; **344**: 801-802 [PMID: 24855239 DOI: 10.1126/science.1255117]

97 **Zeng DQ**, Yu YF, Ou QY, Li XY, Zhong RZ, Xie CM, Hu QG. Prognostic and predictive value of tumor-infiltrating lymphocytes for clinical therapeutic research in patients with non-small cell lung cancer. *Oncotarget* 2016; **7**: 13765-13781 [PMID: 26871598 DOI: 10.18632/oncotarget.7282]

98 **Kumar S**, Skeen MJ, Adiri Y, Yoon H, Vezys VD, Lukacher AE, Evavold BD, Ziegler HK, Boss JM. A cytokine promoter/yellow fluorescent protein reporter transgene serves as an early activation marker of lymphocyte subsets. *Cell Immunol* 2005; **237**: 131-140 [PMID: 16405934 DOI: 10.1016/j.cellimm.2005.11.003]

99 **Piñeiro Fernández J**, Luddy KA, Harmon C, O'Farrelly C. Hepatic Tumor Microenvironments and Effects on NK Cell Phenotype and Function. *Int J Mol Sci* 2019; **20** [PMID: 31450598 DOI: 10.3390/ijms20174131]

100 **Golubovskaya V**, Wu L. Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy. *Cancers (Basel)* 2016; **8** [PMID: 26999211 DOI: 10.3390/cancers8030036]

101 **Chew V**, Tow C, Teo M, Wong HL, Chan J, Gehring A, Loh M, Bolze A, Quek R, Lee VK, Lee KH, Abastado JP, Toh HC, Nardin A. Inflammatory tumour microenvironment is associated with superior survival in hepatocellular carcinoma patients. *J Hepatol* 2010; **52**: 370-379 [PMID: 19720422 DOI: 10.1016/j.jhep.2009.07.013]

102 **Takayama T**, Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J, Shimada K, Sakamoto M, Hirohashi S, Ohashi Y, Kakizoe T. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. *Lancet* 2000; **356**: 802-807 [PMID: 11022927 DOI: 10.1016/s0140-6736(00)02654-4]

103 **Jiang SS**, Tang Y, Zhang YJ, Weng DS, Zhou ZG, Pan K, Pan QZ, Wang QJ, Liu Q, He J, Zhao JJ, Li J, Chen MS, Chang AE, Li Q, Xia JC. A phase I clinical trial utilizing autologous tumor-infiltrating lymphocytes in patients with primary hepatocellular carcinoma. *Oncotarget* 2015; **6**: 41339-41349 [PMID: 26515587 DOI: 10.18632/oncotarget.5463]

104 **Liu P**, Chen L, Zhang H. Natural Killer Cells in Liver Disease and Hepatocellular Carcinoma and the NK Cell-Based Immunotherapy. *J Immunol Res* 2018; **2018**: 1206737 [PMID: 30255103 DOI: 10.1155/2018/1206737]

105 **Gao B**, Jeong WI, Tian Z. Liver: An organ with predominant innate immunity. *Hepatology* 2008; **47**: 729-736 [PMID: 18167066 DOI: 10.1002/hep.22034]

106 **Lin M**, Alnaggar M, Liang S, Wang X, Liang Y, Zhang M, Chen J, Niu L, Xu K. An important discovery on combination of irreversible electroporation and allogeneic natural killer cell immunotherapy for unresectable pancreatic cancer. *Oncotarget* 2017; **8**: 101795-101807 [PMID: 29254205 DOI: 10.18632/oncotarget.21974]

107 **Lin M**, Liang SZ, Wang XH, Liang YQ, Zhang MJ, Niu LZ, Chen JB, Li HB, Xu KC. Clinical efficacy of percutaneous cryoablation combined with allogenic NK cell immunotherapy for advanced non-small cell lung cancer. *Immunol Res* 2017; **65**: 880-887 [PMID: 28508945 DOI: 10.1007/s12026-017-8927-x]

108 **Lin M**, Liang S, Wang X, Liang Y, Zhang M, Chen J, Niu L, Xu K. Cryoablation combined with allogenic natural killer cell immunotherapy improves the curative effect in patients with advanced hepatocellular cancer. *Oncotarget* 2017; **8**: 81967-81977 [PMID: 29137237 DOI: 10.18632/oncotarget.17804]

109 **Alnaggar M**, Lin M, Mesmar A, Liang S, Qaid A, Xu K, Chen J, Niu L, Yin Z. Allogenic Natural Killer Cell Immunotherapy Combined with Irreversible Electroporation for Stage IV Hepatocellular Carcinoma: Survival Outcome. *Cell Physiol Biochem* 2018; **48**: 1882-1893 [PMID: 30092590 DOI: 10.1159/000492509]

110 **Nowakowska P**, Romanski A, Miller N, Odendahl M, Bonig H, Zhang C, Seifried E, Wels WS, Tonn T. Clinical grade manufacturing of genetically modified, CAR-expressing NK-92 cells for the treatment of ErbB2-positive malignancies. *Cancer Immunol Immunother* 2018; **67**: 25-38 [PMID: 28879551 DOI: 10.1007/s00262-017-2055-2]

111 **Yu M**, Luo H, Fan M, Wu X, Shi B, Di S, Liu Y, Pan Z, Jiang H, Li Z. Development of GPC3-Specific Chimeric Antigen Receptor-Engineered Natural Killer Cells for the Treatment of Hepatocellular Carcinoma. *Mol Ther* 2018; **26**: 366-378 [PMID: 29339014 DOI: 10.1016/j.ymthe.2017.12.012]

112 **Liu B**, Liu ZZ, Zhou ML, Lin JW, Chen XM, Li Z, Gao WB, Yu ZD, Liu T. Development of cMETspecific chimeric antigen receptorengineered natural killer cells with cytotoxic effects on human liver cancer HepG2 cells. *Mol Med Rep* 2019; **20**: 2823-2831 [PMID: 31524233 DOI:

10.3892/mmr.2019.10529]
113 **Gardner A**, de Mingo Pulido Á, Ruffell B. Dendritic Cells and Their Role in Immunotherapy. *Front Immunol* 2020; **11**: 924 [PMID: 32508825 DOI: 10.3389/fimmu.2020.00924]
114 **Wang S**, Wang X, Zhou X, Lyerly HK, Morse MA, Ren J. DC-CIK as a widely applicable cancer immunotherapy. *Expert Opin Biol Ther* 2020; **20**: 601-607 [PMID: 32033522 DOI: 10.1080/14712598.2020.1728250]
115 **Zhou Z**, Qin H, Weng L, Ni Y. Clinical efficacy of DC-CIK combined with sorafenib in the treatment of advanced hepatocellular carcinoma. *J BUON* 2019; **24**: 615-621 [PMID: 31128014]
116 **Zhang J**, Li H, Gao D, Zhang B, Zheng M, Lun M, Wei M, Duan R, Guo M, Hua J, Liu Q, Bai J, Liu H, Zheng J, Yao H. A prognosis and impact factor analysis of DC-CIK cell therapy for patients with hepatocellular carcinoma undergoing postoperative TACE. *Cancer Biol Ther* 2018; **19**: 475-483 [PMID: 29400599 DOI: 10.1080/15384047.2018.1433501]
117 **Cao J**, Kong FH, Liu X, Wang XB. Immunotherapy with dendritic cells and cytokine-induced killer cells for hepatocellular carcinoma: A meta-analysis. *World J Gastroenterol* 2019; **25**: 3649-3663 [PMID: 31367163 DOI: 10.3748/wjg.v25.i27.3649]
118 **Guo ZS**, Lu B, Guo Z, Giehl E, Feist M, Dai E, Liu W, Storkus WJ, He Y, Liu Z, Bartlett DL. Vaccinia virus-mediated cancer immunotherapy: cancer vaccines and oncolytics. *J Immunother Cancer* 2019; **7**: 6 [PMID: 30626434 DOI: 10.1186/s40425-018-0495-7]
119 **Park R**, Eshrat F, Al-Jumayli M, Saeed A. Immuno-Oncotherapeutic Approaches in Advanced Hepatocellular Carcinoma. *Vaccines (Basel)* 2020; **8** [PMID: 32784389 DOI: 10.3390/vaccines8030447]
120 **Filmus J**, Capurro M, Rast J. Glypicans. *Genome Biol* 2008; **9**: 224 [PMID: 18505598 DOI: 10.1186/gb-2008-9-5-224]
121 **Nobuoka D**, Yoshikawa T, Sawada Y, Fujiwara T, Nakatsura T. Peptide vaccines for hepatocellular carcinoma. *Hum Vaccin Immunother* 2013; **9**: 210-212 [PMID: 23442593 DOI: 10.4161/hv.22473]
122 **Sawada Y**, Yoshikawa T, Nobuoka D, Shirakawa H, Kuronuma T, Motomura Y, Mizuno S, Ishii H, Nakachi K, Konishi M, Nakagohri T, Takahashi S, Gotohda N, Takayama T, Yamao K, Uesaka K, Furuse J, Kinoshita T, Nakatsura T. Phase I trial of a glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunologic evidence and potential for improving overall survival. *Clin Cancer Res* 2012; **18**: 3686-3696 [PMID: 22577059 DOI: 10.1158/1078-0432.CCR-11-3044]
123 **Shimizu Y**, Suzuki T, Yoshikawa T, Tsuchiya N, Sawada Y, Endo I, Nakatsura T. Cancer immunotherapy-targeted glypican-3 or neoantigens. *Cancer Sci* 2018; **109**: 531-541 [PMID: 29285841 DOI: 10.1111/cas.13485]
124 **Fan J**, Han J, Li J, Gu A, Yin D, Song F, Wang L, Yi Y. The expression and function of immunoglobulin-like transcript 4 in dendritic cells from patients with hepatocellular carcinoma. *Hum Immunol* 2020; **81**: 714-725 [PMID: 33228921 DOI: 10.1016/j.humimm.2020.10.003]
125 **Gu YZ**, Zhao X, Song XR. Ex vivo pulsed dendritic cell vaccination against cancer. *Acta Pharmacol Sin* 2020; **41**: 959-969 [PMID: 32366940 DOI: 10.1038/s41401-020-0415-5]
126 **Wang Q**, Luan W, Warren L, Kadri H, Kim KW, Goz V, Blank S, Isabel Fiel M, Hiotis SP. Autologous Tumor Cell Lysate-Loaded Dendritic Cell Vaccine Inhibited Tumor Progression in an Orthotopic Murine Model for Hepatocellular Carcinoma. *Ann Surg Oncol* 2016; **23**: 574-582 [PMID: 26786094 DOI: 10.1245/s10434-015-5035-9]
127 **Kim JH**, Oh JY, Park BH, Lee DE, Kim JS, Park HE, Roh MS, Je JE, Yoon JH, Thorne SH, Kirn D, Hwang TH. Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. *Mol Ther* 2006; **14**: 361-370 [PMID: 16905462 DOI: 10.1016/j.ymthe.2006.05.008]
128 **Parato KA**, Breitbach CJ, Le Boeuf F, Wang J, Storbeck C, Ilkow C, Diallo JS, Falls T, Burns J, Garcia V, Kanji F, Evgin L, Hu K, Paradis F, Knowles S, Hwang TH, Vanderhyden BC, Auer R, Kirn DH, Bell JC. The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers. *Mol Ther* 2012; **20**: 749-758 [PMID: 22186794 DOI: 10.1038/mt.2011.276]
129 **Heo J**, Reid T, Ruo L, Breitbach CJ, Rose S, Bloomston M, Cho M, Lim HY, Chung HC, Kim CW, Burke J, Lencioni R, Hickman T, Moon A, Lee YS, Kim MK, Daneshmand M, Dubois K, Longpre L, Ngo M, Rooney C, Bell JC, Rhee BG, Patt R, Hwang TH, Kirn DH. Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nat Med* 2013; **19**: 329-336 [PMID: 23396206 DOI: 10.1038/nm.3089]

World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2021 May 24; 12(5): 309-322

DOI: 10.5306/wjco.v12.i5.309

ISSN 2218-4333 (online)

MINIREVIEWS

COVID-19: Where is the treatment?

Bonala Sabeerabi, Sarojamma Vemula, Ramakrishna Vadde, Ganji Purnachandra Nagaraju

ORCID number: Bonala Sabeerabi 0000-0002-0783-0862; Sarojamma Vemula 0000-0003-1966-6753; Ramakrishna Vadde 0000-0002-5223-7208; Ganji Purnachandra Nagaraju 0000-0002-4989-5234.

Author contributions: All authors are equally contributed.

Conflict-of-interest statement: The authors declare no potential conflict of interest for this article.

Manuscript source: Invited manuscript

Specialty type: Oncology

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Bonala Sabeerabi, Department of Biochemistry, Biostandups, Hyderabad 500039, Telangana, India

Sarojamma Vemula, Department of Microbiology, Government Medical College, Anantapur 515001, Andhra Pradesh, India

Ramakrishna Vadde, Department of Biotechnology & Bioinformatics, Yogi Vemana University, Kadapa 516003, Andhra Pradesh, India

Ganji Purnachandra Nagaraju, Department of Hematology and Medical Oncology, Emory University, Atlanta, GA 30322, United States

Corresponding author: Ganji Purnachandra Nagaraju, DSc, PhD, Instructor, Department of Hematology and Medical Oncology, Emory University, 1365 Clifton Road Winship C #3025, Atlanta, GA 30322, United States. pganji@emory.edu

Abstract

Even though the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is related to SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), identifying effective and safe therapeutic strategies remains challenging. In search of finding effective treatments to eradicate the virus and improve disease symptoms, scientists are exploring possible therapies such as anti-viral, anti-malaria, immune therapy, and hormone treatments. However, the efficacy of these treatments was not validated on either SARS-CoV or MERS-CoV. In this study, we have reviewed synthetic evidence achieved through systematic and meta-analysis of therapeutics specific for SARS-CoV-2 and observed that the use of the above-mentioned therapies had no clinical benefits in coronavirus disease 2019 patients and, conversely, displayed side effects.

Key Words: COVID-19; Meta-analysis; Therapeutics; Anti-viral drugs; Immune therapy; Corticosteroids

Core Tip: The outcomes from these studies, supporting the use of anti-corona therapies, remain mostly inconclusive and uninspiring, so far, because of the lack of evidence, methodological flaws, missing data entries, risk of bias, publication bias, heterogeneity of outcomes and the number of subjects included in the respective studies. High-quality

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 7, 2021
Peer-review started: January 14, 2021
First decision: March 1, 2021
Revised: March 4, 2021
Accepted: May 17, 2021
Article in press: May 17, 2021
Published online: May 24, 2021

P-Reviewer: Cassell III AK
S-Editor: Fan JR
L-Editor: A
P-Editor: Li X

data from more stringent studies involving large samples, particularly randomized clinical studies, and caution on when to employ the treatments are needed.

Citation: Sabeerabi B, Vemula S, Vadde R, Nagaraju GP. COVID-19: Where is the treatment? *World J Clin Oncol* 2021; 12(5): 309-322
URL: https://www.wjgnet.com/2218-4333/full/v12/i5/309.htm
DOI: https://dx.doi.org/10.5306/wjco.v12.i5.309

INTRODUCTION

From the establishment of PRISMA guidelines in 2009, it is obvious that the importance and use of meta-analysis is growing at an unprecedented rate in scientific explorations. Meta-analysis is a statistical approach for evaluating the pooled data from various original research studies to provide quantitative, concise, and up to date knowledge[1]. Meta-analysis provides research outcomes *via* scientific synthesize through investigating the size of the effect or overall effect. This statistical analysis played a profound role in providing an evidence-based tool and in clarifying, superficially, paradoxical outcomes in several scientific domains; thus, eliminating controversy and criticism over particular study outcomes[1]. Though the term "meta-analysis" was born in the 1970s, currently the use of this tool has extended from medical sciences to other fields like physiology, conservation, evolution and ecological sciences; this infiltration strongly suggests that meta-analysis is replacing narrative reviews as an alternative, objective and instructive way of recapping biological concepts[2]. Synthesis of evidence from meta-analysis should become a common practice in order to maximize the value of scientific study in primary experimental research. Meta-analysis is very crucial to make progress in biological, medical, policy and conservation applications since these fields are greatly dependent on evidence-based outcomes[1,2]. Meta-analysis has aided in finding patterns, building projections, achieving generalizations and creating evidence-based conclusions in several research branches including oncology[3], obesity[4], pathophysiology[5], drug discovery[6] and diagnostic test accuracy[7]. Moreover, the exploration of corona virus and its therapeutic choices using meta-analysis is growing, even though the amount of original research articles over the topic are limited and these statistical outcomes are creating a corner to reach vast generalizations. Very recently, multiple meta-studies have reported their evidence-based outcomes with traditional medicines, anti-viral drugs, immune boosters, immunotherapy and use of hydroxychloroquine as treatment options in association with corona viral infection[8-12]. These meta-studies hoped to use existing evidence and provide the likability of treatment success on novel corona virus.

In December 2019, an outbreak of coronavirus disease 2019 (COVID-19) in the city of Wuhan, China, spread across the globe within a short period of time and became the latest public health emergency at the international level[13]. As of June 15, 2020, COVID-19 has been recognized in 213 countries and territories, with a total of 7805148 confirmed positive cases and with a total of 431192 fatalities. Infection control and recovery measures are necessary to prevent the current pandemic situation. It has been observed that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can bring asymptomatic, systemic or respiratory disorders in subjects infected with it. COVID-19 disease is characterized by serious upper respiratory illness including lung failure and pneumonia[14], where the cause of disease was COVID-19 virus [World Health Organization (WHO) named on February 11, 2020] and has been identified as a new novel coronavirus, which is now confirmed as SARS-CoV-2[13,14]. Earlier two major outbreaks of corona viruses occurred, namely Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV, posing a great threat to public health; however, these diseases were not deemed as pandemics. The SARS-CoV-2, which emerged in 2002, is a zoonotic corona virus similar to that of SARS-CoV[14,15]. As COVID-19 has triggered enormous human casualties and serious economic losses globally, an understanding of the ongoing situation and the development of strategies to contain the virus's spread are urgently needed. COVID-19 has caused a disturbed lifestyle, colossal human deaths, and pressing industrial losses globally and within a short period. This outbreak calls for urgent and effective measurements, anti-viral

therapeutics, and the establishment of effective strategies to restrain the virus. Several scientific explorations, particularly meta-studies are providing a great amount of evidence to adapt various therapeutic choices including immune therapy, anti-viral drugs and even the use of traditional medicines to treat COVID-19 infection. Hence, in the current review, we aim to review the status of all meta-studies published from 2019-2020 focusing on therapeutic options for COVID-19 to highlight future directions in the development of safe and successful therapeutic agents to prevent the viral disease (Figure 1).

THE PATHOGENESIS OF COVID-19

Though the initial transmission of COVID-19 was reported in Wuhan City, China, the actual source, reservoirs or intermediate carriers of the virus are still unknown. The latest COVID-19 viral genome has 88% similarity with SARS-CoV, which are derived from bats. The similarity also suggests that no birds or reptiles can host this virus except mammals[16]. Though the information on primary reservoirs of COVID-19 remain unclear, the transmission from person-to person *via* virus laden released during sneezing, coughing, or direct contact with infected person was reported[17]. Studies also confirmed that there is no transmission of virus from mother to child during pregnancy[18]. The first step of viral infection is the binding of viral spikes with cell-surface receptors of host cells and subsequent fusion with the plasma membrane, specifically on the epithelial cells of the lungs. Studies have investigated and confirmed that COVID-19 shares similarity in the receptor-binding domain of the SARS-CoV and interacts with angiotensin-converting enzyme 2 receptor (ACE2) present on the upper respiratory tract cells to gain invasion into the host system[17]. Moreover, a very recent study identified a group of human's proteins able to interact with SARS-CoV-2 proteins and these host proteins exhibit a range of functions at the cellular level (including DNA replication, vesicle traffic, mitochondrial, nuclear transport, cytoskeleton, lipid modifications, epigenetic regulators and ubiquitin ligases)[19].

It has been observed that SARS-CoV-2 can bring asymptomatic, systemic or respiratory disorders in subjects infected with it[20,21]. An incubation period of 5.8 d was seen in patients infected with COVID-19 with no symptoms and only after this incubation phase, the symptoms of COVID-19 appear from mild to life-threatening illness within 6 to 41 d, with an average of 14 d[20,21]. The appearance of symptoms after the incubation period depends on age as well as the individual's immune system. The common systemic symptoms of COVID-19 infection are fever, dry cough, fatigue, headache, dyspnoea, gastrointestinal symptoms, lymphopenia and haemoptysis[22]. The life-threating respiratory disorders such as pneumonia, respiratory distress syndrome, acute cardiac injury, serum SARS-CoV-2 viral load (RNAaemia), and prevalence grand-glass opacities in lungs were also reported[22]. Patients infected with COVID-19, who experienced breathing difficulties and pneumonia, also had high levels of pro-inflammatory cytokines and chemokines (granule cell stimulating factor, granulocyte-macrophage colony-stimulating factor, interleukin (IL)1-β, IL1RA, IL7/8/9/10, fibroblast growth factor 2, interferon γ, IP10, MCP1, MIP1α/β, platelet-derived growth factor B, tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor A) in the serum; these cytokines and chemokines are related in promoting disease progression[23]. At present, the world is not prepared to face pandemics like COVID-19 and is suffering from its consequences. Until today, there are no functional therapeutic drugs to treat the COVID-19 viral infection. However, COVID-19 patients are receiving supportive care, oxygen supply *via* ventilators and fluid management to overcome symptoms; nonetheless, there are tremendous efforts for vaccine development are undergoing. The global research community and pharma industries are working closely to find a cure for COVID-19 yet remain unsuccessful due to lack of existing evidence about druggable agents, which can provide a safe and sustainable cure for COVID-19 infection. The one possible way to accelerate the discovery process is to look for relatable drug agents that are primarily used in pneumonia, SARS-CoV and MERS-CoV infections. A wide variety of druggable agents or strategies those are known to work against deadly viruses such human immunodeficiency virus and Ebola can also be adapted; however, a clinical validation is needed. To accelerate the discovery of a druggable agent, a clear validation and generalizations of existing information on corona viruses and their killer is useful. To achieve such goals meta-studies are the only source. Hence, in the following sections, we will highlight the evaluations of meta-studies conducted on various possible drug agents

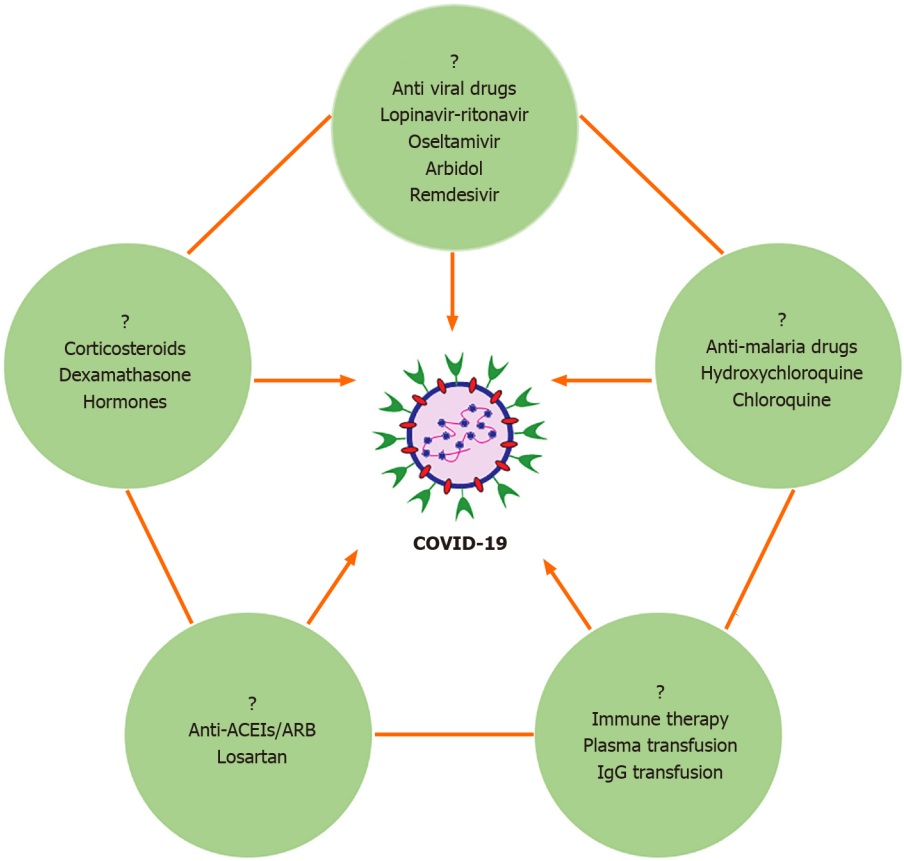


Figure 1 Coronavirus disease 2019 and its possible treatments. COVID-19: Coronavirus disease 2019; ACEI: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin receptor blockers; IgG: Immunoglobulin G.

against COVID-19 to find the most suitable and reliable cure.

Meta-analysis and COVID-19 therapeutics

The synthesis of scientific results from pooled data can help us to compare existing results in order to comprehend epidemiology, mortality, management choices, risk assessment and efficiency of prophylactic strategies against COVID-19. When we set the aim to explore the meta-studies on COVID-19 treatment efficiencies, we were unable to find such studies in the databases. The reason could be due to the lack of sufficient or compelling experimental evidences or clinical investigations to perform meta-analysis on prophylactic strategies against COVID-19. However, a few groups tried to find the pattern in aetiology, comorbidities and pathological variations and risk factors of COVID-19 at length[24-27]. Zhong *et al*[28] tried to perform systematic cum meta-analysis on the efficiency and safety of prophylactic strategies against COVID-19. Since the available data on such a topic was less, they tried to evaluate SARS-CoV and MERS-CoV therapies to find relatable and promising treatment options for SARS-CoV-2 infection[28]. In their meta study, antiviral drugs such as lopinavir/ritonavir, ribavirin and anti-malaria drug hydroxychloroquine based clinical data on SARS-CoV, MERS-CoV and COVID-19 was evaluated and found that, altogether, there was an improved mortality rate and reduced clinical development and radiographical improvement but no clear conclusion on the eradication of virus, the incubation phase, the prevalence of acute respiratory disease syndrome and adverse events[28]. However, a subgroup evaluation confirmed that use of ribavirin and corticosteroids in combination had a positive effect on reducing mortality and hydroxychloroquine, which was demonstrated on radiographical outcomes alone. In addition, use of a combination of lopinavir/ritonavir exhibited better eradication of the virus and improved radiographical appearances with a low prevalence of acute respiratory disease syndrome. Keeping the side effects of the drug combination tested into consideration, the quality of verification on most end results were very low and disappointing[28]. Though the meta-analysis failed to draw direct conclusions, due to the heterogeneity and low quality of evidence and indications, the study is still useful for clinicians to thoroughly acknowledge the dos and don'ts of individual anti-

coronavirus agents on efficacy and safety. On the other hand, Etoom *et al*[29] recently commented on one meta study published by Hu *et al*[30] titled "Prevalence and severity of COVID-19: A systematic review and meta-analysis". The former group thinks a much deeper and careful statistical strategy is needed to evaluate the available evidences on COVID-19. They also suggested that meta-analysis data should be made available, especially in the case of COVID-19, since the number of research explorations are growing immensely and performing new meta-analysis would be easier and quicker[29,30]. We believe, at this stage meta-studies on COVID-19 and therapeutics efficiency might take time because of the lack of sufficient empirical data and since the rate of research investigations are actually yielding low successful outcomes. Nonetheless, we look forward to see an increase in meta-studies aimed at COVID-19 research outcomes. It is noteworthy to mention that, though there were no meta-studies on efficiency of prophylactic strategies against COVID-19, there are a few systematic reviews on the same subject. Meta-analysis and systematic reviews stand atop in estimating the quality of evidence known as "evidence pyramid". Systematic review does not require statistical analysis, but provides a comprehensive synopsis of scientific literature to a specific research question.

Systematic reviews and COVID-19 therapeutics

In order to curtail the current SARS-CoV-2 global crisis, rapid diagnostics and effective therapeutics are the key potential interventions, which are currently occurring. Moreover, the lessons from previous outbreaks have shown that earlier therapeutics can still be questionable for the use in the current pandemic. Among the ongoing clinical investigations, some of them are testing against SARS-CoV and MERS-CoV while the rest are focused on SARS-CoV-2; however, currently, there is no success of effective therapeutics specific to SARS-CoV-2. The ways to eradicate SARS-CoV-2 include anti-viral drugs, immune therapy, immune boosters, vaccines, anti-malarial drugs, monoclonal antibodies and convalescent plasma, which are majorly monitored by pharma and research investigators. The ultimate goal is to develop anti-corona therapeutics; to accelerate such processes, every effort made is accountable and systematic generalization of such progresses play an important role in deciding the efficacy and safety of individual anti-coronavirus agents. In this hour of need what we want is a magic bullet to stop COVID-19; however, it is not easy to identify, testify, validate and get approval for such magic bullet. Constant efforts from biologists, pharmacists and policy makers are needed to evaluate the efficacy of anti-corona therapies and we are running out of time. We have highlighted the evidence collected through systematic analysis on anti-corona therapies to bring a comprehensive, evidence-based evaluation under one roof to further increase the understanding on the current success rate of anti-corona therapies. An overview of some of the most relevant systematic reviews (SR) and meta-analysis (MA) studies conducted on therapeutic strategies specific to COVID-19 are given in Table 1.

Systematic evaluation of anti-viral drugs specific to SARS-CoV-2

The ongoing antiviral drugs against COVID-19 are mostly similar to MERS-CoV and SARS-CoV-1 studies. A recent study conducted a systematic review on the current clinical settings of antiviral therapies against COVID-19[8]. The study also conducted a similar evaluation on MERS-CoV and SARS-CoV-1 studies to filter potential antiviral drugs. In their analysis, only one clinical investigation involving lopinavir-ritonavir in management of COVID-19 was found, where the treatment had no benefit in 199 severe COVID-19 patients[8]. It is noteworthy to mention that other observational studies, where anti-viral drugs such oseltamivir, lopinavir-ritonavir, lianhuaqingwen capsule, arbidol and interferon were used in the management of COVID-19, could not yield positive conclusions due to the lack of data recording and appropriate sample sizes[8]. Most interestingly, the team could not find any clinical settings where the effects of anti-viral drugs were tested against MERS-CoV and SARS-CoV-1 infections [8]. From the above conclusions, we believe that the current pandemic is more challenging to curb and will be difficult and even more challenging to develop an ideal anti-viral drug to manage severe COVID-19 diseases. Another team also aimed to evaluate the prophylaxis of anti-retroviral drugs on COVID-19 using systematic review to generate strong evidence to support anti-viral drugs as the first line of treatment. They reported the availability of 21 observational studies and two randomized trials, which showed the end results on the use of lopinavir-ritonavir on MERS-CoV, SARS-CoV-1, and COVID-19 patients[31]. From their evaluation, it was suggested that there were no clinical benefits from randomized trials, no inconclusive outcomes from observational studies and a low body of evidence across all major end points, indicating that the use of lopinavir-ritonavir anti-viral drugs as the first line of

Table 1 Synthetic of evidence on coronavirus disease 2019 specific therapeutic strategies

Nature of therapeutics	PMID	Study type	Therapeutic	Benefits	Conclusion
Anti-viral drugs	32360583	SR	Lopinavir-ritonavir or ribavirin	Improved mortality rate, radiographical improvement and reduced clinical development	Inconclusive evidences, low quality of evidence and heterogeneity of interventions
	32309809		Lopinavir-ritonavir or Arbidol or Oseltamivir or Lianhuaqingwen capsule or interferon	No benefits in 199 subjects	Side effects. Inconclusive evidence lacks of data recording. Sample size
	32493740	SR & MA			
	32293807	SR	Lopinavir-ritonavir	No clinical benefits	Adverse side effects. Inconclusive outcomes. Low body of evidence. Small sample size
	32506110		Remdesivir	No clinical benefits	Inconclusive outcomes high-quality evidence well-designed studies. Safety
	32378648				
Immune therapy	32406927	SR	Plasma transfusion	Had beneficial outcomes	Side effects. Inconclusive outcomes. Very low-certainty. High risk of bias. Low reporting quality
	32272396		Plasma therapy or hyperimmune immunoglobulin transfusion	Had beneficial outcomes	More evidence. Promising strategy. Sample size. Lack of control group
	32527348				
Anti-malaria drugs	32359203	SR	Hydroxychloroquine/Chloroquine	Had beneficial outcomes	Inconclusive outcomes. Methodological flaws. Risk of bias. Lack of evidence
	32281213			No clinical benefits	Lack of evidence. Safety issues
	32468425				Methodological flaws. Small sample size. Safety issues
	32173110				Lack of evidence. Safety issues. Methodological flaws. Small sample size
	32519281				
Hormone therapy	32283144	SR & MA	Corticosteroids	No clinical benefits	Lack of evidence. Adverse side effects. Methodological flaws. Caution needed
	32409522	SR			
	32372026				
	32391369				
Anti-hypertension drugs	32542337	SR & MA	ACEI/ARB	Had beneficial outcomes	Conflicting results, scarce existing data. Diverse study types. Inconsistent clinical studies, more RCT needed

ACEI: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin-receptor blockers; RCT: Randomised controlled trial; SR: Systematic reviews; MA: Meta-analysis.

treatment is not efficient on COVID-19 patients[31]. The team highlighted that reliability of the proof of end results across MERS-CoV, SARS-CoV-1, and COVID-19 is very low. They also suggested that, in addition to small sample size, the dose, duration and timing of the treatment was not uniform. Moreover, a combination of antiviral drugs along with other interventions may have given rise to the disclosed outcomes [31]. Though both studies differed on trivial aspects of anti-viral drug efficacy against COVID-19, MERS-CoV and SARS-CoV-1 infection, the studies did agree on one common finding, that the use of lopinavir-ritonavir on severe COVID-19 is not efficient. Currently, nearly twenty-five clinical trials are registered and each of the plans are investigating the efficacy and safety of anti-viral agents, including cobicistat, ritonavir, darunavir, lopinavir-ritonavir and tenofovir alafenamide fumarate.

Systematic evaluation of immune therapy specific to SARS-CoV-2

The reason behind choosing immune therapy against COVID-19 is because of the presence of clinical features, such as lymphopenia, increased inflammatory cytokines,

chemokines and reduced IFN-γ expression in T cells, which indicate suppression of the host immune system against sever COVID-19 infections[32]. The strategies to boost the host immune system through neutralizing antibodies or vaccines are focused on provoking the immune system to fight against COVID-19. However, in the case of COVID-19, the most popular known immune therapy is hyperimmune immunoglobulin transfusion or plasma therapy with nearly 48 studies aimed to evaluate hyperimmune immunoglobulin transfusion or plasma therapy for COVID-19 infected people. A rapid systematic review conducted by Valk *et al*[33] assessed the risks and benefits of using plasma transfusion as a potential immune therapy for COVID-19[33]. They reported that the majority of studies identified adverse side effects (grade 3/4) and that the quality of the reported data was low on plasma transfusion. The data deposited on plasma transfusion studies was highly inconsistent, making it difficult to draw outcomes with certainty[33]. Moreover, some of the controlled non-randomised or randomised controlled investigations are still occurring and have not reported any data regarding the harms and benefits of convalescent plasma therapy[33]. Though the plasma therapy looks promising, the current, global systematic analysis it is not efficient and safe. However, the effectiveness and safety of plasma transfusion remain elusive when compared with other immune therapeutic strategies.

Another study performed a systematic review on probable immune therapies for COVID-19. In this study, the authors included evidences from MERS-CoV, SARS-CoV and SARS-CoV-2 infections, in which the safety and efficacy of immunotherapy was investigated[34]. The highlights of the study primarily come from a single ethnic group (in China); evidence was reported in clinical settings, where the use of plasma therapy (300 subjects), hyperimmune immunoglobulin transfusion (80 subjects), thymosin in combination with camrelizumab (120 subjects) and tocilizumab (188 subjects) against COVID-19 were tested[35]. Though the outcomes of the studies with plasma therapy or hyperimmune immunoglobulin transfusion reported clinical improvements in COVID-19 patients, the evidence to support such therapies fails to provide certainty and demands for further investigations on a large-scale and more diverse population[34]. Moreover, the end outcomes of thymosin/camrelizumab combination therapy and tocilizumab against COVID-19 have yet to be tested. However, the study recommends to test the efficacy and safety of interventions like viral-vectors, vaccines, nanoparticles, and monoclonal antibody against COVID-19 infection, since those have been tested for SARS-CoV in non-clinical settings[34]. Another study performed a systematic analysis to determine the efficacy and safety of immune suppressive/stimulating drugs, such as non-steroidal anti-inflammatory drugs, TNF-α inhibitors, IL-6 inhibitors and Janus kinase/signal transducers and activators of transcription pathway inhibitors. These studies reported that no definite supporting evidence is available from clinical investigations; hence, they recommend clinical investigations using such drugs as promising immune therapeutics, since their efficacy was proved in *in vitro* studies[12]. Whatever approach was adapted by the above-discussed systematics, the end outcome, in terms of using immune therapy for COVID-19, remains dark. The literature is filled with reviews discussing the many possible approaches to prevent COVID-19, but what we need at this moment is more evidence-based studies than narrative opinions.

Systematic evaluation of anti-malaria drugs specific to SARS-CoV-2

In an unusual way, the use of anti-malarial drugs to cure viral infections has become quite popular lately. Malaria is caused by a parasite, *Plasmodium,* and COVID-19 disease is caused by the human corona virus; there are no structural or pathological similarities between the virus and the parasite. However, they both definitely increase body temperature upon infection. The earlier use of anti-malaria drug, such as chloroquine, against human corona viral infections in mouse models was reported in 2009[36]. Due to the public health emergency from SARS-CoV-2, every possible means to prevent viral infections was predicted and being tested. Hence, anti-malaria drugs are currently one of the drugs being evaluated for their efficacy and safety against SARS-CoV-2 in humans. A systematic review conducted found evidence regarding the effectiveness of anti-malaria drugs as anti-viral drugs to prevent SARS-CoV-2[37]. The team reported seven clinical trials, which were evaluating hydroxychloroquine/chloroquine as therapy for SARS-CoV-2, as complete. The end outcomes of the study suggest that both hydroxychloroquine/chloroquine were effective compared to supportive care or anti-viral drug treatment of SARS-CoV-2[37]. However, the outcomes are not reliable, since the evaluated studies has methodological flaws and risk for bias, indicating a lack of evidence to support anti-malaria drugs on SARS-CoV-2. However, hopefully, the data from ongoing trials may provide some evidence in the future[37]. Another study found similar outcomes upon systematic revision of prophy-

lactic outcomes of hydroxychloroquine and chloroquine against SARS-CoV-2; here, the team concluded that there is a lack of evidence to support hydroxychloroquine and chloroquine routine use and there are potential safety issues, which need to be further evaluated[38]. Another study screened nearly 663 articles and 12 clinical trials, validated the use of hydroxychloroquine and chloroquine against SARS-CoV-2 and found that some of the studies had better clinical outcomes with hydroxychloroquine or combination of azithromycin/hydroxychloroquine use in COVID-19 patients. However, these studies also had major flaws in their methodology. Moreover, a few studies showed adverse and opposite outcomes with hydroxychloroquine[39]. Similarly, another study reported similar observations and came up with a few recommendations, like employing a proper approach based on Monitored Emergency Use of Unregistered Interventions or WHO guidelines in upcoming clinical settings, especially with the use of anti-malaria drugs[40]. We also believe that a better-quality and stringent studies design and inter-relatable data from clinical trials originating across the globe are needed to clear the air regarding the use of hydroxychloroquine and chloroquine against SARS-CoV-2. It is noteworthy to mention that not only systematic reviews but also meta-analyses confirm the ineffective role of hydroxychloroquine in treating COVID-19 patients[9]. The Meta study confirms that there was no effect on viral eradication and a significant mortality rate was seen in COVID-19 patients treated with hydroxychloroquine[9].

Systematic evaluation of corticosteroids specific to SARS-CoV-2

Steroid hormones exhibit an inhibitory role on inflammation, when used in viral pneumonia; hence, many physicians recommended corticosteroid therapy as a possible treatment for patients with COVID-19. Though corticosteroid does not affect the virus directly, this therapy may help in managing severe inflammation and regulate homeostasis. Thousands of people infected with COVID-19 were treated with corticosteroid alone or in combination with anti-viral drugs. Meta-analysis and systematic reviews conducted on finding evidence in support of corticosteroid therapy for SARS-CoV-2 suggested that hormone therapy is ineffective and provokes adverse side effects.

A study by Yang *et al*[41] found that only patients with severe COVID-19 require hormone therapy and routine use of corticosteroid; these patients showed an increased mortality rate, bacterial infection and low blood potassium levels[41]. A caution must be taken while considering corticosteroid as a therapeutic option for mild symptoms [42]. Overall, the study finds inconclusive results, such as sample size, risk of bias in outcomes and the lack of data from multi-centre clinical trials[41]. A different meta-analysis ruled out the safety and efficacy of corticosteroids in SARS-CoV-2 infections. When they tested the virus' clearing effect by corticosteroids, they observed a slow virus clearing rate in two studies[43]. The meta-analysis concludes that there was no improvements in the death rate or the length of stay, which was accompanied by adverse effects[43]. Due to predominance of observational trials in the meta-study, a demand for confirmation from randomized trials to overcome the publication bias is needed[43]. Additional studies also concluded that the current evidence does not fully recommend the use of corticosteroids in SARS-CoV-2 infections; however, a few outcomes recommend the use of methylprednisolone to decrease the mortality rate in severe SARS-CoV-2[44,45].

Other notable mentions

While we discussed the most popular strategies employed in treating COVID-19, it is also important to explore other strategies employed in curing SARS-CoV-2 infection, such as remdesivir, anti-hypertension drugs and Traditional Chinese Medicine. Remdesivir is a broad-spectrum, anti-viral nucleotide analogue that has gained significant attention lately. In preclinical studies, remdesivir has been known to block a range of corona viruses and improve lung function therapeutically; however, the efficacy of remdesivir in COVID-19 patients remains short and scattered. The drug remdesivir received approval to be used under "Emergency Use Authorization" against severe COVID-19 cases, but is still awaiting approval by Food and Drug Administration. A recent systematic review conducted assessed the current evidence on the efficacy and safety of remdesivir and found favorable evidence as a first line treatment option in SARS-CoV-2[46]. The study reported that in order to confirm and recommend remdesivir as high quality and bias free, evidence from clinical settings is needed. Moreover, clinical settings should qualify with larger sample sizes, constrictive design and well-recorded data to synthesize an effective con-clusion[46, 47]. Furthermore, the future is hopeful with these ongoing trials and these studies may provide effective evidence on the benefits of remdesivir in COVID-19[47]. The second

choice that was opted to treat SARS-CoV-2 infection was inhibitors of the angiotensin receptor or ACE, a possible way to block viral interaction with receptors on lung epithelia cells. Meta studies on Angiotensin-converting enzyme inhibitors/angiotensin-receptor blockers (ACEIs/ARBs) inhibitor-based treatment on SARS-CoV-2 concluded that using inhibitors of ACEIs/ARBs can be continued but large studies such as randomised controlled trial are needed and additional evaluation on the relationship between polymorphism of ACE2 and its inhibitor is a must in the future investigations[48,49]. On the bright side, the upcoming outcomes from clinical trials (NCT04312009 and NCT04311177) using anti-ARB drug (losartan) in COVID-19 hold promising insights. The third choice that was opted to treat SARS-CoV-2 infection was a combination of Western medicine with TCM. One study aimed to investigate the benefits and harms of herbal medicine and Western medicine over COVID-19[50]. The amalgamated therapy rapidly increased the overall effective rate with better clinical outcomes in COVID-19 patients with zero side effects. However, additional evidence from randomized clinical trials may help to validate the benefits or harms of integrated medicine in the treatment of COVID-19[50]. Another study also showed that integrated medicine has beneficial effects when compared to Western medicine alone. The combination therapy did not yield any adverse effects in COVID-19 subjects. The number of studies included quality of data but poor methodologies were adapted in tested studies; a demand for more evidence with good quality to make definite decisions about combination therapy is needed in the future[11]. Effective vaccines are curial in the long-term to prevent rapid transmission of SARS-CoV-2 infections but developing vaccines is time consuming. However, the current crisis is pushing the limits of vaccine development and a few vaccines are being investigated in clinical trials right now. Table 2 provides the details of vaccines developed and their current clinical state specific to SARS-CoV-2. The preceding vaccines belong to DNA, RNA, inactivated viruses, recombinant viral spike proteins, dendritic cells, minigenes and viral vector-based systems. A vaccine developed by Oxford University and AstraZeneca is the most progressive one to enter phase III study settings; this vaccine serves as hope for a promising cure for SARS-CoV-2. Clinical investigations, clinical clearance and approval from the governing body are prerequisites for any drug, vaccines or therapeutic strategies created. Currently, apart from the ones discussed earlier, a wide range of anti-viral drugs, vaccines, cell therapies and anti-bacterial therapeutics are ongoing to determine the safety and efficacy specific to SARS-CoV-2.

SARS-CoV-2 specific drug targets under clinical investigation

Even though the success rate was low and search for suitable, relatable therapeutic drugs against SARS-CoV-2 continues with extensive hard work. In this section we focus on some of the possible upcoming therapeutics which are under early as well late stages of clinical monitoring. A pilot study pursing the benefits of using amniotic fluid (NCT04319731) or mesenchymal stromal cells from card tissues (NCT04399889, NCT04345601 and NCT04276987) for patients with COVID-19 and use of amniotic fluid was an approved strategies to minimize inflammation, tissues damage in humans. Use of rhDNase1 inhalation to trap neutrophils increased in circulation due to elevated inflammation is under evaluation against SARS-CoV-2 infection (NCT04409925). Anti-IL-6 antibody named Sarilumab has entered clinical evaluation as a potential mediator to interrupt cytokine-linked respiratory injury caused by SARS-CoV-2 infection (NCT04386239). Mouth or nose cleaning and gargling with povidone-iodine solution is currently undergoing clinical examination (NCT04393792), since povidone-iodine killed SARS-CoV-2 virus effectively in *in vitro* studies[51]. A phase III study aimed to rule out dosing with Sildenafil tablets in SARS-CoV-2, since it was approved by WHO for the prevention of pulmonary arterial hypertension (NCT04304313). A Tyrosine kinase inhibitor, Imatinib mesylate blocked inflammatory responses in invitro, *in vivo* and in few clinical trials, is under further examination in a phase III trial on SARS-CoV-2 and hoping to observe reduction in disease severity and inflammation (NCT04422678). Baricitinib, anti-Janus kinase inhibitor was approved to treat rheumatoid arthritis earlier and preclinical studies confirm that it can lower or prevent entry of viruses in to epithelia cells and reduce cytokine release, is under phase III clinical monitoring to use on SARS-CoV-2 (NCT04320277). The use of repurposed bacterial mucosal vaccines Bactek-MV130 in the form of spray is undergoing phase III trial to provide benefits for COVID-19 induced mild pneumonia (NCT04363814). Based on preclinical evidences where increased circulatory Vascular Endothelial Growth Factor was seen in COVID-19 subjects, anti-vascular endothelial growth factor drug namely Bevacizumab (FAD approved to treat certain cancers), is being tested in critical or severe patients with COVID-19 pneumonia (NCT04275414). Ifenprodil, a drug used to inactivate activated neutrophils and T-cells is under phase

Table 2 Vaccines specific to severe acute respiratory syndrome coronavirus 2 under clinical investigation and their development status

Vaccine name	Vaccine type	Status	Registration ID	Date of registration	Developer
2019-nCOV	Adenovirus vaccine	Phase II	ChiCTR2000031781	10-Apr-20	Academy of Military Medical Sciences
Ad5-nCoV	Adenovirus vaccine	Phase II	NCT04341389	10-Apr-20	CanSino Biologics
AV-COVID-19	Autologous dendritic cells	Phase IB/II	NCT04386252	13-May-20	Aivita Biomedical, Inc
BBIBP-CorV	Inactivated virus	Phase I/II	ChiCTR2000032459	29-Apr-20	Beijing Institute of Biological Products & Sinopharm
BNT162	mRNA vaccine	Phase I/II	NCT04380701	8-May-20	BioNTech and Pfizer
ChAdOx1	Adenovirus vaccine	Phase II/III	NCT04400838	26-May-20	University of Oxford
Covax-19™	SARS-CoV-2 spike protein	Phase I	NCT04428073	11-Jun-20	GeneCure Biotechnologies
COVID-19/aAPC	Antigen presenting cells	Phase I	NCT04299724	9-Mar-20	Shenzhen Geno-Immune Medical Institute
INO-4800	DNA vaccine	Phase I	NCT04336410	7-Apr-20	Inovio Pharmaceuticals
LV-SMENP-DC	Lentiviral vector system	Phase I/II	NCT04276896	19-Feb-20	Shenzhen Geno-Immune Medical Institute
mRNA-1273	mRNA vaccine	Phase II	NCT04405076	28-May-20	ModernaTX, Inc
NVX-CoV2373	Recombinant Spike Protein	Phase I/II	NCT04368988	30-Apr-20	Novavax
PiCoVacc	Inactivated virus + adjuvant	Phase I/II	NCT04352608	20-Apr-20	Sinovac
V- SARS	Heat-inactivated plasma	Phase I/II	NCT04380532	8-May-20	Immunitor LLC
Vero cells	Inactivated virus	Phase I/II	ChiCTR2000031809	11-Apr-20	Wuhan Institute of Biological Products & Sinopharm

LLC: Lewis lung carcinoma; COVID-19: Coronavirus disease 2019; SARS: Severe acute respiratory syndrome.

IIb/III trial to discover the safety and efficacy in the treatment of SARS-CoV-2 infection (NCT04382924). There is no recorded evidence from all the trials motioned above, so we do not know whether anti-inflammatory drugs, rheumatoid arthritis and anti-septic solutions have any benefits in COVID-19 patients. Noteworthy to mention that the above detailed investigations are based on the evidence gathered from preclinical studies and the benefits of using these repurposed medications against COVID-19 need evidence from these study outcomes. Moreover, the safety and efficacy of these drugs on COVID-19 are forthcoming and we hope the outcomes from these studies provide conclusive, bias free evidences. It seems the new players under investigation are mainly to treat SARS-CoV-2 symptoms and have zero effect on viral load, except for povidone-iodine, which was known to eradicate SARS-CoV-2. The world is eagerly dependent on scientific and pharma community for the discovery of magic bullet to treat COVID-19.

SARS-CoV-2 therapeutic challenges and anticipations

In spite of the worldwide distress, no drug or vaccines is available under approval to treat SARS-CoV-2 infection. So far, clinical reports and synthetic evidence show that reusing of existing anti-viral, anti-malaria, immune stimulators and corticosteroids on COVID-19 disease has been unsatisfactory and unsafe. In order to escalate the identification and validation of anti-SARS-CoV-2 medicine, use of computer based high through put data analysis in finding suitable drug targets is recommended. A broad range of anti-viral agents in preclinical and clinical settings must be evaluated for their safety and efficacy against SARS-CoV-2. We believe that use of repurposed medicines for COVID-19 are going to be part of short-term strategy. In spite of the fact that these relatable treatment options have been prioritized to treat SARS-CoV-2 and the outcomes are biased. These uncovering's highlight an immediate call for novel anti-corona medicines specific to SARS-CoV-2 virus. In the current urgency there are several challenges and overcoming those challenges is crucial in developing novel anti-SARS-CoV-2 medications. One such hurdle is collection of evidence from

preclinical experiments, which is expensive and time-dependent. In the absence of preclinical data, computer-based analysis of target profiling allows either to validate or invalidate the use of predicted drug; permits to accelerate invention of novel and beneficial therapeutics against SARS-CoV-2. Though the operating cost is reduced, the safety profile of those computer-generated targets must be validated at least in clinical settings. Another limitation faced by scientist is the availability of suitable study models (cells, mice and primates) to investigate the virus-host interaction and evaluate the potency of anti-SARS-CoV-2 drugs. In addition, BSL3 Laboratories with suitable study models are very few and conducting experiments in such environments is technically difficult. The development of host-based and/or SARS-CoV-2 based clinical inventions must be prioritized since only one or two of drugs will pass through clinical settings. Toxicity, dosage, availability of drug delivery routes and some other limitations make these drugs to pass through the clinical stage. Normally, the development and clinical approval of vaccine needs more than 10 years of time and efforts, however, vaccine development programs against SARS-CoV-2 are breaking the convectional norms and working hard to launch safe and effective SARS-CoV-2 vaccines as early as possible. It can be more challenging to treat COVID-19 diseases, if the virus develops genetic mutations and/or drug resistance during treatment. Studies have reported presence of 93 possible mutations and among them majority of mutations were missense mutations and the genome of SARS-CoV-2 was found to be highly conserved[52]. Also, patients with underlying health issues such as diabetes, cancer, renal failure and women with pregnancy need exceptional care. In addition, management of COVID-19 patients has become challenging due to lack of sufficient medical staff, availability of drugs, subject recognition's, isolation and implication of control procedures and delivery of personalized care towards COVID-19 patients. New Zealand and few Asian countries managed to contain COVID-19 transmission effectively while compared with most of developed countries like United States and Europe. As the epidemic advances, hunger amid poor countries, influence of lock down on mental status of children and adults may increases. To avoid upcoming foreseeable future, systematic and unified approach is vital for managing COVID-19 epidemic. A single drug or a combination approach to preventing COVID-19 disease is needed to prevent mortality, restore the normal lifestyle and economic growth world-wide. The ideal drug must be able to kill virus load with zero or less toxicity in humans, affordable with minimum production time.

CONCLUSION

In this real-time crisis, a need for high-quality, bias-free, and effective evidence in eradicating the SARS-CoV-2 virus and reducing disease-associated symptoms with zero side effects is needed. Currently, the journal database is filled with narrative research and speculations with hypothetical reasoning; data on evidence-based research is limited. A shift in evidence originating from original research investigations and clinical studies with high-quality evidence is needed and this evidence may aid in progressing towards the development of effective and safe therapeutic options for SARS-CoV-2. Moreover, the systematic reviews and meta-studies are providing substantial evidence and clearing the air of bias for the public, physicians, and scientists. Drugs like hydroxychloroquine/chloroquine or remdesivir have gained a lot of attention in the public and the media; these drugs have also gained support from experts and are now being characterized as effective therapeutic drugs for SARS-CoV-2 but scientifically it is unwise to recommend these drugs to treat SARS-CoV-2 without caution. Currently, there are no effective anti-viral agents, immune therapy, or vaccines against SARS-CoV-2 but the future is filled with only hope. The current scientific evidence hold inconclusive outcomes because of the low number of studies conducted, low sample size, flawed study designs, publication bias, heterogeneity, missing data records, quality of evidence, ethnicity and presence of adverse effects. However, these flaws cannot stop current and future investigations to identify, characterize and validate possible therapeutic innovations specific to SARS-CoV-2.

Future directions

So far, considering the trajectory of COVID-19 pandemic, the primary need to control the transmission of the disease is to practice physical distancing, wearing masks, keeping hands off from surfaces, and washing them with proper detergent. These rules have somewhat provided the needed protection. The public is well aware of the facts to restrain themselves from being exposed. Developing surfaces or masks that

can kill the virus within seconds once contact made could be a way to prevent highly transmissive virus-laden droplets that are just released. Coming to the scientific role in finding a cure, it is very crucial to understand the genetic profile of the virus at the molecular level and use this knowledge to build safe tools to screen, identify and develop therapeutics for the prevention and treatment of SARS-CoV-2. Currently, no laboratory in the world has whole virus to study in detail. Hence, it is only fitting to build the tools and resources that can be utilized for evaluating therapeutic efficacy. The journey to finding a vaccine or an anti-viral therapy specific to SARS-CoV-2 must start from the basic research integrated with advanced technologies such as artificial intelligence, computational biology, nanotechnology, and genome-wide association studies. The efforts made in the past to find the cure for COVID-19 though failed but provided immense knowledge on what not to practice and open new doors of possibility. Currently, applying a single therapeutic strategy, or even a combination of strategies is not enough to cure diseases including cancers and infectious diseases. In the end, prevention is better than a cure.

REFERENCES

1 **Gurevitch J**, Koricheva J, Nakagawa S, Stewart G. Meta-analysis and the science of research synthesis. *Nature* 2018; **555**: 175-182 [PMID: 29517004 DOI: 10.1038/nature25753]

2 **Nakagawa S**, Noble DW, Senior AM, Lagisz M. Meta-evaluation of meta-analysis: ten appraisal questions for biologists. *BMC Biol* 2017; **15**: 18 [PMID: 28257642 DOI: 10.1186/s12915-017-0357-7]

3 **Xu C**, Chen YP, Du XJ, Liu JQ, Huang CL, Chen L, Zhou GQ, Li WF, Mao YP, Hsu C, Liu Q, Lin AH, Tang LL, Sun Y, Ma J. Comparative safety of immune checkpoint inhibitors in cancer: systematic review and network meta-analysis. *BMJ* 2018; **363**: k4226 [PMID: 30409774 DOI: 10.1136/bmj.k4226]

4 **Simmonds M**, Llewellyn A, Owen CG, Woolacott N. Predicting adult obesity from childhood obesity: a systematic review and meta-analysis. *Obes Rev* 2016; **17**: 95-107 [PMID: 26696565 DOI: 10.1111/obr.12334]

5 **Fu Y**, Lee CH, Chi CC. Association of Psoriasis With Inflammatory Bowel Disease: A Systematic Review and Meta-analysis. *JAMA Dermatol* 2018; **154**: 1417-1423 [PMID: 30422277 DOI: 10.1001/jamadermatol.2018.3631]

6 **Cipriani A**, Furukawa TA, Salanti G, Chaimani A, Atkinson LZ, Ogawa Y, Leucht S, Ruhe HG, Turner EH, Higgins JPT, Egger M, Takeshima N, Hayasaka Y, Imai H, Shinohara K, Tajika A, Ioannidis JPA, Geddes JR. Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: a systematic review and network meta-analysis. *Lancet* 2018; **391**: 1357-1366 [PMID: 29477251 DOI: 10.1016/S0140-6736(17)32802-7]

7 **Shin IS**. Recent Research Trends in Meta-analysis. *Asian Nurs Res (Korean Soc Nurs Sci)* 2017; **11**: 79-83 [PMID: 28688503 DOI: 10.1016/j.anr.2017.05.004]

8 **Yousefifard M**, Zali A, Mohamed Ali K, Madani Neishaboori A, Zarghi A, Hosseini M, Safari S. Antiviral therapy in management of COVID-19: a systematic review on current evidence. *Arch Acad Emerg Med* 2020; **8**: e45 [PMID: 32309809]

9 **Singh AK**, Singh A, Singh R, Misra A. "Hydroxychloroquine in patients with COVID-19: A Systematic Review and meta-analysis. *Diabetes Metab Syndr* 2020; **14**: 589-596 [PMID: 32417708 DOI: 10.1016/j.dsx.2020.05.017]

10 **Kermali M**, Khalsa RK, Pillai K, Ismail Z, Harky A. The role of biomarkers in diagnosis of COVID-19 - A systematic review. *Life Sci* 2020; **254**: 117788 [PMID: 32475810 DOI: 10.1016/j.lfs.2020.117788]

11 **Liu M**, Gao Y, Yuan Y, Yang K, Shi S, Zhang J, Tian J. Efficacy and Safety of Integrated Traditional Chinese and Western Medicine for Corona Virus Disease 2019 (COVID-19): a systematic review and meta-analysis. *Pharmacol Res* 2020; **158**: 104896 [PMID: 32438037 DOI: 10.1016/j.phrs.2020.104896]

12 **Russell B**, Moss C, George G, Santaolalla A, Cope A, Papa S, Van Hemelrijck M. Associations between immune-suppressive and stimulating drugs and novel COVID-19-a systematic review of current evidence. *Ecancermedicalscience* 2020; **14**: 1022 [PMID: 32256705 DOI: 10.3332/ecancer.2020.1022]

13 **Dariya B**, Nagaraju GP. Understanding novel COVID-19: Its impact on organ failure and risk assessment for diabetic and cancer patients. *Cytokine Growth Factor Rev* 2020; **53**: 43-52 [PMID: 32409230 DOI: 10.1016/j.cytogfr.2020.05.001]

14 **Ahn DG**, Shin HJ, Kim MH, Lee S, Kim HS, Myoung J, Kim BT, Kim SJ. Current Status of Epidemiology, Diagnosis, Therapeutics, and Vaccines for Novel Coronavirus Disease 2019 (COVID-19). *J Microbiol Biotechnol* 2020; **30**: 313-324 [PMID: 32238757 DOI: 10.4014/jmb.2003.03011]

15 **Rothan HA**, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. *J Autoimmun* 2020; **109**: 102433 [PMID: 32113704 DOI: 10.1016/j.jaut.2020.102433]

16 **Lu R**, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma

J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, Tan W. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 2020; **395**: 565-574 [PMID: 32007145 DOI: 10.1016/S0140-6736(20)30251-8]

17 **Wu P**, Hao X, Lau EHY, Wong JY, Leung KSM, Wu JT, Cowling BJ, Leung GM. Real-time tentative assessment of the epidemiological characteristics of novel coronavirus infections in Wuhan, China, as at 22 January 2020. *Euro Surveill* 2020; **25** [PMID: 31992388 DOI: 10.2807/1560-7917.ES.2020.25.3.2000044]

18 **Chen H**, Guo J, Wang C, Luo F, Yu X, Zhang W, Li J, Zhao D, Xu D, Gong Q, Liao J, Yang H, Hou W, Zhang Y. Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: a retrospective review of medical records. *Lancet* 2020; **395**: 809-815 [PMID: 32151335 DOI: 10.1016/S0140-6736(20)30360-3]

19 **Malle L**. A map of SARS-CoV-2 and host cell interactions. *Nat Rev Immunol* 2020; **20**: 351 [PMID: 32303698 DOI: 10.1038/s41577-020-0318-1]

20 **Li Q**, Guan X, Wu P, Wang X, Zhou L, Tong Y, Ren R, Leung KSM, Lau EHY, Wong JY, Xing X, Xiang N, Wu Y, Li C, Chen Q, Li D, Liu T, Zhao J, Liu M, Tu W, Chen C, Jin L, Yang R, Wang Q, Zhou S, Wang R, Liu H, Luo Y, Liu Y, Shao G, Li H, Tao Z, Yang Y, Deng Z, Liu B, Ma Z, Zhang Y, Shi G, Lam TTY, Wu JT, Gao GF, Cowling BJ, Yang B, Leung GM, Feng Z. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. *N Engl J Med* 2020; **382**: 1199-1207 [PMID: 31995857 DOI: 10.1056/NEJMoa2001316]

21 **Wang W**, Tang J, Wei F. Updated understanding of the outbreak of 2019 novel coronavirus (2019-nCoV) in Wuhan, China. *J Med Virol* 2020; **92**: 441-447 [PMID: 31994742 DOI: 10.1002/jmv.25689]

22 **Habibzadeh P**, Stoneman EK. The Novel Coronavirus: A Bird's Eye View. *Int J Occup Environ Med* 2020; **11**: 65-71 [PMID: 32020915 DOI: 10.15171/ijoem.2020.1921]

23 **Huang C**, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497-506 [PMID: 31986264 DOI: 10.1016/S0140-6736(20)30183-5]

24 **Yang J**, Zheng Y, Gou X, Pu K, Chen Z, Guo Q, Ji R, Wang H, Wang Y, Zhou Y. Prevalence of comorbidities and its effects in patients infected with SARS-CoV-2: a systematic review and meta-analysis. *Int J Infect Dis* 2020; **94**: 91-95 [PMID: 32173574 DOI: 10.1016/j.ijid.2020.03.017]

25 **Rodriguez-Morales AJ**, Cardona-Ospina JA, Gutiérrez-Ocampo E, Villamizar-Peña R, Holguin-Rivera Y, Escalera-Antezana JP, Alvarado-Arnez LE, Bonilla-Aldana DK, Franco-Paredes C, Henao-Martinez AF, Paniz-Mondolfi A, Lagos-Grisales GJ, Ramírez-Vallejo E, Suárez JA, Zambrano LI, Villamil-Gómez WE, Balbin-Ramon GJ, Rabaan AA, Harapan H, Dhama K, Nishiura H, Kataoka H, Ahmad T, Sah R; Latin American Network of Coronavirus Disease 2019-COVID-19 Research (LANCOVID-19). Electronic address: https://www.lancovid.org. Clinical, laboratory and imaging features of COVID-19: A systematic review and meta-analysis. *Travel Med Infect Dis* 2020; **34**: 101623 [PMID: 32179124 DOI: 10.1016/j.tmaid.2020.101623]

26 **Kisely S**, Warren N, McMahon L, Dalais C, Henry I, Siskind D. Occurrence, prevention, and management of the psychological effects of emerging virus outbreaks on healthcare workers: rapid review and meta-analysis. *BMJ* 2020; **369**: m1642 [PMID: 32371466 DOI: 10.1136/bmj.m1642]

27 **Alqahtani JS**, Oyelade T, Aldhahir AM, Alghamdi SM, Almehmadi M, Alqahtani AS, Quaderi S, Mandal S, Hurst JR. Prevalence, Severity and Mortality associated with COPD and Smoking in patients with COVID-19: A Rapid Systematic Review and Meta-Analysis. *PLoS One* 2020; **15**: e0233147 [PMID: 32392262 DOI: 10.1371/journal.pone.0233147]

28 **Zhong H**, Wang Y, Zhang ZL, Liu YX, Le KJ, Cui M, Yu YT, Gu ZC, Gao Y, Lin HW. Efficacy and safety of current therapeutic options for COVID-19 - lessons to be learnt from SARS and MERS epidemic: A systematic review and meta-analysis. *Pharmacol Res* 2020; **157**: 104872 [PMID: 32360583 DOI: 10.1016/j.phrs.2020.104872]

29 **Etoom M**, Alwardat S, Alwardat M. Issues for conducting meta-analyses in COVID-19. Commentary on "Prevalence and severity of corona virus disease 2019 (COVID-19): A systematic review and meta-analysis". *J Clin Virol* 2020; **128**: 104389 [PMID: 32387969 DOI: 10.1016/j.jcv.2020.104389]

30 **Hu Y**, Sun J, Dai Z, Deng H, Li X, Huang Q, Wu Y, Sun L, Xu Y. Prevalence and severity of corona virus disease 2019 (COVID-19): A systematic review and meta-analysis. *J Clin Virol* 2020; **127**: 104371 [PMID: 32315817 DOI: 10.1016/j.jcv.2020.104371]

31 **Ford N**, Vitoria M, Rangaraj A, Norris SL, Calmy A, Doherty M. Systematic review of the efficacy and safety of antiretroviral drugs against SARS, MERS or COVID-19: initial assessment. *J Int AIDS Soc* 2020; **23**: e25489 [PMID: 32293807 DOI: 10.1002/jia2.25489]

32 **Pedersen SF**, Ho YC. SARS-CoV-2: a storm is raging. *J Clin Invest* 2020; **130**: 2202-2205 [PMID: 32217834 DOI: 10.1172/JCI137647]

33 **Valk SJ**, Piechotta V, Chai KL, Doree C, Monsef I, Wood EM, Lamikanra A, Kimber C, McQuilten Z, So-Osman C, Estcourt LJ, Skoetz N. Convalescent plasma or hyperimmune immunoglobulin for people with COVID-19: a rapid review. *Cochrane Database Syst Rev* 2020; **5**: CD013600 [PMID: 32406927 DOI: 10.1002/14651858.CD013600]

34 **AminJafari A**, Ghasemi S. The possible of immunotherapy for COVID-19: A systematic review. *Int Immunopharmacol* 2020; **83**: 106455 [PMID: 32272396 DOI: 10.1016/j.intimp.2020.106455]

35 **Zhang Q**, Wang Y, Qi C, Shen L, Li J. Clinical trial analysis of 2019-nCoV therapy registered in

China. *J Med Virol* 2020; **92**: 540-545 [PMID: 32108352 DOI: 10.1002/jmv.25733]
36 **Keyaerts E**, Li S, Vijgen L, Rysman E, Verbeeck J, Van Ranst M, Maes P. Antiviral activity of chloroquine against human coronavirus OC43 infection in newborn mice. *Antimicrob Agents Chemother* 2009; **53**: 3416-3421 [PMID: 19506054 DOI: 10.1128/AAC.01509-08]
37 **Chowdhury MS**, Rathod J, Gernsheimer J. A Rapid Systematic Review of Clinical Trials Utilizing Chloroquine and Hydroxychloroquine as a Treatment for COVID-19. *Acad Emerg Med* 2020; **27**: 493-504 [PMID: 32359203 DOI: 10.1111/acem.14005]
38 **Shah S**, Das S, Jain A, Misra DP, Negi VS. A systematic review of the prophylactic role of chloroquine and hydroxychloroquine in coronavirus disease-19 (COVID-19). *Int J Rheum Dis* 2020; **23**: 613-619 [PMID: 32281213 DOI: 10.1111/1756-185X.13842]
39 **Das S**, Bhowmick S, Tiwari S, Sen S. An Updated Systematic Review of the Therapeutic Role of Hydroxychloroquine in Coronavirus Disease-19 (COVID-19). *Clin Drug Investig* 2020; **40**: 591-601 [PMID: 32468425 DOI: 10.1007/s40261-020-00927-1]
40 **Cortegiani A**, Ingoglia G, Ippolito M, Giarratano A, Einav S. A systematic review on the efficacy and safety of chloroquine for the treatment of COVID-19. *J Crit Care* 2020; **57**: 279-283 [PMID: 32173110 DOI: 10.1016/j.jcrc.2020.03.005]
41 **Yang Z**, Liu J, Zhou Y, Zhao X, Zhao Q. The effect of corticosteroid treatment on patients with coronavirus infection: a systematic review and meta-analysis. *J Infect* 2020; **81**: e13-e20 [PMID: 32283144 DOI: 10.1016/j.jinf.2020.03.062]
42 **Ye Z**, Wang Y, Colunga-Lozano LE, Prasad M, Tangamornsuksan W, Rochwerg B, Yao L, Motaghi S, Couban RJ, Ghadimi M, Bala MM, Gomaa H, Fang F, Xiao Y, Guyatt GH. Efficacy and safety of corticosteroids in COVID-19 based on evidence for COVID-19, other coronavirus infections, influenza, community-acquired pneumonia and acute respiratory distress syndrome: a systematic review and meta-analysis. *CMAJ* 2020; **192**: E756-E767 [PMID: 32409522 DOI: 10.1503/cmaj.200645]
43 **Li H**, Chen C, Hu F, Wang J, Zhao Q, Gale RP, Liang Y. Impact of corticosteroid therapy on outcomes of persons with SARS-CoV-2, SARS-CoV, or MERS-CoV infection: a systematic review and meta-analysis. *Leukemia* 2020; **34**: 1503-1511 [PMID: 32372026 DOI: 10.1038/s41375-020-0848-3]
44 **Veronese N**, Demurtas J, Yang L, Tonelli R, Barbagallo M, Lopalco P, Lagolio E, Celotto S, Pizzol D, Zou L, Tully MA, Ilie PC, Trott M, López-Sánchez GF, Smith L. Use of Corticosteroids in Coronavirus Disease 2019 Pneumonia: A Systematic Review of the Literature. *Front Med (Lausanne)* 2020; **7**: 170 [PMID: 32391369 DOI: 10.3389/fmed.2020.00170]
45 **Zhang JJY**, Lee KS, Ang LW, Leo YS, Young BE. Risk Factors for Severe Disease and Efficacy of Treatment in Patients Infected With COVID-19: A Systematic Review, Meta-Analysis, and Meta-Regression Analysis. *Clin Infect Dis* 2020; **71**: 2199-2206 [PMID: 32407459 DOI: 10.1093/cid/ciaa576]
46 **Nasir M**, Talha KA, Islam T, Saha SK, Selina F, Parveen RA. Use of Remdesivir in the Management of COVID-19: A Systematic Review on Current Evidences. *Mymensingh Med J* 2020; **29**: 481-487 [PMID: 32506110]
47 **Li Z**, Wang X, Cao D, Sun R, Li C, Li G. Rapid review for the anti-coronavirus effect of remdesivir. *Drug Discov Ther* 2020; **14**: 73-76 [PMID: 32378648 DOI: 10.5582/ddt.2020.01015]
48 **Grover A**, Oberoi M. A systematic review and meta-analysis to evaluate the clinical outcomes in COVID-19 patients on angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. *Eur Heart J Cardiovasc Pharmacother* 2021; **7**: 148-157 [PMID: 32542337 DOI: 10.1093/ehjcvp/pvaa064]
49 **Zhang X**, Yu J, Pan LY, Jiang HY. ACEI/ARB use and risk of infection or severity or mortality of COVID-19: A systematic review and meta-analysis. *Pharmacol Res* 2020; **158**: 104927 [PMID: 32422341 DOI: 10.1016/j.phrs.2020.104927]
50 **Ang L**, Song E, Lee HW, Lee MS. Herbal Medicine for the Treatment of Coronavirus Disease 2019 (COVID-19): A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *J Clin Med* 2020; **9** [PMID: 32456123 DOI: 10.3390/jcm9051583]
51 **Bidra AS**, Pelletier JS, Westover JB, Frank S, Brown SM, Tessema B. Rapid In-Vitro Inactivation of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Using Povidone-Iodine Oral Antiseptic Rinse. *J Prosthodont* 2020; **29**: 529-533 [PMID: 32511851 DOI: 10.1111/jopr.13209]
52 **Phan T**. Genetic diversity and evolution of SARS-CoV-2. *Infect Genet Evol* 2020; **81**: 104260 [PMID: 32092483 DOI: 10.1016/j.meegid.2020.104260]

World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2021 May 24; 12(5): 323-334

DOI: 10.5306/wjco.v12.i5.323

ISSN 2218-4333 (online)

MINIREVIEWS

Immune response evaluation criteria in solid tumors for assessment of atypical responses after immunotherapy

Davide Ippolito, Cesare Maino, Maria Ragusi, Marco Porta, Davide Gandola, Cammillo Talei Franzesi, Teresa Paola Giandola, Sandro Sironi

ORCID number: Davide Ippolito 0000-0002-2696-7047; Cesare Maino 0000-0002-5742-802X; Maria Ragusi 0000-0002-7076-9208; Marco Porta 0000-0002-1833-6587; Davide Gandola 0000-0002-4505-4892; Cammillo Talei Franzesi 0000-0003-1728-7653; Teresa Paola Giandola 0000-0001-8750-0531; Sandro Sironi 0000-0002-4469-5073.

Author contributions: All authors contributed equally to this work and have read and approved the final manuscript.

Conflict-of-interest statement: All authors declare no conflict-of-interest.

Manuscript source: Invited

Davide Ippolito, Cesare Maino, Maria Ragusi, Marco Porta, Davide Gandola, Cammillo Talei Franzesi, Teresa Paola Giandola, Department of Diagnostic Radiology, H. S. Gerardo Monza, School of Medicine, University of Milano-Bicocca, Monza 20900, Italy

Sandro Sironi, Diagnostic Radiology, University of Milano-Bicocca, Bergamo 24127, Italy

Corresponding author: Davide Ippolito, MD, Adjunct Professor, Doctor, Department of Diagnostic Radiology, H. S. Gerardo Monza, School of Medicine, University of Milano-Bicocca, Via Pergolesi 33, Monza 20900, Italy. davide.atena@tiscali.it

Abstract

In 2017, immune response evaluation criteria in solid tumors (iRECIST) were introduced to validate radiologic and clinical interpretations and to better analyze tumor's response to immunotherapy, considering the different time of following and response, between this new therapy compared to the standard one. However, even if the iRECIST are worldwide accepted, to date, different aspects should be better underlined and well reported, especially in clinical practice. Clinical experience has demonstrated that in a non-negligible percentage of patients, it is challenging to determine the correct category of response (stable disease, progression disease, partial or complete response), and consequently, to define which is the best management for those patients. Approaching radiological response in patients who underwent immunotherapy, a new uncommon kind of target lesions behavior was found. This phenomenon is mainly due to the different mechanisms of action of immunotherapeutic drug. Therefore, new groups of response have been described in clinical practice, defined as "atypical responses," and categorized into three new groups: pseudoprogression, hyperprogression, and dissociated response. This review summarizes and reports these patterns, helping clinicians and radiologists get used to atypical responses, in order to identify patients that respond best to treatment.

Key Words: Response evaluation criteria in solid tumors; Tumor response; Pseudoprogression; Hyperprogression; Dissociated response

manuscript

Specialty type: Oncology

Country/Territory of origin: Italy

Peer-review report's scientific quality classification
Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 24, 2021
Peer-review started: January 24, 2021
First decision: March 8, 2021
Revised: March 23, 2021
Accepted: April 23, 2021
Article in press: April 23, 2021
Published online: May 24, 2021

P-Reviewer: Peck-Radosavljevic M, Rajer M, Sun Y
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Yuan YY

Core Tip: Atypical responses are frequent events in the immunotherapy era. On these bases, it is fundamental to summarize and recap the most common and important response manifestations to help clinicians in everyday practice. Here, we present the three most common clinical and radiological patterns of response to immunotherapy: pseudoprogression, hyperprogression, and dissociated response, reporting important studies to identify the different behavior and guarantee the best management, strengthening the communication skills between specialists.

Citation: Ippolito D, Maino C, Ragusi M, Porta M, Gandola D, Franzesi CT, Giandola TP, Sironi S. Immune response evaluation criteria in solid tumors for assessment of atypical responses after immunotherapy. *World J Clin Oncol* 2021; 12(5): 323-334
URL: https://www.wjgnet.com/2218-4333/full/v12/i5/323.htm
DOI: https://dx.doi.org/10.5306/wjco.v12.i5.323

INTRODUCTION

In the last few years, new therapies such as immunotherapy have been experimented with and introduced into clinical practice for the treatment of oncologic patients. Immunotherapy is a type of treatment that involves the immune system to fight cancer, targeting malignant cells and providing a precise immune response through tumor antigen recognition[1].

There are different types of immunotherapy, so different types of cancer responses can be achieved. All of them are bound by a fundamental principle: Immunotherapy is different from standard therapies (*i.e.* chemotherapy, radiotherapy, or oncologic surgery) because it helps the self-response to cancer[2].

For these reasons, the standard criteria for monitoring the success of therapy in oncologic patients are not sufficient. All scores, including the World Health Organization classification and the response evaluation criteria in solid tumors (RECIST 1.1.), do not consider that fighting cancer for immunotherapy requires a synergy between tumor cells and host cells[3,4]. To obviate this essential issue, since 2004, different criteria were developed to analyze these responses such as immune-related response criteria, immune-related RECIST, and finally in 2017 immuno-RECIST (iRECIST)[5-8]. These new criteria aim to consider the variety and the time of response to immunotherapy compared with standard therapy, and to standardize and validate the radiologic and clinical interpretation[9].

However, immunotherapy raises different questions such as: why is the target lesion increased at first control after immunotherapy and reduced at its end? Why is the target legion bigger at the end of treatment, but the patient's conditions improve? Why do some metastases disappear, and others become bigger? These different phenomena are called pseudoprogression, hyperprogression, and dissociate response, respectively, and belong to the new lexicon of cancer response to immunotherapeutic agents[10,11].

Radiologists and clinicians should be confident with these patterns (Figure 1) and the interpretation of these data to better understand and manage oncologic patients who have undergone immunotherapy.

In this setting, the present review aims to critically analyze and summarize the most common type of responses to immunotherapy and to drive the knowledge of correct radiologic and clinical interpretation of iRECIST, strengthening the communication skills between specialists.

PSEUDOPROGRESSION

Pseudoprogression is defined as the phenomenon characterized by an initial increase in primary tumor size or new lesions appearance, after starting immunotherapy, followed by a decrease in tumor burden[12-15]. Pseudoprogression should not be considered a true tumor progression but an infiltration and recruitment of various immune cells, such as T or B lymphocytes in the tumor core[16]. Two biological hypotheses have been proposed to explain the phenomenon of pseudoprogression

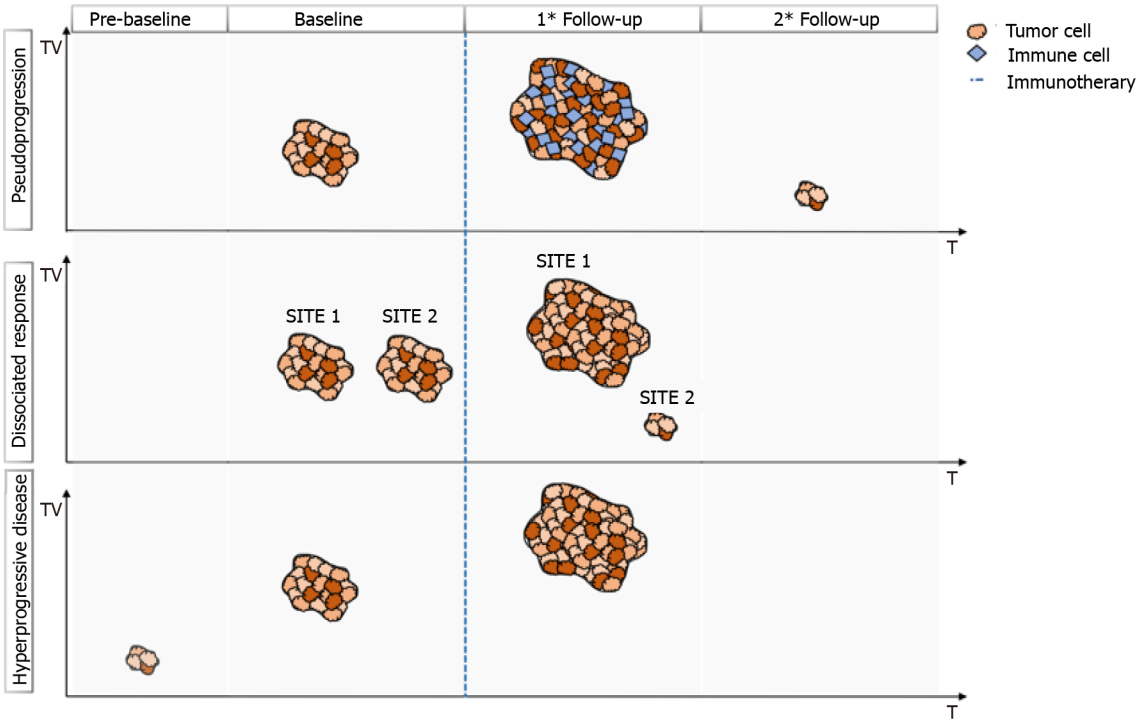


Figure 1 Graphical summary of different responses to immunotherapy. Pseudoprogression: increase of longest diameter > 20% at first follow-up, followed by a decrease of > 30% at subsequent follow-up. Dissociated response: increase of some target lesions of > 20% and reduction of at least another target lesion > 30% at follow-up. Hyperprogression: significant increase of target lesion at first follow-up; a baseline study is needed to correctly assess the hyperprogression. T: Time; TV: Tumor volume.

observed in patients treated with immuno-oncology agents. The first hypothesis concerns tumors' continuous growth until the activation of an effective antitumoral immune response; the second one suggests that an immune-cell influx could occur in the tumoral microenvironment caused by the reactivation of the immune system, leading to inflammation and a transient increase of tumor burden[15].

A study by Cohen *et al*[17] described the case of a patient with melanoma brain metastasis, who was treated with pembrolizumab, presenting a pseudoprogression of brain lesions revealed through magnetic resonance imaging (MRI) and biopsy. The MRI showed an enlargement of central nervous system lesions with diffuse perilesional edema, while the histologic evaluation revealed tumor cells surrounded by reactive astrocytosis, scattered inflammatory cells, and microglial cells, which was consistent with the abovementioned response to treatment rather than tumor growth.

Rocha *et al*[18] described the case of a patient with end-stage squamous cell lung cancer, who was treated with nivolumab and exhibited pseudoprogression of the liver lesions, proved by the biopsy. The tissue sample revealed extensive areas of necrosis, no viable tumor cells, and lymphocyte infiltration. In the liver biopsy, the number of $CD4^-$, $CD8^-$ and $CD103^-$ cells were increased, the ratio of $CD4^+/CD8^+$ T cells was decreased, and $CD68^+$ staining indicated a higher proportion of macrophages, suggesting an inflammatory response rather than disease progression. Moreover, other cases have shown necrosis, hemorrhage, edema, and immune cell infiltration in lesions with pseudoprogression[14,15,19,20]. Therefore, the infiltration of immune cells, such as $CD4^+$, $CD8^+$ cells and macrophages, represents the major mechanism of pseudoprogression, consequently including edema, hemorrhage, and necrosis[12].

An unconventional pattern of response to immunotherapy was first described with the development of cytotoxic T-lymphocyte antigen 4 inhibitors in melanoma, with a patient experiencing enlargement of a cutaneous lesion during the first weeks of treatment, followed by prolonged stabilization[15]. Since then, pseudoprogression has been used to describe an objective response obtained after initial progression disease and has been observed in other cancer types[16] (Figure 2).

The occurrence of pseudoprogression was confirmed in large trials, which allows treatment beyond progression; its incidence, reported in different tumor types, has never exceeded 10% of patients[21]. However, a recent study determined that the incidence of atypical response is about 20%, including the development of new lesions, and the increase greater than 10% in the total sum of the longest dimension[22].

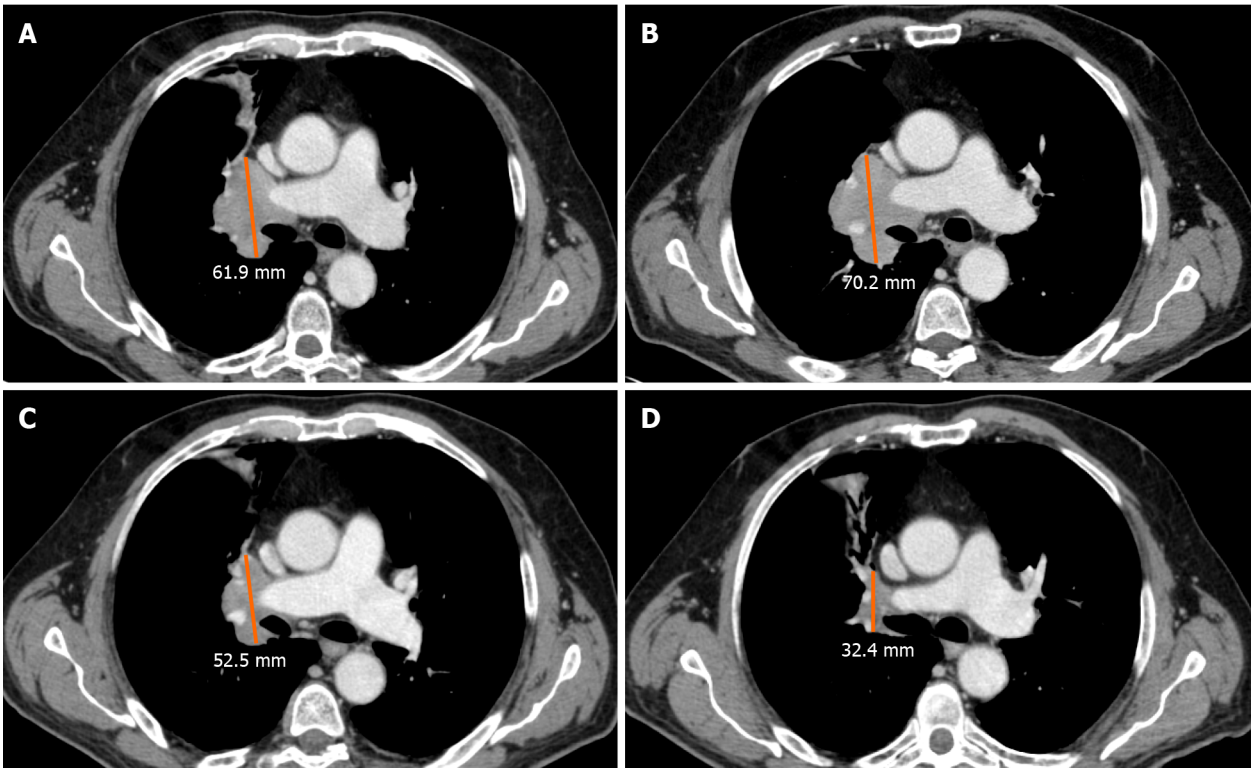


Figure 2 Axial computed tomography images in the portal-venous phase of a 69 y/o male, ex-smoker with non-small lung cell carcinoma, during second-line therapy with Atezolizumab. A: Pre-treatment imaging show the right peri-hilar lesion; B: During follow-up after 4 wk the lesion increase in size; C and D: During the following computed tomography scans (8 and 12 wk) a significant decrease in longest diameter was achieved, confirming a final response to treatment with the presence of intercurrent (B) pseudoprogression.

Pseudoprogression has been described in different types of tumors, mainly in melanoma patients but also in non-small lung cell carcinoma (NSCLC) (Figure 3), renal cancer (RCC), urothelial cancer, uveal melanoma, Merkel cell carcinoma, mesothelioma, Hodgkin lymphoma, and head and neck squamous cell carcinoma (HNSCC) [23] and it can also occur in metastatic lesions and some oncologic patients with pleural effusion and ascites[14,24-29].

The reported incidence of pseudoprogression in clinical trials was 2.78%-9.69% for melanoma, 1.81%-5.77% for NSCLC, 2.86%-8.82% for RCC, 1.49%-7.14% for urothelial carcinoma, 11.11% for uveal melanoma, 1.79% for HNSCC, 1.14% for Merkel cell carcinoma, and 6.90% for mesothelioma[12].

Clinical and biological characteristics of different tumors, the demographic characteristics of patients, and the different types of immunotherapy agents used might explain the different incidence of pseudoprogression in various types of solid tumors. In addition, according to some case reports, there might be some sites of pseudoprogression specific to the tumor type after immunotherapy, such as brain metastasis pseudoprogression of lung cancer and RCC[30,31].

Interestingly, for patients treated beyond progression, no increase in immune-related toxicity was reported. Furthermore, patients experiencing pseudoprogression had longer overall survival (OS) compared with standard progressive disease (PD), suggesting that patients who present with pseudoprogression can effectively obtain benefit from treatment beyond progression[23].

The iRECIST guidelines proposed two specific response patterns: unconfirmed PD (iUPD) and confirmed PD (iCPD). The iUPD is defined as PD for the RECIST v1.1 criteria that is not confirmed at the follow-up imaging assessment within 4-8 wk. The iCPD is defined as the appearance of a new lesion or further growth of the sum of measures of target lesions of 5 mm or greater at the diagnostic follow-up after the iUPD within 4-8 wk, or as an increase in a non-target lesion, that was initially categorized as iUPD. If no change in tumor size nor extent from iUPD occurs, then the time point response would again be iUPD. Complete response (iCR), partial response (iPR), and stable disease (iSD) were assigned based on the RECIST 1.1. Moreover, if after iSD, iPR, or iCR, PD takes place again, we consider it as iUPD and reset the bar again through the application of the so-called "dynamic time point"[7]. To resume, iUPD can be assigned multiple times as long as iCPD is not confirmed at the next

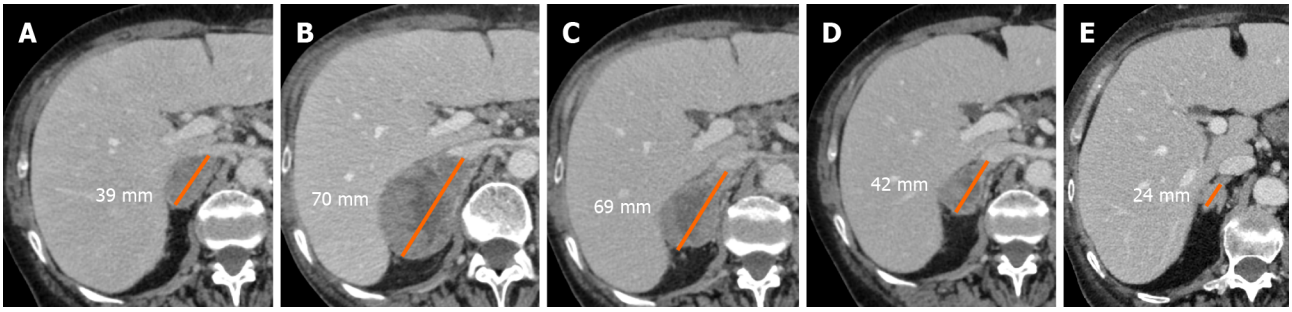


Figure 3 Axial computed tomography images in the portal-venous phase of a 65 y/o female, with non-small lung cell carcinoma and a programmed death-ligand 1 expression > 90%, during first-line treatment with Pembrolizumab. A: In the baseline examination, computed tomography (CT) shows a metastatic lesion in the right adrenal gland; B: After 4 wk of treatment, the lesion becomes bigger, with a total increment of 21%, referred to as unconfirmed progression; C-E: during following CT scans, the target lesion shows a progressive dimensional reduction with a total decrease of 46%. These variations in the size of the lesion, during immunotherapy, was in line with pseudoprogression.

assessment and iRECIST requires the confirmation of progression to rule out or confirm pseudoprogression.

The iRECIST guidelines proposed a status of iUPD, which would allow the continuation of treatment and follow-up more closely to better benefit patients. This approach allows the identification, understanding, and better characterization of atypical responses, such as delayed responses that occur after pseudoprogression[7].

To differentiate pseudoprogression from true progression, the iRECIST guidelines recommend that clinical trials should only include patients who are clinically stable to continue treatments until the next assessment (≥ 4 wk later). In these cases, the next imaging assessment should be performed no longer than 8 wk later, to ensure that patients remain fit for rescue therapies[7].

Among the potential useful methods to identify pseudoprogression in tumors treated with immunotherapy and to differentiate it from the true progression of the disease, the combination of biopsy and histopathologic examination is considered the gold standard, although it presents some disadvantages due to the invasive nature of the procedure. Compared to biopsy, the radiographic follow-up presents incomparable advantages in the monitoring of pseudoprogression. It could be used in any type of tumor with measurable lesions and it is convenient, non-invasive, and can avoid prematurely discontinued immunotherapy for pseudoprogression. In addition to radiological computed tomography (CT) follow-up, other proposed methods to determine pseudoprogression included MRI and positron emission tomography (PET)/CT, which can distinguish inflammatory cell infiltration from the enlarged tumor tissue, at least theoretically. In recent literature, the circulating tumor's DNA and interleukin-8 serum levels were purposed in the follow-up of oncologic patients to quickly identify a possible pseudoprogression[12].

A summary of the most important studies focusing on pseudoprogression is reported in Table 1.

HYPERPROGRESSION

Hyperprogressive disease (HPD) is considered fast tumor growth, after starting immunotherapy, regarding the absolute mass. However, compared with the other atypical patterns, HPD relies on its intrinsic definition in the "expected" response, and consequently, a specific description is currently missing. For example, empiric doubling of tumor volume or by using linear growth in tumor diameter have been proposed to identify the HPD and, as a matter of fact, recently published papers reported different ways to define HPD and different thresholds to stratify pa-tients[32-34]. Moreover, considering that HPD can be shown in different cancer types, a standardized definition is needed.

Different cellular and genetic triggering events were studied to better define and understand HPD. The first described is linked to cytotoxic agents used before immunotherapy, probably causing a decreased effect of the last one[35] due to clones' selection able to escape therapy. On the other hand, new immunotherapeutic agents can bind other than targeted receptors and allow rapid tumor growth. Finally, different genetic mutations, such as the most common one Janus kinase 1/2 mutation,

Table 1 Incidence of atypical response in different cancer types and treatments, according to the most recent literature

Response	Cancer type	Treatment	Incidence (% range)
Pseudoprogression	Melanoma	Ipilimumab	7.4-9.7
		Tremelimumab	2.8-6.3
		PD1/PD-L1 inhibitors	3.7-8.3
		Pembrolizumab	3.7-7.3
	RCC	PD1/PD-L1 inhibitors	4.9-14.8
		Atezolizumab	2.9
	NSCLC	PD1/PD-L1 inhibitors	1.9-6.9
		Atezolizumab	2.8
	Urothelial	Atezolizumab	1.5-6.8
		Durvalumab	7.1
		PD1/PD-L1 inhibitors	8.9
	HNSCC	Pembrolizumab	1.8
		PD1/PD-L1 inhibitors	1.3
	Mesothelioma	Tremelimumab	6.9
Hyperprogression	NSCLC	PD1/PD-L1 inhibitors	8.0-14.0
	Gastric	PD1/PD-L1 inhibitors	21.0-29.4
	RCC	PD1/PD-L1 inhibitors	7.0-46.0
	Melanoma	PD1/PD-L1 inhibitors	1.2
Dissociated response	NSCLC	PD1/PD-L1 inhibitors	7-5-10

HNSCC: Head and neck squamous cell carcinoma; NSCLC: Non-small lung cell carcinoma; PD1: Programmed cell death 1; PD-L1: Programmed death-ligand 1; RCC: Renal cell carcinoma.

can be directly linked to HPD, generating resistance to immunotherapy and resulting in a fast tumor volume increase. The tumor microenvironment can be strictly involved in HPD, especially by immune cell infiltration, as reported in previous papers[36-38].

From a radiological point of view, to identify HPD, at least one imaging exam should be obtained before and one after starting immunotherapy, to correctly establish an increase in tumor volume higher than the expected one[39,40].

Even if the iRECIST algorithm is the most widely applied in clinical practice, it does not suggest evaluating the pretreatment imaging data to identify the tumor growth rate (TGR), and suspected hyperprogressive patients should be followed-up for at least 12 wk for definitive confirmation[39]. The identification of HPD poses a challenge for the iRECIST, which fail to capture pre- and post-treatment tumor growth kinetics (TGK) at early times of disease, and consequently, different parameters such as "RECIST progression at the first evaluation"[39], TGR[40], TGK ratio (ratio of the slope of tumor growth before treatment and the slope of tumor growth on treatment), time to treatment failure (TTF)[41], and the combination of clinical and radiological criteria [42] have been proposed.

A recent study by Gomes da Morais *et al*[43], combining four different definitions for HPD previously proposed, found no overall significant differences between baseline and post-baseline tumor growth rate (P = 0.93). Finally, the authors confirmed that the progression-free survival (PFS) was shorter in patients with HPD compared with non-HPD ones.

A metanalysis published by Kim *et al*[44] evaluated a total of 217 HPD cases of 1519 cancer patients. Considering the lack in HPD definition, its incidence ranged from 1% to 30%, in line with Frelaut *et al*[23], reporting a range from 7% and 29%. Authors identified age (> 65 years), gender (female), aggressive primary tumor (high recurrence rate, > 2 metastatic sites), histological and immunological profiling (*i.e.* low programmed death-ligand 1 expression, epidermal growth factor receptor, mouse double minute 2 homology and DNA (cytosine-5)-methyltransferase 3A alterations) as predictive factors for HPD.

Analyzing the most important recent studies, Park *et al*[45] identified HPD in 18 patients (14.4%) with head and neck cancer, underlying that younger age, a primary tumor of the oral cavity, and previous locoregional irradiation are significant predictors of HPD. Moreover, patients with HPD showed a shorter median PFS and OS.

To date, different published papers have investigated the importance of HPD in lung cancer patients. Kim *et al*[46] observed HPD in 55 (20.9%), 54 (20.5%), and 98 (37.3%) patients according to the TGK, TGR, and TTF, underlying that HPD was associated with worse PFS and OS. The same results in terms of incidence were reported in previous retrospective studies by Ferrara *et al*[40] (14%), Lo Russo *et al*[42] (26%), Kim *et al*[46] (21%), as summarized in the review by Kim *et al*[44]. More recently, Kas *et al*[47], with a retrospective study including 406 patients, suggested a new definition for HPD in patients with NSCLC, based on ΔTGR.

Aoki *et al*[48] and Sasaki *et al*[49] studied the importance of HPD in gastric cancer patients reporting an incidence of 29.4% and 21% after nivolumab treatment, respectively. Both studies reported a slight decrease in PFS and OS in patients with HPD.

Kim *et al*[50] reported that HPD exists in a fraction of hepatocellular carcinoma (HCC) patients who received programmed cell death protein 1 (PD-1) blockade: Analyses of the baseline immune profile and on-treatment tumor growth dynamics could promote optimal patient selection and earlier identification of rapid tumor growth induced by PD-1 inhibitors in HCC patients[50].

Zheng *et al*[51] reviewed patients with RCC under immunotherapy, finding that the incidence of HPD ranged between 7% and 74% without any strong suggestive factors associated.

Regarding melanoma, immunotherapy treatment is not extensively reported in the literature. A recent retrospective study by Hao *et al*[52] and Schuiveling *et al*[53], enrolling 168 patients, reported a 1.2% incidence of HPD.

According to the RECIST working group, a CT scan 8 wk after the first treatment is needed to evaluate early response[7]. In line with the guidelines, if progression is not confirmed, the follow-up should be continued as previously planned, while in case of suspected progression at first-imaging follow-up, a confirmatory CT 4 wk later should be required. Moreover, considering the importance of pre-baseline imaging, a CT scan at least 1 mo before starting immunotherapy should be evaluated to define the tumor volume and consider it in further evaluations. During the anamnestic questionnaire, special attention should be addressed to pre-immunotherapy treatments, specifically regarding conventional cytotoxic agents, as aforementioned[39,40]. Radiological assessment, both CT- and MRI-based, is fundamental to determine the growth rate; however, the true positive rate can be weakened by pseudoprogression in case of pre-baseline missing, because it is not possible to distinguish between the two patterns.

On these bases, a complete assessment based on clinical and radiological findings, along with a careful evaluation of pre-baseline imaging, is needed to correctly stratify patients suspected of HPD, to define the best clinical approach possible to increase PFS and OS. The difficulties to standardize the HPD definition by using radiological criteria firstly rely on the various types of cancer to deal with and, consequently, on the different imaging techniques considered as the reference standard for staging and re-staging patients.

A summary of the most important studies focusing on hyperprogression is reported in Table 1.

DISSOCIATED RESPONSE

Besides the mixed pattern of response arising from traditional platinum-based chemotherapy, the development of immunotherapy has led to the introduction of the concept of dissociated response (DR).

DR has been recently described as a concomitant increase in the size of some target lesions or the appearance of new lesions, accompanied by regression of other ones[54]. A combination of factors may explain the biological mechanisms of a dissociated tumor response. Tumor heterogeneity within an individual patient and differences in tissue penetration of anti-cancer drugs have been proposed as potential reasons for DR [55]. Tumoral cells can undergo clonal evolution from a single progenitor cell into more aggressive and therapy-resistant cells, due to genomic instability of solid cancer cells.

This genotypic and phenotypic heterogeneity is an unfavorable prognostic factor for cells' survival, and it can explain the DR, particularly when using targeted therapies due to their selective pressure on tumor evolution. Moreover, the heterogeneity of the immune environment of the lesions can actively influence therapeutic response and therefore explain different responses[56] (Figure 4).

In literature few studies reported on the incidence of DR, ranging from 7.5% to 10% [54,55,57]. Using fluorodeoxyglucose PET/CT, Humbert *et al*[58] recently pub-lished a prospective study including 50 patients with NSCLC treated with pembrolizumab in first-line therapy or with nivolumab in second-line therapy, showing that 10% of the population had a DR.

DR has been associated with different prognoses compared to progressive or non-PD. Tazdait *et al*[54] observed similar survival between patients with the non-PD and those with the atypical response, even if pseudoprogression and DR were not evaluated separately. On the contrary, the higher survival of patients with DR, compared to those with PD, was confirmed both by Tazdait *et al*[54] and Tozuka *et al* [55], suggesting that the prognosis of patients with DR is probably intermediate between those with PD and those with the non-PD.

In the literature, several different definitions of DR were encountered; in particular, it is still not clear if a concomitant progression and reduction of different lesions are sufficient to consider as DR, or if it is necessary to reach at least 20% of PD and 30% of PR[54,55,57]. On PET/CT, DR definition should be inspired by PET Response Criteria In solid tumor (PERCIST) and defined as a concomitant relative decrease > 30% in some tumor lesions metabolism and relative metabolic increase > 30% in others.

An important issue is the optimal duration of treatment due to the potential of late treatment effect and the rare phenomenon of pseudoprogression. Many clinicians choose to continue treatment beyond progression with immunotherapy according to the RECIST[59]. As the progressing lesions might represent pseudoprogression, the monitoring and management of patients with the DR should be similar to that of patients with pseudoprogression, if the patient is clinically stable. A recent study shows that continuing immunotherapy post-DR had significantly better survival than discontinuing therapy[57].

Besides, continuing immune checkpoint inhibitor treatment plus local ablative therapy targeted to progressing lesions could be a valid alternative to immunotherapy alone in case of single progressive lesions[56]. However, if the patient is clinically deteriorating the interruption of immune checkpoint inhibitor treatment and switching to another therapy, or clinical trial participation, should be considered[60].

The high number of atypical responses such as pseudoprogression and DR suggest that in most cases the RECIST 1.1 underestimates the benefit of treatment with immunotherapy and the new iRECIST are certainly superior in the evaluation of responses. The iRECIST consider consistently pseudoprogression, while DR is not considered[7,11], suggesting that they may not correctly describe the clinical benefit from immunotherapy[61].

Considering the different interpretations of DR given by the different authors, a more uniform definition of this phenomenon is crucial to assess the correct prognosis of patients with DR compared to progressive and non-PD after immunotherapy. As suggested by Humbert and Chardin[56], DR on CT exam should be inspired by RECIST 1.1, defined as a concomitant decrease in size > 30% in some lesions and increase in size > 20% in others (and/or presence of new lesions), while on PET/CT, DR should be motivated by PERCIST criteria, defined as a concomitant decrease > 30% in some tumor lesions metabolism and metabolic increase > 30% in others (and/or new hypermetabolic lesions).

A summary of the most important studies focusing on DR is reported in Table 1.

To conclude, DR should be considered in the iRECIST in addition to or separately from a PD, partial response, and stable disease, through radiological evaluation, for a more precise evaluation of tumor response to the immunotherapy.

CONCLUSION

iRECIST can help to correctly categorize the classes of response to immunotherapy treatment by dividing patients into four main groups (iSD, iPR, iCR, iPD), according to the radiological target lesion modifications, achieved along the time, and the standard solid response criteria (RECIST 1.1). Recently, other different kinds of response have been described in literature after immunotherapy treatment, defined as atypical responses, categorized in three patterns: pseudoprogression, hyperprogression, and

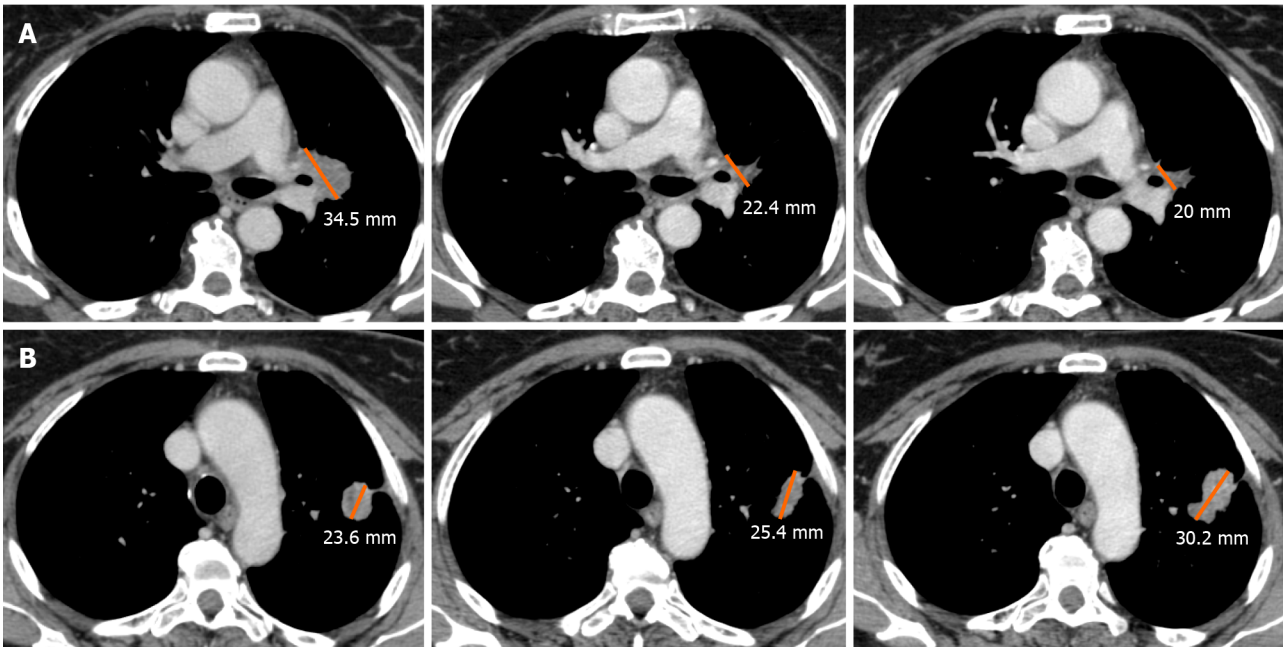


Figure 4 Axial computed tomography images in the portal-venous phase of a 57 y/o female ex-smoker with non-small lung cell carcinoma during second-line treatment with Pembrolizumab. Images show a dissociated response of two target lesions. A: The left peri-hilar lesion progressively decreased in size during follow-up, if compared to the pre-treatment computed tomography scan (after 3 wk and after 9 wk of immunotherapy from left to right, respectively); B: The second target lesion in left lung firstly regressed after 3 wk of immunotherapy showing, then a progression during the follow-up period (from left to right, respectively).

dissociated response. The correct knowledge of these new atypical patterns should be correctly assessed by both radiologists and clinicians, through the deep investigation of clinical anamnesis and imaging findings to guarantee the best management.

REFERENCES

1 **Liu M**, Guo F. Recent updates on cancer immunotherapy. *Prec Clin Med* 2018; **1**: 65-74 [PMID: 30687562 DOI: 10.1093/pcmedi/pby011]

2 **Kwak JJ**, Tirumani SH, Van den Abbeele AD, Koo PJ, Jacene HA. Cancer immunotherapy: imaging assessment of novel treatment response patterns and immune-related adverse events. *Radiographics* 2015; **35**: 424-437 [PMID: 25763727 DOI: 10.1148/rg.352140121]

3 **Eisenhauer EA**, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, Kaplan R, Lacombe D, Verweij J. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; **45**: 228-247 [PMID: 19097774 DOI: 10.1016/j.ejca.2008.10.026]

4 **Carter BW**, Bhosale PR, Yang WT. Immunotherapy and the role of imaging. *Cancer* 2018; **124**: 2906-2922 [PMID: 29671876 DOI: 10.1002/cncr.31349]

5 **Wolchok JD**, Hoos A, O'Day S, Weber JS, Hamid O, Lebbé C, Maio M, Binder M, Bohnsack O, Nichol G, Humphrey R, Hodi FS. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res* 2009; **15**: 7412-7420 [PMID: 19934295 DOI: 10.1158/1078-0432.CCR-09-1624]

6 **Nishino M**, Giobbie-Hurder A, Gargano M, Suda M, Ramaiya NH, Hodi FS. Developing a common language for tumor response to immunotherapy: immune-related response criteria using unidimensional measurements. *Clin Cancer Res* 2013; **19**: 3936-3943 [PMID: 23743568 DOI: 10.1158/1078-0432.CCR-13-0895]

7 **Seymour L**, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S, Lin NU, Litière S, Dancey J, Chen A, Hodi FS, Therasse P, Hoekstra OS, Shankar LK, Wolchok JD, Ballinger M, Caramella C, de Vries EGE; RECIST working group. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. *Lancet Oncol* 2017; **18**: e143-e152 [PMID: 28271869 DOI: 10.1016/S1470-2045(17)30074-8]

8 **Le Lay J**, Jarraya H, Lebellec L, Penel N. irRECIST and iRECIST: the devil is in the details. *Ann Oncol* 2017; **28**: 1676-1678 [PMID: 28383646 DOI: 10.1093/annonc/mdx168]

9 **Inno A**, Lo Russo G, Salgarello M, Corrao G, Casolino R, Galli G, Modena A, Romano L, Pusceddu S, Greco FG, Garassino MC, Gori S. The evolving landscape of criteria for evaluating tumor response in the era of cancer immunotherapy: From Karnofsky to iRECIST. *Tumori* 2018; **104**: 88-95 [PMID:

29714647 DOI: 10.1177/0300891618766173]
10 **Lennartz S**, Diederich S, Doehn C, Gebauer B, Grünwald V, Notohamiprodjo M, Sommer W, Schlemmer HP, Persigehl T. Radiological Monitoring of Modern Immunotherapy: A Novel Challenge for Interdisciplinary Patient Care. *Rofo* 2020; **192**: 235-245 [PMID: 31994155 DOI: 10.1055/a-1015-6869]
11 **Persigehl T**, Lennartz S, Schwartz LH. iRECIST: how to do it. *Cancer Imaging* 2020; **20**: 2 [PMID: 31900236 DOI: 10.1186/s40644-019-0281-x]
12 **Jia W**, Gao Q, Han A, Zhu H, Yu J. The potential mechanism, recognition and clinical significance of tumor pseudoprogression after immunotherapy. *Cancer Biol Med* 2019; **16**: 655-670 [PMID: 31908886 DOI: 10.20892/j.issn.2095-3941.2019.0144]
13 **Nishino M**. Pseudoprogression and Measurement Variability. *JCO* 2016; **34**: 3480-3481 [PMID: 27458299 DOI: 10.1200/JCO.2016.67.6759]
14 **Hochmair MJ**, Schwab S, Burghuber OC, Krenbek D, Prosch H. Symptomatic pseudo-progression followed by significant treatment response in two lung cancer patients treated with immunotherapy. *Lung Cancer* 2017; **113**: 4-6 [PMID: 29110847 DOI: 10.1016/j.lungcan.2017.08.020]
15 **Di Giacomo AM**, Danielli R, Guidoboni M, Calabrò L, Carlucci D, Miracco C, Volterrani L, Mazzei MA, Biagioli M, Altomonte M, Maio M. Therapeutic efficacy of ipilimumab, an anti-CTLA-4 monoclonal antibody, in patients with metastatic melanoma unresponsive to prior systemic treatments: clinical and immunological evidence from three patient cases. *Cancer Immunol Immunother* 2009; **58**: 1297-1306 [PMID: 19139884 DOI: 10.1007/s00262-008-0642-y]
16 **Chiou VL**, Burotto M. Pseudoprogression and Immune-Related Response in Solid Tumors. *J Clin Oncol* 2015; **33**: 3541-3543 [PMID: 26261262 DOI: 10.1200/JCO.2015.61.6870]
17 **Cohen JV**, Alomari AK, Vortmeyer AO, Jilaveanu LB, Goldberg SB, Mahajan A, Chiang VL, Kluger HM. Melanoma Brain Metastasis Pseudoprogression after Pembrolizumab Treatment. *Cancer Immunol Res* 2016; **4**: 179-182 [PMID: 26701266 DOI: 10.1158/2326-6066.CIR-15-0160]
18 **Rocha P**, Hardy-Werbin M, Naranjo D, Taus Á, Rodrigo M, Zuccarino F, Roth R, Wood O, Ottensmeier CH, Arriola E. CD103+CD8+ Lymphocytes Characterize the Immune Infiltration in a Case With Pseudoprogression in Squamous NSCLC. *J Thorac Oncol* 2018; **13**: e193-e196 [PMID: 29775806 DOI: 10.1016/j.jtho.2018.05.008]
19 **Tabei T**, Tsuura Y, Kobayashi K. Pseudoprogression: A case of metastatic renal clear cell carcinoma treated with nivolumab: Letter to the Editor. *Pathol Int* 2018; **68**: 627-629 [PMID: 30151940 DOI: 10.1111/pin.12714]
20 **Tanizaki J**, Hayashi H, Kimura M, Tanaka K, Takeda M, Shimizu S, Ito A, Nakagawa K. Report of two cases of pseudoprogression in patients with non-small cell lung cancer treated with nivolumab-including histological analysis of one case after tumor regression. *Lung Cancer* 2016; **102**: 44-48 [PMID: 27987588 DOI: 10.1016/j.lungcan.2016.10.014]
21 **Borcoman E**, Nandikolla A, Long G, Goel S, Le Tourneau C. Patterns of Response and Progression to Immunotherapy. *Am Soc Clin Oncol Educ Book* 2018; **38**: 169-178 [PMID: 30231380 DOI: 10.1200/EDBK_200643]
22 **Thomas R**, Somarouthu B, Alessandrino F, Kurra V, Shinagare AB. Atypical Response Patterns in Patients Treated With Nivolumab. *AJR Am J Roentgenol* 2019; 1-5 [PMID: 30917022 DOI: 10.2214/AJR.18.20938]
23 **Frelaut M**, du Rusquec P, de Moura A, Le Tourneau C, Borcoman E. Pseudoprogression and Hyperprogression as New Forms of Response to Immunotherapy. *BioDrugs* 2020; **34**: 463-476 [PMID: 32394415 DOI: 10.1007/s40259-020-00425-y]
24 **Swami U**, Smith M, Zhang J. Central Nervous System Pseudoprogression With Nivolumab in a Patient With Squamous Cell Lung Cancer Followed by Prolonged Response. *J Thorac Oncol* 2018; **13**: e183-e184 [PMID: 30166022 DOI: 10.1016/j.jtho.2018.05.001]
25 **Yoshimura A**, Takumi C, Tsuji T, Hamashima R, Shiotsu S, Yuba T, Urata Y, Hiraoka N. Pulmonary pleomorphic carcinoma with pseudoprogression during nivolumab therapy and the usefulness of tumor markers: A case report. *Clin Case Rep* 2018; **6**: 1338-1341 [PMID: 29988633 DOI: 10.1002/ccr3.1627]
26 **Wong AS**, Thian YL, Kapur J, Leong CN, Kee P, Lee CT, Lee MB. Pushing the limits of immune-related response: a case of "extreme pseudoprogression". *Cancer Immunol Immunother* 2018; **67**: 1105-1111 [PMID: 29728723 DOI: 10.1007/s00262-018-2167-3]
27 **Chae YK**, Wang S, Nimeiri H, Kalyan A, Giles FJ. Pseudoprogression in microsatellite instability-high colorectal cancer during treatment with combination T cell mediated immunotherapy: a case report and literature review. *Oncotarget* 2017; **8**: 57889-57897 [PMID: 28915720 DOI: 10.18632/oncotarget.18361]
28 **Liu G**, Chen T, Li R, Zhu L, Liu D, Ding Z. Well-controlled pleural effusion indicated pseudoprogression after immunotherapy in lung cancer: A case report. *Thorac Cancer* 2018; **9**: 1190-1193 [PMID: 29978949 DOI: 10.1111/1759-7714.12799]
29 **Sweis RF**, Zha Y, Pass L, Heiss B, Chongsuwat T, Luke JJ, Gajewski TF, Szmulewitz R. Pseudoprogression manifesting as recurrent ascites with anti-PD-1 immunotherapy in urothelial bladder cancer. *J Immunother Cancer* 2018; **6**: 24 [PMID: 29618376 DOI: 10.1186/s40425-018-0334-x]
30 **Doherty MK**, Jao K, Shepherd FA, Hazrati LN, Leighl NB. Central Nervous System Pseudoprogression in a Patient Treated with PD-1 Checkpoint Inhibitor. *J Thorac Oncol* 2015; **10**: e100-e101 [PMID: 26398825 DOI: 10.1097/JTO.0000000000000587]

31 **Lewis GD**, Jonasch E, Shah AY, Fuller GN, Farach AM, Butler EB, Teh BS. Renal cell carcinoma brain metastasis with pseudoprogression and radiation necrosis on nivolumab after previous treatment with stereotactic radiosurgery: An illustrative case report and review of the literature. *Pract Radiat Oncol* 2018; **8**: e262-e265 [PMID: 29706304 DOI: 10.1016/j.prro.2018.03.003]

32 **Saâda-Bouzid E**, Defaucheux C, Karabajakian A, Coloma VP, Servois V, Paoletti X, Even C, Fayette J, Guigay J, Loirat D, Peyrade F, Alt M, Gal J, Le Tourneau C. Hyperprogression during anti-PD-1/PD-L1 therapy in patients with recurrent and/or metastatic head and neck squamous cell carcinoma. *Ann Oncol* 2017; **28**: 1605-1611 [PMID: 28419181 DOI: 10.1093/annonc/mdx178]

33 **Boland JL**, Zhou Q, Martin M, Callahan MK, Konner J, O'Cearbhaill RE, Friedman CF, Tew W, Makker V, Grisham RN, Hensley ML, Zecca N, Iasonos AE, Snyder A, Hyman DM, Sabbatini P, Aghajanian C, Cadoo KA, Zamarin D. Early disease progression and treatment discontinuation in patients with advanced ovarian cancer receiving immune checkpoint blockade. *Gynecol Oncol* 2019; **152**: 251-258 [PMID: 30470581 DOI: 10.1016/j.ygyno.2018.11.025]

34 **Faure M**, Rochigneux P, Olive D, Taix S, Brenot-Rossi I, Gilabert M. Hyperprogressive Disease in Anorectal Melanoma Treated by PD-1 Inhibitors. *Front Immunol* 2018; **9**: 797 [PMID: 29725330 DOI: 10.3389/fimmu.2018.00797]

35 **Grasselly C**, Denis M, Bourguignon A, Talhi N, Mathe D, Tourette A, Serre L, Jordheim LP, Matera EL, Dumontet C. The Antitumor Activity of Combinations of Cytotoxic Chemotherapy and Immune Checkpoint Inhibitors Is Model-Dependent. *Front Immunol* 2018; **9**: 2100 [PMID: 30356816 DOI: 10.3389/fimmu.2018.02100]

36 **Xiong D**, Wang Y, Singavi AK, Mackinnon AC, George B, You M. Immunogenomic Landscape Contributes to Hyperprogressive Disease after Anti-PD-1 Immunotherapy for Cancer. *iScience* 2018; **9**: 258-277 [PMID: 30439581 DOI: 10.1016/j.isci.2018.10.021]

37 **Wang F**, Li B, Wei Y, Zhao Y, Wang L, Zhang P, Yang J, He W, Chen H, Jiao Z, Li Y. Tumor-derived exosomes induce $PD1^+$ macrophage population in human gastric cancer that promotes disease progression. *Oncogenesis* 2018; **7**: 41 [PMID: 29799520 DOI: 10.1038/s41389-018-0049-3]

38 **Fung KY**, Nguyen PM, Putoczki T. The expanding role of innate lymphoid cells and their T-cell counterparts in gastrointestinal cancers. *Mol Immunol* 2019; **110**: 48-56 [PMID: 29174430 DOI: 10.1016/j.molimm.2017.11.013]

39 **Champiat S**, Ferrara R, Massard C, Besse B, Marabelle A, Soria JC, Ferté C. Hyperprogressive disease: recognizing a novel pattern to improve patient management. *Nat Rev Clin Oncol* 2018; **15**: 748-762 [PMID: 30361681 DOI: 10.1038/s41571-018-0111-2]

40 **Ferrara R**, Mezquita L, Texier M, Lahmar J, Audigier-Valette C, Tessonnier L, Mazieres J, Zalcman G, Brosseau S, Le Moulec S, Leroy L, Duchemann B, Lefebvre C, Veillon R, Westeel V, Koscielny S, Champiat S, Ferté C, Planchard D, Remon J, Boucher M-E, Gazzah A, Adam J, Bria E, Tortora G, Soria J-C, Besse B, Caramella C. Hyperprogressive Disease in Patients With Advanced Non–Small Cell Lung Cancer Treated With PD-1/PD-L1 Inhibitors or With Single-Agent Chemotherapy. *JAMA Oncol* 2018; **4**: 1543 [PMID: 30193240 DOI: 10.1001/jamaoncol.2018.3676]

41 **Kato S**, Goodman A, Walavalkar V, Barkauskas DA, Sharabi A, Kurzrock R. Hyperprogressors after Immunotherapy: Analysis of Genomic Alterations Associated with Accelerated Growth Rate. *Clin Cancer Res* 2017; **23**: 4242-4250 [PMID: 28351930 DOI: 10.1158/1078-0432.CCR-16-3133]

42 **Lo Russo G**, Moro M, Sommariva M, Cancila V, Boeri M, Centonze G, Ferro S, Ganzinelli M, Gasparini P, Huber V, Milione M, Porcu L, Proto C, Pruneri G, Signorelli D, Sangaletti S, Sfondrini L, Storti C, Tassi E, Bardelli A, Marsoni S, Torri V, Tripodo C, Colombo MP, Anichini A, Rivoltini L, Balsari A, Sozzi G, Garassino MC. Antibody-Fc/FcR Interaction on Macrophages as a Mechanism for Hyperprogressive Disease in Non-small Cell Lung Cancer Subsequent to PD-1/PD-L1 Blockade. *Clin Cancer Res* 2019; **25**: 989-999 [PMID: 30206165 DOI: 10.1158/1078-0432.CCR-18-1390]

43 **Gomes de Morais AL**, Cardenas JM, de Miguel Luken MJ, Boni V, Moreno I, Ao G, Liu RH, de Hoyos FB, Cubillo A, Calvo E. Comparative assessment of different radiological criteria to identify paradoxical hyperprogression (HPD) to IO drugs. *JCO* 2020; **38**: e15229-e15229 [DOI: 10.1200/JCO.2020.38.15_suppl.e15229]

44 **Kim JY**, Lee KH, Kang J, Borcoman E, Saada-Bouzid E, Kronbichler A, Hong SH, de Rezende LFM, Ogino S, Keum N, Song M, Luchini C, van der Vliet HJ, Shin JI, Gamerith G. Hyperprogressive Disease during Anti-PD-1 (PDCD1) / PD-L1 (CD274) Therapy: A Systematic Review and Meta-Analysis. *Cancers (Basel)* 2019; **11** [PMID: 31683809 DOI: 10.3390/cancers11111699]

45 **Park JH**, Chun SH, Lee Y-G, Chang H, Lee K-W, Kim HR, Shin SH, An HJ, Lee KE, Hwang IG, Ahn M-J, Kim S-B, Keam B. Hyperprogressive disease and its clinical impact in patients with recurrent and/or metastatic head and neck squamous cell carcinoma treated with immune-checkpoint inhibitors: Korean cancer study group HN 18–12. *J Cancer Res Clin Oncol* 2020; **146**: 3359-3369 [PMID: 32671504 DOI: 10.1007/s00432-020-03316-5]

46 **Kim CG**, Kim KH, Pyo KH, Xin CF, Hong MH, Ahn BC, Kim Y, Choi SJ, Yoon HI, Lee JG, Lee CY, Park SY, Park SH, Cho BC, Shim HS, Shin EC, Kim HR. Hyperprogressive disease during PD-1/PD-L1 blockade in patients with non-small-cell lung cancer. *Ann Oncol* 2019; **30**: 1104-1113 [PMID: 30977778 DOI: 10.1093/annonc/mdz123]

47 **Kas B**, Talbot H, Ferrara R, Richard C, Lamarque JP, Pitre-Champagnat S, Planchard D, Balleyguier C, Besse B, Mezquita L, Lassau N, Caramella C. Clarification of Definitions of Hyperprogressive Disease During Immunotherapy for Non-Small Cell Lung Cancer. *JAMA Oncol* 2020; **6**: 1039-1046 [PMID: 32525513 DOI: 10.1001/jamaoncol.2020.1634]

48 **Aoki M**, Shoji H, Nagashima K, Imazeki H, Miyamoto T, Hirano H, Honma Y, Iwasa S, Okita N, Takashima A, Kato K, Higuchi K, Boku N. Hyperprogressive disease during nivolumab or irinotecan treatment in patients with advanced gastric cancer. *ESMO Open* 2019; **4**: e000488 [PMID: 31231567 DOI: 10.1136/esmoopen-2019-000488]

49 **Sasaki A**, Nakamura Y, Mishima S, Kawazoe A, Kuboki Y, Bando H, Kojima T, Doi T, Ohtsu A, Yoshino T, Kuwata T, Akimoto T, Shitara K. Predictive factors for hyperprogressive disease during nivolumab as anti-PD1 treatment in patients with advanced gastric cancer. *Gastric Cancer* 2019; **22**: 793-802 [PMID: 30627987 DOI: 10.1007/s10120-018-00922-8]

50 **Kim CG**, Kim C, Yoon SE, Kim KH, Choi SJ, Kang B, Kim HR, Park SH, Shin EC, Kim YY, Kim DJ, Chung HC, Chon HJ, Choi HJ, Lim HY. Hyperprogressive disease during PD-1 blockade in patients with advanced hepatocellular carcinoma. *J Hepatol* 2021; **74**: 350-359 [PMID: 32810553 DOI: 10.1016/j.jhep.2020.08.010]

51 **Zheng Z**, Wu K, Yao Z, Mu X, Wu H, Zhao W, Cheng L, Liu Z. Hyperprogressive disease in patients with advanced renal cell carcinoma: a new pattern of post-treatment cancer behavior. *Immunol Res* 2020; **68**: 204-212 [PMID: 32651873 DOI: 10.1007/s12026-020-09138-4]

52 **Hao C**, Tian J, Liu H, Li F, Niu H, Zhu B. Efficacy and safety of anti-PD-1 and anti-PD-1 combined with anti-CTLA-4 immunotherapy to advanced melanoma: A systematic review and meta-analysis of randomized controlled trials. *Medicine (Baltimore)* 2017; **96**: e7325 [PMID: 28658143 DOI: 10.1097/MD.0000000000007325]

53 **Schuiveling M**, Tonk EHJ, Verheijden RJ, Suijkerbuijk KPM. Hyperprogressive disease rarely occurs during checkpoint inhibitor treatment for advanced melanoma. *Cancer Immunol Immunother* 2021; **70**: 1491-1496 [PMID: 32929554 DOI: 10.1007/s00262-020-02716-3]

54 **Tazdait M**, Mezquita L, Lahmar J, Ferrara R, Bidault F, Ammari S, Balleyguier C, Planchard D, Gazzah A, Soria JC, Marabelle A, Besse B, Caramella C. Patterns of responses in metastatic NSCLC during PD-1 or PDL-1 inhibitor therapy: Comparison of RECIST 1.1, irRECIST and iRECIST criteria. *Eur J Cancer* 2018; **88**: 38-47 [PMID: 29182990 DOI: 10.1016/j.ejca.2017.10.017]

55 **Tozuka T**, Kitazono S, Sakamoto H, Yoshida H, Amino Y, Uematsu S, Yoshizawa T, Hasegawa T, Uchibori K, Yanagitani N, Horiike A, Horai T, Seike M, Gemma A, Nishio M. Dissociated responses at initial computed tomography evaluation is a good prognostic factor in non-small cell lung cancer patients treated with anti-programmed cell death-1/ligand 1 inhibitors. *BMC Cancer* 2020; **20**: 207 [PMID: 32164651 DOI: 10.1186/s12885-020-6704-z]

56 **Humbert O**, Chardin D. Dissociated Response in Metastatic Cancer: An Atypical Pattern Brought Into the Spotlight With Immunotherapy. *Front Oncol* 2020; **10**: 566297 [PMID: 33072599 DOI: 10.3389/fonc.2020.566297]

57 **Zhou H**, Sun Y, Xiu W, Han J, Zhong L, Suo J, Wei H, Wang Y, Zhu J. Overall survival benefit of continuing immune checkpoint inhibitors treatment post dissociated response in patients with advanced lung cancer. *J Cancer Res Clin Oncol* 2020; **146**: 2979-2988 [PMID: 32518972 DOI: 10.1007/s00432-020-03282-y]

58 **Humbert O**, Cadour N, Paquet M, Schiappa R, Poudenx M, Chardin D, Borchiellini D, Benisvy D, Ouvrier MJ, Zwarthoed C, Schiazza A, Ilie M, Ghalloussi H, Koulibaly PM, Darcourt J, Otto J. ^{18}FDG PET/CT in the early assessment of non-small cell lung cancer response to immunotherapy: frequency and clinical significance of atypical evolutive patterns. *Eur J Nucl Med Mol Imaging* 2020; **47**: 1158-1167 [PMID: 31760467 DOI: 10.1007/s00259-019-04573-4]

59 **Reinhorn D**, Jacobi O, Icht O, Dudnik E, Rotem O, Zer A, Goldstein DA. Treatment beyond progression with immune checkpoint inhibitors in non-small-cell lung cancer. *Immunotherapy* 2020; **12**: 235-243 [PMID: 32189549 DOI: 10.2217/imt-2019-0131]

60 **Billan S**, Kaidar-Person O, Gil Z. Treatment after progression in the era of immunotherapy. *Lancet Oncol* 2020; **21**: e463-e476 [PMID: 33002442 DOI: 10.1016/S1470-2045(20)30328-4]

61 **Gandara DR**, von Pawel J, Mazieres J, Sullivan R, Helland Å, Han JY, Ponce Aix S, Rittmeyer A, Barlesi F, Kubo T, Park K, Goldschmidt J, Gandhi M, Yun C, Yu W, Matheny C, He P, Sandler A, Ballinger M, Fehrenbacher L. Atezolizumab Treatment Beyond Progression in Advanced NSCLC: Results From the Randomized, Phase III OAK Study. *J Thorac Oncol* 2018; **13**: 1906-1918 [PMID: 30217492 DOI: 10.1016/j.jtho.2018.08.2027]

World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2021 May 24; 12(5): 335-341

DOI: 10.5306/wjco.v12.i5.335

ISSN 2218-4333 (online)

MINIREVIEWS

Hepatic Langerhans cell histiocytosis: A review

Zhiyan Fu, Hua Li, Mustafa Erdem Arslan, Peter F Ells, Hwajeong Lee

ORCID number: Zhiyan Fu 0000-0002-9541-9968; Hua Li 0000-0001-7481-3942; Mustafa Erdem Arslan 0000-0002-0683-7421; Peter F Ells 0000-0003-2687-0242; Hwajeong Lee 0000-0001-7005-6278.

Author contributions: All authors have contributed to the manuscript and agree with the final version of the manuscript; Fu Z and Lee H are credited with significant contribution to the drafting of the work, literature review of all the sections discussed, the revision of critically important intellectual content, final approval of the published version, and agreement of accountability for all aspects of the work; Li H and Arslan ME are credited with literature review of all sections, and agreement of accountability for all parts of the work; Ells PF is credited with assisting in final content language polishing, and agreement of accountability for all aspects of the work.

Conflict-of-interest statement: The authors have nothing to disclose.

Zhiyan Fu, Hua Li, Mustafa Erdem Arslan, Hwajeong Lee, Department of Pathology and Laboratory Medicine, Albany Medical Center, Albany, NY 12208, United States

Peter F Ells, Department of Gastroenterology, Albany Medical Center, Albany, NY 12208, United States

Corresponding author: Hwajeong Lee, MD, Associate Professor, Department of Pathology and Laboratory Medicine, Albany Medical Center, 47 New Scotland Ave., MC81, Albany, NY 12208, United States. leeh5@amc.edu

Abstract

Hepatic Langerhans cell histiocytosis (LCH) is characterized by proliferation and accumulation of Langerhans cells in the liver, causing liver dysfunction or forming a mass lesion. The liver can be involved in isolation, or be affected along with other organs. A common clinical hepatic presentation is cholestasis with pruritis, fatigue and direct hyperbilirubinemia. In late stages, there may be hypoalbuminemia. Liver biopsy may be required for the diagnosis of hepatic LCH. Histologic finding may be diverse, including lobular Langerhans cell infiltrate with mixed inflammatory background, primary biliary cholangitis-like pattern, sclerosing cholangitis-like pattern, and even cirrhosis at later stages. Because of its non-specific injury patterns with broad differential diagnosis, establishing a diagnosis of hepatic LCH can be challenging. Hepatic LCH can easily be missed unless this diagnosis is considered at the time of biopsy interpretation. A definitive diagnosis relies on positive staining with CD1a and S100 antigen. Liver involvement is a high risk feature in LCH. The overall prognosis of hepatic LCH is poor. Treating at an early stage may improve the outcome. Systemic chemotherapy is the mainstay of treatment and liver transplantation may be offered. New molecular markers involved in pathogenesis of LCH are being explored with a potential for targeted therapy. However, further studies are needed to improve outcome.

Key Words: Langerhans cell; Liver; Cholangitis; CD1a

Core Tip: Hepatic Langerhans cell histiocytosis (LCH) is characterized by proliferation and accumulation of Langerhans cells in the liver, causing liver dysfunction or forming

Manuscript source: Invited manuscript

Specialty type: Oncology

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 2, 2021
Peer-review started: February 2, 2021
First decision: March 8, 2021
Revised: March 19, 2021
Accepted: April 25, 2021
Article in press: April 25, 2021
Published online: May 24, 2021

P-Reviewer: Soldera J
S-Editor: Fan JR
L-Editor: A
P-Editor: Wu YXJ

a mass lesion. There are diverse injury patterns on liver biopsy, including lobular Langerhans cell infiltrate with mixed inflammatory background, primary biliary cholangitis-like pattern, sclerosing cholangitis-like pattern, and even cirrhosis at later stage. The overall prognosis of hepatic LCH is poor. New molecular markers involved in pathogenesis of LCH are being explored with a potential for targeted therapy.

Citation: Fu Z, Li H, Arslan ME, Ells PF, Lee H. Hepatic Langerhans cell histiocytosis: A review. *World J Clin Oncol* 2021; 12(5): 335-341
URL: https://www.wjgnet.com/2218-4333/full/v12/i5/335.htm
DOI: https://dx.doi.org/10.5306/wjco.v12.i5.335

INTRODUCTION

Langerhans cell histiocytosis (LCH) is characterized by proliferation and accumulation of dendritic antigen-presenting histiocytes, Langerhans cells, in tissue. Langerhans cells express CD1a, S-100 and langerin proteins, and show Birbeck granules on ultrastructural examination[1]. LCH is a rare disease with an annual incidence of about 5 cases *per* 1 million population. The disease is more common in Caucasian population of northern European descent, with a male predominance. It can affect any age group but most cases occur in children[1,2]. The etiology is unknown. However, there is a strong association between pulmonary LCH and smoking[3,4].

Some authors have considered that LCH is an abnormal reactive process to an inciting event, such as viral infection, given the fact that multiple cytokines are involved in the process and LCH has been reported to regress spontaneously when it's an isolated lesion[5,6]. However, the revised 4th edition World Health Organization[1] classified LCH as a clonal neoplastic process. Recently, Murakami *et al*[7] proposed that both *BRAF* mutation and Interleukin-1 Loop amplification play important roles in the pathogenesis of LCH[7]. More recently, other groups found that *BRAF*V600E mutation or alternative activating MAPK pathway gene mutations are almost universally identified in LCH[8,9]. These studies provide a potential for molecular alteration-based targeted therapy for LCH.

LCH demonstrates a variable clinical picture and course. It can involve a single organ system (SS-LCH) or multiple organ systems (MS-LCH). While any organ can be affected, bone is the most frequent site occurring in 80% of cases of LCH; it can be unifocal or multifocal. A third of LCH cases involve the skin and a quarter involve the pituitary gland. Other organs, such as liver, spleen, lungs, lymph nodes, gastrointestinal tract, and rarely parotid glands and nails, can also be involved[10].

When LCH involves a single organ, the course may be indolent and have a favorable survival. Su *et al*[11] reported 100% 5-year survival in pediatric LCH patients when the disease involved only bone[11]. In a long-term (over 17 years) follow-up study of pediatric LCH, SS-LCH showed a 100% regression rate and low relapse *vs* 73% regression in MS-LCH[12].

LCH involving the skin, bone, lymph nodes or pituitary gland is considered "low-risk" because of its good response to treatment. Involvement of the lungs, hematopoietic system and spleen, and liver is considered "high risk" with an unfavorable outcome[1,6,12,13].

In this review, we focus on hepatic LCH and provide a brief overview of its clinical presentation, laboratory/imaging findings and current treatment. Its pathologic findings and differential diagnosis are also reviewed.

DIAGNOSIS OF HEPATIC LCH

The diagnosis of hepatic LCH in a patient with known LCH requires one or more of the followings: (1) hepatomegaly, defined as a liver edge greater than 3 cm below the costal margin at the mid clavicular line (confirmed by ultrasound); (2) liver dysfunction defined either by abnormal serum biochemical tests including bilirubin greater than 3 times the upper limit of normal, protein less than 55 g/L, albumin less than 3 g/dL, transaminases [alanine aminotransferase (ALT)/ aspartate aminotransferase (AST)] greater than 3 times normal, or by clinical entities including an

intrahepatic nodular mass or ascites or edema, not as a result of other causes; or (3) histopathological findings of active disease[14,15].

INCIDENCE OF HEPATIC LCH

The liver may be affected in isolation[16-19], or involved along with other organ systems such as lymph nodes, skin and lungs.

In pediatric LCH, the frequency of liver involvement is variable but may be high (15%-60%). It carries a poor prognosis. In adult LCH, liver involvement is relatively uncommon with an incidence of 16%-27%. However, it is probably under recognized. One study of multisystemic LCH in adults reported liver involvement in 87%[14,18,20].

CLINICAL MANIFESTATION

There are 2 stages of hepatic LCH: An early stage with liver parenchymal infiltration by Langerhans cells and a late stage with biliary sclerosis[18,20]. Clinical manifestations differ based on the stage.

A common presentation is jaundice with direct hyperbilirubinemia and hypoalbuminemia. Patients may present with fatigue and pruritus[21]. Clinically, hepatic LCH may mimic other conditions such as chronic destructive cholangitis, metabolic disease, hepatitis, neoplasia obstructing biliary tract and inherited deficient conjugation of bilirubin. It can also present as Reye syndrome, chronic inflammatory bowel disease, or hemochromatosis[14]. In late stages, patients may present with severe sclerosing cholangitis and liver failure. A subset of patients progresses to develop cirrhosis, which may lead to portal hypertension and secondary hypersplenism[22,23].

LABORATORY/IMAGE FINDINGS

Laboratory tests show abnormal serum liver tests with mild to moderately elevated ALT and AST. Cholestatic biochemical profiles are seen with increased total bilirubin, γ glutamyl transferases and anaplastic lymphoma kinase phosphatase. The albumin level is often low. The prothrombin time may be prolonged due to decreased clotting factor. Depending on liver function, clotting factors may be depleted. Complete blood count is usually normal, although the platelet count may be low in patients with portal hypertension and splenomegaly. Abdominal contrast-enhanced computed tomography may demonstrate hepatomegaly with solitary or multiple hypodense hepatic nodules, which can be confluent[18]. At a late stage, magnetic resonance imaging may show biliary tree abnormalities[20].

PATHOLOGY FINDINGS

Liver biopsy is the cornerstone of the diagnosis of LCH and disease staging. Liver biopsy can show various injury patterns. In an early stage, lobular Langerhans cell infiltrate mixed with lymphocytes can be seen. The Langerhans cells may form focal aggregates or be multinucleated[18]. In addition to lymphocytes, mature eosinophils, neutrophils and plasma cells can also be noted. A definitive diagnosis can be rendered based on positive immunohistochemical staining with CD1a and S100 antigen of the Langerhans cells.

At a later stage, Langerhans cells may infiltrate the bile ducts and cause sclerosing cholangitis. In some cases, histologic features of sclerosing cholangitis may be seen in a biopsy without identifiable Langerhans cells[17,22,24]. This may be due to selective involvement of the major bile ducts by Langerhans cells; large ducts are unlikely to be sampled in the usual needle biopsy[25]. Chronic non-suppurative destructive cholangitis injury pattern has been also reported[26].

Notably, hepatic LCH may mimic primary biliary cholangitis (PBC). Rush *et al*[19] reported hepatic LCH presenting as a small noncaseating granuloma in the portal tract with rare multinucleated epithelioid giant cells within the portal inflammatory infiltrate. The patient underwent a liver transplantation for the presumed diagnosis of advanced anti-mitochondrial antibody (AMA)-negative PBC. However, the disease

recurred in the allograft. The diagnosis of hepatic LCH was finally rendered three years after transplantation in the allograft biopsy. Four years later (7 years after the transplantation), the patient lost the liver graft. LCH diagnosis was confirmed in the explanted allograft liver[19].

Similarly, our group reported a case of hepatic LCH that histologically and pathologically mimicked PBC. A 65-year-old man presented with intermittent pruritus with cholestatic biochemistry profile for years. The liver biopsy showed portal histiocytic aggregate (non-necrotizing granuloma) encasing a damaged bile duct (Figure 1). Tests for autoantibodies including AMA were negative, therefore AMA-negative PBC was suspected. One month later, multiple skin lesions developed and a diagnosis of LCH was rendered on a skin biopsy. In light of the positive skin biopsy, the previous liver biopsy was re-examined. The histiocytes surrounding the duct were positive for CD1a and S100, confirming the diagnosis of hepatic LCH, retrospectively[21].

These two cases highlight the difficulty of rendering a diagnosis of hepatic LCH without appropriate clinical context.

In summary, hepatic LCH may present as a non-specific inflammatory process with varying injury pattern. Therefore, the diagnosis of hepatic LCH can easily be missed when this diagnosis is not considered. In addition, its patchy nature and limited sampling may further hinder the diagnosis of hepatic LCH[21]. In late stage MS-LCH, even though the clinical picture may be strongly indicative of hepatic LCH, it may be difficult to render a definitive diagnosis on the initial biopsy and a repeat biopsy may be necessary[6].

DIFFERENTIAL DIAGNOSIS

There are a variety of differential diagnoses for hepatic LCH due to its nonspecific presentations and morphologic features.

The morphological and clinical findings of hepatic LCH may resemble primary sclerosing cholangitis (PSC). A classic "beaded" appearance of extrahepatic biliary tree by endoscopic retrograde cholangiopancreatography and periductal onion-skin type fibrosis with negative stains for S100 and CD1a may be helpful in the diagnosis of PSC.

Granulomatous reaction with ductular proliferation raises a differential diagnosis of PBC. The presence of a dense portal lymphoplasmacytic infiltrate, mild degree of interface activity, and negativity for S100 and CD1a in conjunction with positivity for AMA can be helpful for PBC diagnosis. However, as demonstrated above, it can be very difficult to differentiate hepatic LCH from AMA negative PBC in a small liver biopsy, especially in the absence of appropriate clinical context.

In hepatic LCH, the presence of granulomatous inflammation also raises a differential diagnosis of infection. Grocott (or Gomori) methenamine silver and acid-fast bacteria stains may help to rule out infectious etiology, although they have low sensitivity[27-30]. Tissue culture may be helpful in these scenarios. Other differential diagnosis includes sarcoidosis, foreign-body type giant cell reactions, drug-induced liver injury, and non-Hodgkin's and Hodgkin's lymphoma.

Additionally, myeloproliferative disorders and myeloid leukemias can express CD1a and/or S100 protein, mimicking LCH. However, these are distinguished by sinusoidal infiltrative pattern of the neoplastic cells[25].

TREATMENT

Depending on clinical course and degree of organ dysfunction, variable treatment options are offered for LCH. Usually, patients with single site SS-LCH are observed only or offered monotherapy, such as oral 6-mercaptopurine and methotrexate, indomethacin, bisphosphonates, and hydroxyurea. Patients with MS-LCH usually benefit from systemic therapy, such as the vinblastine-prednisone combination [6,15, 20].

NOVEL MOLECULAR TARGETS

Recently, molecular markers such as *BRAFV600E* and *MAP2K1* have been identified in LCH pathogenesis. These markers may serve as molecular targets for precision therapy in the future. For instance, vemurafenib, a *BRAF* inhibitor, had prolonged

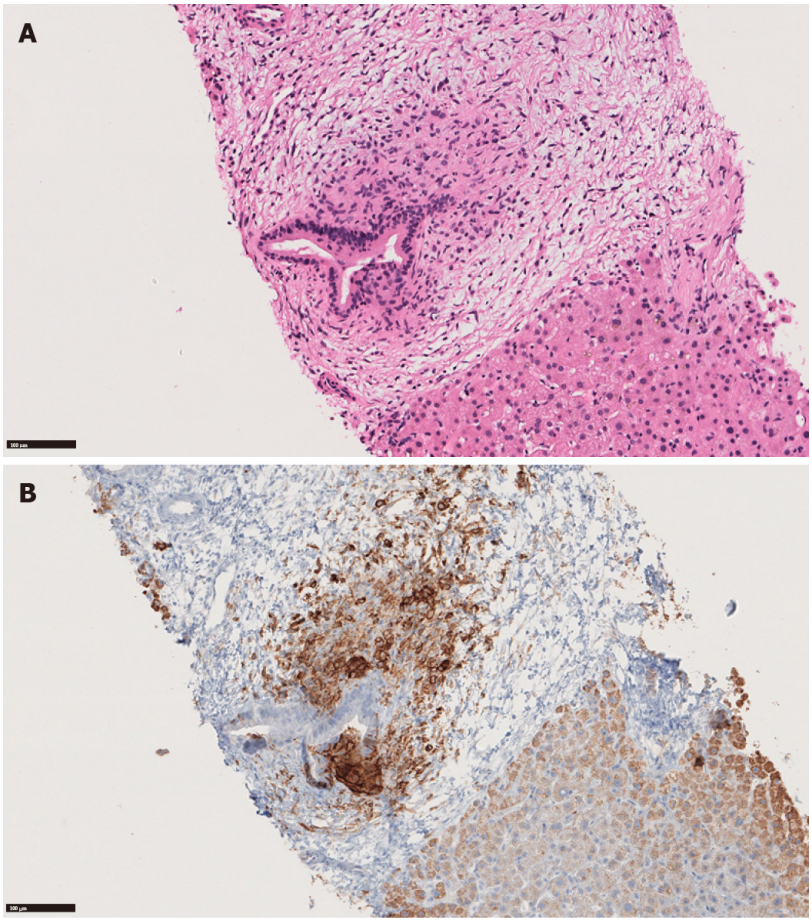


Figure 1 Hepatic Langerhans cell histiocytosis mimicking primary biliary cholangitis. A: Portal non-necrotizing granulomatous inflammation encasing a duct (Hematoxylin and eosin, 200 ×); B: CD1a immunostain highlights Langerhans cells surrounding the duct (CD1a, 200 ×). Image A was originally published in Cureus. Citation: Li H, Ells P, Arslan ME, Robstad KA, Lee H. Hepatic Langerhans Cell Histiocytosis (LCH) Presenting as a Harbinger of Multisystem LCH. *Cureus* 2020; 12: e8591 [PMID: 32676232 DOI: 10.7759/cureus.8591]. Copyright © The Authors 2020. Published by Cureus, Inc.[21].

efficacy in patients with *BRAF V600*-mutant LCH[31]. In addition, MEK inhibitors showed near-universal responses in patients with histiocytoses, including LCH, regardless of tumor genotype[32]. However, prospective clinical trials would be required to determine optimal duration of therapy and to explore a potential for combination with other targeted or cytotoxic therapies.

The optimal treatment for hepatic LCH remains to be determined. Currently, systemic chemotherapy is the mainstay of treatment. Yi *et al*[33] reported that earlier systemic chemotherapy has led to a relatively better outcome in hepatic LCH. However, the therapy was not effective once cirrhosis developed[33]. Therefore, early diagnosis and treatment of hepatic LCH would be crucial. Unfortunately, a subset of hepatic LCH patients develops severe sclerosing cholangitis that progresses to cirrhosis. In this case, liver transplantation may be the treatment of choice and should be considered early in the disease course[34]. Tang *et al*[35] reported a case of hepatic LCH that was successfully treated by liver transplantation with subsequent tacrolimus and mycophenolate mofetil as immunosuppressants[35].

Further collaborative studies regarding the biology, clinical presentation and outcome of hepatic LCH would be required to explore and refine variable treatment options.

PROGNOSIS

In LCH, liver involvement has a significant bearing on survival. The overall prognosis of hepatic LCH is poor with a fatality rate of 30%-50% (versus < 10% without liver involvement) and median survival of 9 years[6,18,20]. The 3-year survival rate of LCH with liver involvement is 51.8%, compared with that of 96.7% without liver involvement[6]. In Abdallah *et al*[20]'s study, 30% of patients died due to sclerosing

cholangitis complicated by secondary cirrhosis[20]. Therefore, it is very important to identify liver involvement at an early reversible stage. Early detection and treatment may improve the outcome[6,18].

CONCLUSION

While hepatic LCH is relatively uncommon, it portends a poor prognosis. Early detection and treatment of hepatic LCH may allow a better prognosis. Liver biopsy plays an important role in the diagnosis and management of hepatic LCH. Unfortunately, the histomorphology is non-specific and needs to be differentiated from other granulomatous processes, such as PBC or infection. A definitive diagnosis requires confirmatory immunohistochemical staining with CD1a and S100. Currently, systemic chemotherapy is the mainstay of treatment for hepatic LCH. Further studies are required to explore other treatment modalities including molecularly targeted therapy.

REFERENCES

1 **Swerdlow SH**, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (Revised 4th Edition). Lyon: IARC, 2017: 470-472

2 **Pileri SA**, Grogan TM, Harris NL, Banks P, Campo E, Chan JK, Favera RD, Delsol G, De Wolf-Peeters C, Falini B, Gascoyne RD, Gaulard P, Gatter KC, Isaacson PG, Jaffe ES, Kluin P, Knowles DM, Mason DY, Mori S, Müller-Hermelink HK, Piris MA, Ralfkiaer E, Stein H, Su IJ, Warnke RA, Weiss LM. Tumours of histiocytes and accessory dendritic cells: an immunohistochemical approach to classification from the International Lymphoma Study Group based on 61 cases. *Histopathology* 2002; **41**: 1-29 [PMID: 12121233 DOI: 10.1046/j.1365-2559.2002.01418.x]

3 **Vassallo R**, Ryu JH, Schroeder DR, Decker PA, Limper AH. Clinical outcomes of pulmonary Langerhans'-cell histiocytosis in adults. *N Engl J Med* 2002; **346**: 484-490 [PMID: 11844849 DOI: 10.1056/NEJMoa012087]

4 **Bernstrand C**, Cederlund K, Sandstedt B, Ahström L, Lundell M, Dahlquist G, Henter JI. Pulmonary abnormalities at long-term follow-up of patients with Langerhans cell histiocytosis. *Med Pediatr Oncol* 2001; **36**: 459-468 [PMID: 11260569 DOI: 10.1002/mpo.1110]

5 **Tillotson CV**, Anjum F, Patel BC. Langerhans Cell Histiocytosis. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 [PMID: 28613635]

6 **Henter JI**, Tondini C, Pritchard J. Histiocyte disorders. *Crit Rev Oncol Hematol* 2004; **50**: 157-174 [PMID: 15157664 DOI: 10.1016/j.critrevonc.2004.01.002]

7 **Murakami I**, Matsushita M, Iwasaki T, Kuwamoto S, Kato M, Nagata K, Horie Y, Hayashi K, Imamura T, Morimoto A, Imashuku S, Gogusev J, Jaubert F, Takata K, Oka T, Yoshino T. Interleukin-1 loop model for pathogenesis of Langerhans cell histiocytosis. *Cell Commun Signal* 2015; **13**: 13 [PMID: 25889448 DOI: 10.1186/s12964-015-0092-z]

8 **Chakraborty R**, Burke TM, Hampton OA, Zinn DJ, Lim KP, Abhyankar H, Scull B, Kumar V, Kakkar N, Wheeler DA, Roy A, Poulikakos PI, Merad M, McClain KL, Parsons DW, Allen CE. Alternative genetic mechanisms of BRAF activation in Langerhans cell histiocytosis. *Blood* 2016; **128**: 2533-2537 [PMID: 27729324 DOI: 10.1182/blood-2016-08-733790]

9 **Héritier S**, Hélias-Rodzewicz Z, Chakraborty R, Sengal AG, Bellanné-Chantelot C, Thomas C, Moreau A, Fraitag S, Allen CE, Donadieu J, Emile JF. New somatic BRAF splicing mutation in Langerhans cell histiocytosis. *Mol Cancer* 2017; **16**: 115 [PMID: 28679432 DOI: 10.1186/s12943-017-0690-z]

10 **Narayanasamy Rajavelu T**, Abimannane A, Chinnaiah Govindhareddy DK, Kayal S, Kar R. Langerhans' Cell Histiocytosis Masquerading as Caroli's Disease. *J Pediatr Hematol Oncol* 2020; **42**: e620-e622 [PMID: 31033792 DOI: 10.1097/MPH.0000000000001495]

11 **Su M**, Gao YJ, Pan C, Chen J, Tang JY. Outcome of children with Langerhans cell histiocytosis and single-system involvement: A retrospective study at a single center in Shanghai, China. *Pediatr Hematol Oncol* 2018; **35**: 385-392 [PMID: 30693828 DOI: 10.1080/08880018.2018.1545814]

12 **İnce D**, Demirağ B, Özek G, Erbay A, Ortaç R, Oymak Y, Kamer S, Yaman Y, Kundak S, Vergin C. Pediatric langerhans cell histiocytosis: single center experience over a 17-year period. *Turk J Pediatr* 2016; **58**: 349-355 [PMID: 28276205 DOI: 10.24953/turkjped.2016.04.001]

13 **Gadner H**, Grois N, Arico M, Broadbent V, Ceci A, Jakobson A, Komp D, Michaelis J, Nicholson S, Pötschger U, Pritchard J, Ladisch S; Histiocyte Society. A randomized trial of treatment for multisystem Langerhans' cell histiocytosis. *J Pediatr* 2001; **138**: 728-734 [PMID: 11343051 DOI: 10.1067/mpd.2001.111331]

14 **Haupt R**, Minkov M, Astigarraga I, Schäfer E, Nanduri V, Jubran R, Egeler RM, Janka G, Micic D, Rodriguez-Galindo C, Van Gool S, Visser J, Weitzman S, Donadieu J; Euro Histio Network. Langerhans cell histiocytosis (LCH): guidelines for diagnosis, clinical work-up, and treatment for patients till the age of 18 years. *Pediatr Blood Cancer* 2013; **60**: 175-184 [PMID: 23109216 DOI:

10.1002/pbc.24367]
15 **Rodriguez-Galindo C**, Allen CE. Langerhans cell histiocytosis. *Blood* 2020; **135**: 1319-1331 [PMID: 32106306 DOI: 10.1182/blood.2019000934]
16 **Finn LS**, Jaffe R. Langerhans' cell granuloma confined to the bile duct. *Pediatr Pathol Lab Med* 1997; **17**: 461-468 [PMID: 9185224]
17 **Kaplan KJ**, Goodman ZD, Ishak KG. Liver involvement in Langerhans' cell histiocytosis: a study of nine cases. *Mod Pathol* 1999; **12**: 370-378 [PMID: 10229501]
18 **Araujo B**, Costa F, Lopes J, Castro R. Adult langerhans cell histiocytosis with hepatic and pulmonary involvement. *Case Rep Radiol* 2015; **2015**: 536328 [PMID: 25977828 DOI: 10.1155/2015/536328]
19 **Rush N**, Saxena R, Lin J. Langerhans Cell Histiocytosis With Solitary Hepatic Involvement in an Adult. *AJSP Rev Rep* 2020; **25**: 40-44 [DOI: 10.1097/PCR.0000000000000358]
20 **Abdallah M**, Généreau T, Donadieu J, Emile JF, Chazouillères O, Gaujoux-Viala C, Cabane J. Langerhans' cell histiocytosis of the liver in adults. *Clin Res Hepatol Gastroenterol* 2011; **35**: 475-481 [PMID: 21550330 DOI: 10.1016/j.clinre.2011.03.012]
21 **Li H**, Ells P, Arslan ME, Robstad KA, Lee H. Hepatic Langerhans Cell Histiocytosis (LCH) Presenting as a Harbinger of Multisystem LCH. *Cureus* 2020; **12**: e8591 [PMID: 32676232 DOI: 10.7759/cureus.8591]
22 **Hatemi I**, Baysal B, Senturk H, Behzatoglu K, Bozkurt ER, Ozbay G. Adult Langerhans cell histiocytosis and sclerosing cholangitis: a case report and review of the literature. *Hepatol Int* 2010; **4**: 653-658 [PMID: 21063491 DOI: 10.1007/s12072-010-9205-3]
23 **Pirovino M**, Jeanneret C, Läng RH, Luisier J, Bianchi L, Spichtin H. Liver cirrhosis in histiocytosis X. *Liver* 1988; **8**: 293-298 [PMID: 3264377 DOI: 10.1111/j.1600-0676.1988.tb01007.x]
24 **Liu DG**, Zhang YX, Li F. Multisystem Langerhans cell histiocytosis with liver dysfunction as the first presentation: A case report. *Oncol Lett* 2012; **3**: 391-394 [PMID: 22740918 DOI: 10.3892/ol.2011.462]
25 **Jaffe R**. Liver involvement in the histiocytic disorders of childhood. *Pediatr Dev Pathol* 2004; **7**: 214-225 [PMID: 15022067 DOI: 10.1007/s10024-003-9876-z]
26 **Haas S**, Theuerkauf I, Kühnen A, Wickesberg A, Fischer HP. [Langerhans' cell histiocytosis of the liver. Differential diagnosis of a rare chronic destructive sclerosing cholangitis]. *Pathologe* 2003; **24**: 119-123 [PMID: 12673501 DOI: 10.1007/s00292-002-0565-x]
27 **Zink AR**, Nerlich AG. Molecular strain identification of the Mycobacterium tuberculosis complex in archival tissue samples. *J Clin Pathol* 2004; **57**: 1185-1192 [PMID: 15509681 DOI: 10.1136/jcp.2003.015719]
28 **Fukunaga H**, Murakami T, Gondo T, Sugi K, Ishihara T. Sensitivity of acid-fast staining for Mycobacterium tuberculosis in formalin-fixed tissue. *Am J Respir Crit Care Med* 2002; **166**: 994-997 [PMID: 12359660 DOI: 10.1164/rccm.2111028]
29 **Reza Kermanshahi T**, Rhatigan R. Comparison between PAS and GMS stains for the diagnosis of onychomycosis. *J Cutan Pathol* 2010; **37**: 1041-1044 [PMID: 19922486 DOI: 10.1111/j.1600-0560.2009.01468.x]
30 **Azar MM**, Slotkin R, Abi-Raad R, Liu Y, Grant MH, Malinis MF. Gomori Methenamine Silver Stain on Bronchoalveolar Lavage Fluid Is Poorly Sensitive for Diagnosis of *Pneumocystis jiroveci* Pneumonia in HIV-Negative Immunocompromised Patients and May Lead to Missed or Delayed Diagnoses. *Arch Pathol Lab Med* 2020 [PMID: 31904277 DOI: 10.5858/arpa.2019-0394-OA]
31 **Diamond EL**, Subbiah V, Lockhart AC, Blay JY, Puzanov I, Chau I, Raje NS, Wolf J, Erinjeri JP, Torrisi J, Lacouture M, Elez E, Martínez-Valle F, Durham B, Arcila ME, Ulaner G, Abdel-Wahab O, Pitcher B, Makrutzki M, Riehl T, Baselga J, Hyman DM. Vemurafenib for BRAF V600-Mutant Erdheim-Chester Disease and Langerhans Cell Histiocytosis: Analysis of Data From the Histology-Independent, Phase 2, Open-label VE-BASKET Study. *JAMA Oncol* 2018; **4**: 384-388 [PMID: 29188284 DOI: 10.1001/jamaoncol.2017.5029]
32 **Diamond EL**, Durham BH, Ulaner GA, Drill E, Buthorn J, Ki M, Bitner L, Cho H, Young RJ, Francis JH, Rampal R, Lacouture M, Brody LA, Ozkaya N, Dogan A, Rosen N, Iasonos A, Abdel-Wahab O, Hyman DM. Efficacy of MEK inhibition in patients with histiocytic neoplasms. *Nature* 2019; **567**: 521-524 [PMID: 30867592 DOI: 10.1038/s41586-019-1012-y]
33 **Yi X**, Han T, Zai H, Long X, Wang X, Li W. Liver involvement of Langerhans' cell histiocytosis in children. *Int J Clin Exp Med* 2015; **8**: 7098-7106 [PMID: 26221247]
34 **Braier J**, Ciocca M, Latella A, de Davila MG, Drajer M, Imventarza O. Cholestasis, sclerosing cholangitis, and liver transplantation in Langerhans cell Histiocytosis. *Med Pediatr Oncol* 2002; **38**: 178-182 [PMID: 11836717 DOI: 10.1002/mpo.1306]
35 **Tang Y**, Zhang Z, Chen M, Ju W, Wang D, Ji F, Ren Q, Guo Z, He X. Severe sclerosing cholangitis after Langerhans cell histiocytosis treated by liver transplantation: An adult case report. *Medicine (Baltimore)* 2017; **96**: e5994 [PMID: 28248858 DOI: 10.1097/MD.0000000000005994]

World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2021 May 24; 12(5): 342-354

DOI: 10.5306/wjco.v12.i5.342

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Basic Study

Thymoquinone anticancer activity is enhanced when combined with royal jelly in human breast cancer

Maya M Moubarak, Nour Chanouha, Najwa Abou Ibrahim, Hala Khalife, Hala Gali-Muhtasib

ORCID number: Maya M Moubarak 0000-0003-1961-4435; Nour Chanouha 0000-0002-4209-5488; Najwa Abou Ibrahim 0000-0001-6051-0174; Hala Khalife 0000-0001-8565-3585; Hala Gali-Muhtasib 0000-0001-6840-3015.

Author contributions: Moubarak MM carried out lab work as part of her MSc thesis, performed analysis and interpretation of data (*e.g.*, biostatistics, statistical analysis and editing), and wrote the first draft of the manuscript; Chanouha N performed initial lab work and determined dose responses and performed data analysis; Abou Ibrahim N brought the importance of royal jelly to the attention of the corresponding author, provided it, and contributed intellectually to the study; Khalife H reviewed the manuscript and contributed in the critical appraisal of data; Gali-Muhtasib H conceived the project, supervised the work, and edited the manuscript draft; All authors have read and approved the final manuscript.

Supported by The Lebanese National Council for Scientific Research and the American University of Beirut, No.103482; and the Undergraduate Research Experience of the Faculty of Arts and Sciences, American University

Maya M Moubarak, Nour Chanouha, Department of Biology, American University of Beirut, Beirut 1107-2020, Lebanon

Najwa Abou Ibrahim, Hala Khalife, Rammal Rammal Laboratory (ATAC group), Faculty of Sciences I, Hadath 1003, Lebanon

Hala Gali-Muhtasib, Department of Biology and Center for Drug Discovery, American University of Beirut, Beirut 1107-2020, Lebanon

Corresponding author: Hala Gali-Muhtasib, PhD, Professor, Department of Biology and Center for Drug Discovery, American University of Beirut, Bliss Street, Biology Bldg, Room 207, PO Box 11-0236, Riad El Solh, Lebanon, Beirut 1107-2020, Lebanon. amro@aub.edu.lb

Abstract

BACKGROUND

Breast cancer is the most common cause of the majority of cancer-related deaths in women, among which triple-negative breast cancer is the most aggressive type of breast cancer diagnosed with limited treatment options. Thymoquinone (TQ), the main bioactive constituent of *Nigella sativa*, has been extensively studied as a potent anticancer molecule against various types of cancers. Honeybee products such as the royal jelly (RJ), the nutritive secretion fed to honeybee queens, exhibit a variety of biological activities besides its anticancer effect. However, the anticancer activity of the combination of TQ and RJ against breast cancer is still unknown.

AIM

To investigate cytotoxicity of RJ in FHs 74 Int cells and the anticancer effects of TQ, RJ, and their combinations in the MDA-MB-231 cell line.

METHODS

Cells were treated with TQ, RJ, and their combinations for 24 h. Using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, we determined the half-maximal inhibitory concentration of TQ. Trypan blue and 3-(4,5 dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were then performed to assess the cell viability in response to different treatment conditions. Cell death and cycle regulation were investigated using propidium iodide deoxyribonucleic acid staining followed by flow cytometry in response to a single dose of TQ, RJ, and their combination. Immunostaining for cleaved caspase 3 and Ki67 expression

of Beirut.

Conflict-of-interest statement: Authors declare no conflict of interest for this manuscript.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Manuscript source: Invited manuscript

Specialty type: Oncology

Country/Territory of origin: Lebanon

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 28, 2021
Peer-review started: January 28, 2021
First decision: March 1, 2021
Revised: March 13, 2021
Accepted: April 26, 2021
Article in press: April 26, 2021
Published online: May 24, 2021

P-Reviewer: Dahmen U
S-Editor: Liu M
L-Editor: Filipodia

was used to determine apoptosis induction and changes in cell proliferation.

RESULTS

TQ alone inhibited cell viability in a dose-dependent manner at concentrations below and above the half-maximal inhibitory concentration. RJ exhibited relatively nontoxic effects against MDA-MB-231 cells and FHs 74 Int small intestinal cells at concentrations below 5 μg/mL. High doses of RJ (200 μg/mL) had greater toxicity against MDA-MB-231 cells. Interestingly, the inhibition of cell viability was most pronounced in response to 15 μmol/L TQ and 5 μg/mL RJ. A dose of 15 μmol/L TQ caused a significant increase in the PreG1 population, while a more pronounced effect on cell viability inhibition and PreG1 increase was observed in response to TQ and RJ combinations. TQ was the main inducer of caspase 3-dependent apoptosis when applied alone and in combination with RJ. In contrast, no significant regulation of Ki67 expression was observed, indicating that the decrease in cell viability was due to apoptosis induction rather than to inhibition of cell proliferation.

CONCLUSION

This study is the first to report enhanced anticancer effects of TQ and RJ combination against MDA-MB-231 breast cancer cells, which could confer an advantage for cancer therapy.

Key Words: Anticancer activity; Breast cancer cells; Drug combination; Natural products; Royal jelly; Thymoquinone

Core Tip: Royal jelly enhances thymoquinone (TQ) anticancer activity against breast cancer. TQ induces the apoptotic response in breast cancer cells while royal jelly when combined with TQ potentiates the reduction in cell viability more than each drug alone.

Citation: Moubarak MM, Chanouha N, Abou Ibrahim N, Khalife H, Gali-Muhtasib H. Thymoquinone anticancer activity is enhanced when combined with royal jelly in human breast cancer. *World J Clin Oncol* 2021; 12(5): 342-354
URL: https://www.wjgnet.com/2218-4333/full/v12/i5/342.htm
DOI: https://dx.doi.org/10.5306/wjco.v12.i5.342

INTRODUCTION

According to the global cancer project GLOBOCAN 2020, 19.3 million new cancer cases and 10 million cancer deaths have occurred in 2020[1]. The World Health Organization predicted that the global burden of cancer would double to about 29-37 million new cancer cases by 2040. Their report estimated the trend of future cancer cases by which breast cancer will account for 2778850 cases in 2040[2]. Breast cancer is the most common cancer among women worldwide[3] and is responsible for the majority of cancer-related deaths among women[4]. Invasive ductal carcinoma (IDC) is the most frequent type of breast tumor, followed by invasive lobular carcinoma (ILC). Together they make up 90% of breast cancers, while the remaining 10% is caused by particular types of none-ILC/none-IDC tumors[5-7]. Triple-negative breast cancer is an aggressive type of breast cancer with limited treatment options[8,9].

The human breast cancer cell line MDA-MB-231, a highly invasive and poorly differentiated triple-negative breast cancer (TNBC), was employed in our study. Being one of the most commonly used breast cancer cell lines in medical research, MDA-MB-231 derives from pleural effusion in metastatic mammary adenocarcinomas[10]. The absence of estrogen receptor, progesterone receptor, and human epidermal receptor 2 expression renders them nonresponsive to hormonal treatments[11]. However, these cells possess high invasive capacity and metastatic potential as they degrade the extracellular matrix of tissues and metastasize into lung, bone, or brain-specific cancers [12-14]. Conventional treatment of breast cancer comprises surgical procedures,

P-Editor: Yuan YY

radiotherapy, chemotherapy, endocrine (hormonal) therapy, and targeted and immunotherapies[15]. Despite the therapeutic impact of conventional treatments, they exert numerous side effects. Thus, extensive research has been conducted on alternative treatments utilizing plant-derived natural products with relatively non-toxic effects and high therapeutic potential.

Thymoquinone (TQ), the main bioactive constituent of *Nigella sativa* L. (Ranunculaceae), modulates the hallmarks of cancer[16] in addition to its cytoprotective[17,18], immunomodulatory, anti-oxidant, and anti-inflammatory activities[19,20]. Previous studies reported that TQ alone and in combination with natural and chemical agents act to inhibit breast cancer[21-23]. For instance, TQ in combination with piperine, lowered vascular endothelial growth factor expression, enhanced serum interferon-γ levels and apoptosis induction, and shifted the immune response toward T helper1 responses against EMT6 epithelial breast cancer[24]. TQ was shown to induce apoptosis when used alone or combined with amoxifen and suppressed the growth, viability, and invasion of breast cancer cell lines[25,26] through the regulation of the Akt signaling pathway[27].

Royal jelly (RJ), the nutritive secretion secreted from the mandibular and hypopharyngeal glands of worker bees *Apis mellifera* (Hymenoptera, Apidae), is the only food of the queen bee at larval and adult life and is responsible for fertility and prolonged life span[28,29]. Similar to TQ, RJ exerts various biological activities, including wound healing[30], anti-oxidant[26,31], immunomodulatory, and anti-inflammatory[32] activities, and anti-hypercholesterolemic[33,34], anti-hypertensive[35], anti-aging[36,37], and anticancer activities[38,39]. RJ was found to inhibit the proliferation of estradiol-induced cell proliferation of MCF-7 breast cancer cells[38] and reduce the volume of the 4T1 breast mammary tumor[39]. RJ also inhibited the proliferation of human colorectal adenocarcinoma cells[40], neuroblastoma[41], and vascular endothelial growth factor-induced migration, proliferation, and tube formation in human umbilical vein endothelial cells[42].

The poor prognosis of TNBC and its ability to resist chemotherapy and meta-stasize [43,44] made combination therapy a necessary option. TQ was successfully combined with several agents to enhance its anticancer therapeutic efficacy; however, TQ anticancer activity was not tested in combination with RJ. In a previous study, we showed that TQ exerted a dose-dependent antitumor effect against a panel of human colon cancer cell lines with minimal cytotoxicity against FHs 74 Int non-tumorigenic human intestinal cells. Here, to assess the cytotoxic effects of RJ, FHs 74 Int intestinal cell line was used as a model of non-tumorigenic epithelial cells. In our study, we investigated the anticancer activity of TQ and RJ combination *in vitro* against the MDA-MB-231 human TNBC cell line. Significant inhibitory effect of TQ and RJ combination was revealed by the enhanced cell death effects in MDA-MB-231 cell line. TQ was the main inducer of apoptosis mediating cell death mechanisms by inducing caspase 3 dependent apoptosis in a dose-dependent manner.

MATERIALS AND METHODS

Materials

MDA-MB-231 human breast cancer and FHs 74 Int human small intestinal cell lines were purchased from ATCC (Manassas, VA, United States). Dulbecco's Modified Eagle Medium (DMEM) and DMEM-F12 cell culture media were purchased from Lonza (Verviers, Belgium). TQ, trypsin-ethylenediamine tetraacetic acid, Dulbecco's phosphate-buffered saline (PBS), horse serum, fetal bovine serum (FBS), penicillin-streptomycin, dimethyl sulfoxide (DMSO), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), trypan blue and methanol were purchased from Sigma-Aldrich (St. Louis, MO, United States). Insulin used for FHs 74 Int cell line culture (Actrapid 100 IU/mL) was purchased from the pharmacy at the American University of Beirut Medical Center. 4′,6-diamidino-2-phenylindole stain was purchased from Abcam (Cambridge, United Kingdom). Rabbit caspase 3 polyclonal antibody (9662) was purchased from Cell Signaling Technology (Danvers, MA, United States). Goat anti-rabbit polyclonal secondary antibody, Alexa Fluor 568 (A11011) was purchased from Invitrogen, Thermo Fisher Scientific (Carlsbad, CA, United States). Rabbit Ki67 monoclonal primary antibody (Cell Marque 275R-15) and donkey anti-rabbit Cy3 secondary antibody (Jackson 711-165-152) were provided by Dr. Noel Ghanem, Professor of Biology, American University of Beirut. Crude RJ was purchased from the bee farm at Rashaya al-Wadi, Lebanon, located at 1200-1600 m above sea level. A variety of seasonal plants predominate at this altitude and contribute to the diet of

bees, among which are Brassicaceae (Nasturtium), Anacardiaceae (Rhus), Ulmaceae (Ulmus), Rosaceae (Rosa), and Apiaceae (Eryngium). RJ was collected during the summer season of 2018-19 and stored at -20 °C.

Cell culture conditions

MDA-MB-231 was used as the model of an aggressive breast cancer cell line, while FHs 74 Int cell line was used as the model of a non-tumorigenic epithelial cell line. Both cell lines were cultured in their respective media in two-dimensional monolayer conditions. MDA-MB-231 cells were grown in DMEM cell culture media supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin (penicillin-streptomycin with penicillin at 10000 units and streptomycin at 10 mg/mL), and 10 μg/mL insulin was added to grow FHs 74 Int cells. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

Dissolution of RJ

Fresh RJ was supplied to our lab in the form of a solid extract. Knowing that RJ contains both polar and non-polar compounds, we considered mixing the crude RJ with DMSO, a polar aprotic solvent that is miscible with dH_2O and that is capable of dissolving hydrophilic and hydrophobic compounds[45,46]. Different proportions of dH_2O to DMSO were used to determine the ratio that produces the best solubility. Complete solubility was only obtained upon dissolving 20 mg of RJ in a solution of 800 μL dH_2O and 200 μL DMSO solution at 37 °C for 30 min along with vortexing every 10 min. Therefore, this protocol was used to prepare fresh RJ stocks prior to every treatment. Fresh RJ stock was then used to prepare dilutions needed for experiments.

Drug preparation and treatment

Directly before use, fresh stock of the purified synthetic compound TQ of 0.1 mol/L concentration was prepared by dissolving 16.4 mg of TQ crystals in 1 mL methanol. TQ stock was then diluted in respective media to obtain different TQ concentrations ranging from 1 μmol/L to 100 μmol/L used in cell treatment. RJ fresh stock was prepared by dissolving 20 mg in 200 μL DMSO mixed with 800 μL distilled water solution at 37 °C for 30 min. Intermediate concentrations of RJ ranging between 0.01 and 200 μg/mL were then prepared by serial dilutions from stock and used in cell treatment. In all experiments, treatment with TQ and RJ each alone or in combination was performed at 50% cell confluency. Treatment with TQ-RJ combination was done by adding TQ and RJ, each alone in wells containing their respective media and incubating cells with this mixture at different concentrations for 24 h.

MTT cell viability assay

All cells were seeded in 96-well plates at a density of 10000 cells/well, then treated for 24 h. Cell viability was then assessed by MTT that measures the ability of metabolically active cells to convert tetrazolium salt into violet formazan crystals. Cells were incubated with 120 μL of MTT solution (5 mg/mL prepared in 1 × PBS) for 3 h at 37 °C. Afterward, the solution containing the MTT dye was removed and replaced with 100 μL isopropanol to dissolve the formazan crystal. MTT optical density was then measured using a microplate reader enzyme-linked immuno-sorbent assay at 595 nm. Cellular viability was expressed as a percentage of metabolically active cells in treated conditions relative to control. Cell viability was reported as an average of three independent experiments, each condition in sextuplicate. RJ inhibitory effect was not determined by MTT due to its interference with the colorimetric absorbance measures of the MTT assay (data not shown).

Trypan blue exclusion assay

FHs 74 Int cells were seeded in 24-well-plates at a density of 70000 cells/well, while MDA-MB-231 were seeded in 12-well plates at a density of 20000 cells/well. Following treatment of cells for 24 h, alive and dead cells were collected. Samples were centrifuged at 1300 rpm for 5 min. Then, pellets were resuspended in DMEM growth medium, and trypan blue was added to the cell suspension in a 1:1 ratio. Next, cells were counted using a hemocytometer under the Axiovert inverted microscope at 10 × magnification. Cells stained blue were counted as dead, and results are expressed as a percentage of total cells. Cell viability was reported as an average of three independent experiments, each condition in duplicates.

Combination index analysis

The interaction between TQ and RJ was assessed using the Chou-Talalay plot[47]. Combination indices (CI) were calculated from the mean affected fraction at each drug combination using CompuSyn software (CompuSyn, Inc. Paramus, NJ, United States). CI > 1, CI = 1, and CI < 1 indicate antagonistic, additive, and synergistic effects, respectively.

Cell cycle analysis

MDA-MD-231 cells were seeded in 6-well plates at a density of 80000 cells/well. Cells were treated with 0.1 μg/mL RJ and 15 μmol/L TQ each alone. After 24 h, cells were collected and washed twice with 1 × PBS, fixed in 70% ice-cold ethanol, and stored at −20 °C for at least 1 d. Subsequently, cells were washed twice with 1 × PBS and incubated for 30 min at 37 °C with 100 μL of propidium iodide (PI) solution [6 μL RNase, 30 μL PI (1 mg/mL)]. Supernatants were then transferred to flow tubes with 200 μL PBS added. Cell cycle analysis was performed using the Fluorescence-activated cell sorting scan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, United States) and the Cell Quest software (Becton-Dickinson) was used to analyze the distribution of cells in the different phases of the cell cycle.

Immunofluorescence assay

MDA-MB-231 cells were plated on coverslips in 12-well plates at a density of 60000 cells/well. The medium was then removed, and the cells were treated with either TQ, RJ, or combinations. After treatment, the cells were washed twice with 1 × PBS and fixed at room temperature for 20 min in 4% formaldehyde. The formaldehyde was then removed, and the cells were washed three times in PBS before permeabilization in 0.5% Triton solution for 5 min. After two successive washes in PBS, cells were blocked in blocking buffer with FBS for 1 h at room temperature. Apoptosis was assessed using the caspase 3 antibody, which was subsequently diluted (1:500) in 3% bovine serum albumin and incubated separately with the cells overnight at 4 °C. The primary antibody was removed the next day, and the cells were washed three times in PBS supplemented with 0.1% Tween 20 before incubation for 1 h with goat anti-rabbit secondary antibody diluted (1:200) in 3% bovine serum albumin at room temperature. Finally, the secondary antibody was removed, and the cells were washed three times in PBS with 0.1% Tween 20 before staining the nuclei with 4′,6-diamidino-2-phenylindole and mounting on a glass slide. To evaluate cell proliferation, the same immunostaining protocol was followed for Ki67 immunofluorescence with minor modifications, including the preparation of Ki67 primary antibody and donkey anti-rabbit Cy3 secondary antibody solutions in blocking buffer with donkey serum at dilution 1:500 and 1:200 ratios, respectively. Also, cells were washed three times in PBS only after the removal of the primary and secondary antibodies. Imaging and visualization were performed using the microscope Zeiss Axio. For cleaved caspase 3 and Ki67 biomarkers, an equal number of representative images were taken for each slide in all conditions. The percentage of apoptotic cells expressing cleaved caspase 3 was then calculated while Ki67 immunofluorescence intensity was measured by ZEN lite Digital Imaging Software.

Statistical analysis

Unless otherwise stated, data are presented as mean ± standard error of the mean of three independent experiments with statistical analysis performed by one way analysis of variance (non-parametric) multiple comparison test on Graph Pad Prism V.7. Software (La Jolla, CA, United States). Statistical significance was set with a 95% confidence interval at $P < 0.05$.

Confocal Imaging

Cells were visualized and imaged by Axiovert inverted microscope from Zeiss at 10 × magnification. Confocal images were taken on Confocal Microscope Zeiss LSM710 at 40 × oil immersion magnification.

RESULTS

Cytotoxicity of TQ and RJ on human breast cancer cells

In a previous study, we showed that TQ doses up to 60 μmol/L exert minimal cytotoxic effects on normal intestinal cells[48]. Post 24 h treatment, TQ at concen-

trations below 15 μmol/L did not exert any statistically significant toxicity on MDA-MB-231 cell line relative to the control. Cell viability decreased remarkably, reaching 47% at 20 μmol/L, revealing that the half-maximal inhibitory concentration (IC_{50}) value was around 20 μmol/L (Figure 1A). The decline in cell viability was more pronounced with increasing TQ concentrations, indicating that TQ exhibited significant anticancer activity against MDA-MB-231 human breast cancer cells in a dose dependent manner. RJ exerted mild inhibitory effects at low doses of RJ (below 5 μg/mL) on FHs 74 Int non-tumorigenic human intestinal cells and MDA-MB-231 human breast cancer cells. However, at higher doses ranging from 10 μg/mL to 200 μg/mL of RJ, a more pronounced decrease in cell viability was observed, suggesting that very high doses of RJ are toxic to FHs 74 Int non-tumorigenic cells with a greater toxicity being exerted on breast cancer cells (Figure 1B and C). The IC_{50} of RJ was estimated to be 216 μg/mL in MDA-MB-231 cells, while it was much higher (292 μg/mL) in the FHs 74 Int cell line. Based on these results, relatively non-toxic doses of RJ ranging between 0.1-5.0 μg/mL were combined with TQ in subsequent experiments.

Anticancer effect of TQ, RJ, and their combinations against breast cancer

Next, we determined the effects of the combination of increasing doses of both RJ and TQ on cell viability. As shown in Figure 2A and B, no significant reduction in MDA-MB-231 viability was detected upon the treatment with RJ alone at doses of 0.1 μg/mL and 5 μg/mL or with TQ alone at doses below 10 μmol/L. Treatment with 5 μg/mL RJ, when combined with 5 μmol/L or 7.5 μmol/L of TQ caused 21% and 29% inhibition in cell viability, respectively (Figure 2B). The anti-tumor effects were more pronounced upon treatment with higher TQ doses. A dose of 10 μmol/L TQ in combination with 0.1 μg/mL RJ or 5 μg/mL RJ yielded a significant decrease in MDA-MB-231 cell viability by 40% and 58%, respectively. Treatment with 5 μg/mL RJ in combination with 10 μmol/L or 15 μmol/L of TQ decreased cell viability by 58% and 74%, respectively. These findings confirm the more potent anti-tumor effects upon combination treatment with higher RJ doses compared to each drug alone. The inhibition of cell viability (58% inhibition) by this combination treatment was greater than the sum of inhibition observed by each compound alone (6% and 12% inhibition by TQ and RJ alone, respectively), suggesting a synergistic effect. CIs were then calculated using CompuSyn software, confirming the synergistic interaction between both compounds in all the combinations tested with a CI value < 1. Anti-tumor effect was most pronounced (CI = 0.584) upon the combination of 5 μg/mL RJ with 10 μmol/L TQ (Figure 2C). Therefore, RJ enhances TQ anti-tumor activity against breast cancer by inducing dose dependent cell death effects.

TQ alone and in combination with RJ increases PreG1 population in breast cancer cells

To confirm further cell death and determine whether the inhibition of cell viability by RJ and TQ treatment of MDA-MB-231 cells was associated with changes in cell cycle regulation, cell cycle analysis using PI deoxyribonucleic acid staining with flow cytometry was performed. Cell death was significantly enhanced in response to TQ alone and when TQ was combined with 0.1 μg/mL RJ, a relatively non-cytotoxic dose (Figure 3A and B). In comparison with the control, the PreG1 population increased significantly upon the treatment with 15 μmol/L TQ alone, while a greater elevation was obtained when this dose of TQ was combined with 0.1 μg/mL RJ (Figure 3B). The increase in the PreG1 population was associated with a notable reduction in the G0/G1 and G2/M populations.

TQ and RJ combinations induce apoptotic cell death in breast cancer cells

To identify the mechanism of action responsible for the enhanced cell death effect of TQ and RJ combination treatment, we assessed the apoptotic effects of each compound alone and their combinations in MDA-MB-231 cell line. Insignificant increase in apoptosis levels was reported upon treatment with 0.1 μg/mL or 5 μg/mL of RJ. Confocal micrographs showed enhancement of apoptosis in response to the TQ and combination treatment, as evidenced by the increase in apoptotic nuclear bodies in MDA-MB-231 cells (Figure 4A). A significant increase in active caspase 3 expression was observed in response to treatment with 10 μmol/L and 15 μmol/L TQ alone, yielding a respective increase of 52% and 73% of caspase 3 expression in MDA-MB-231 cells (Figure 4B). Similar results were obtained upon treatment with 0.1 μg/mL RJ in combination with 10 μmol/L and 15 μmol/L TQ, while a more pronounced apoptotic effect was observed in response to treatment with 5 μg/mL RJ in combination with 10

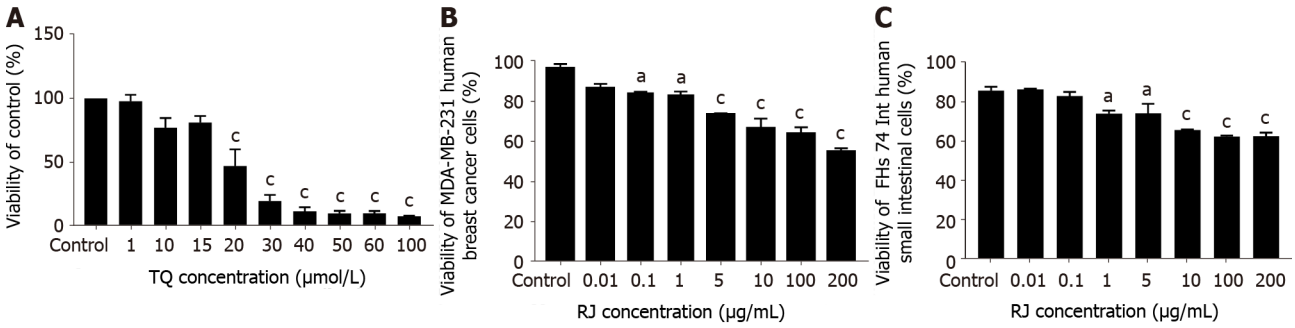
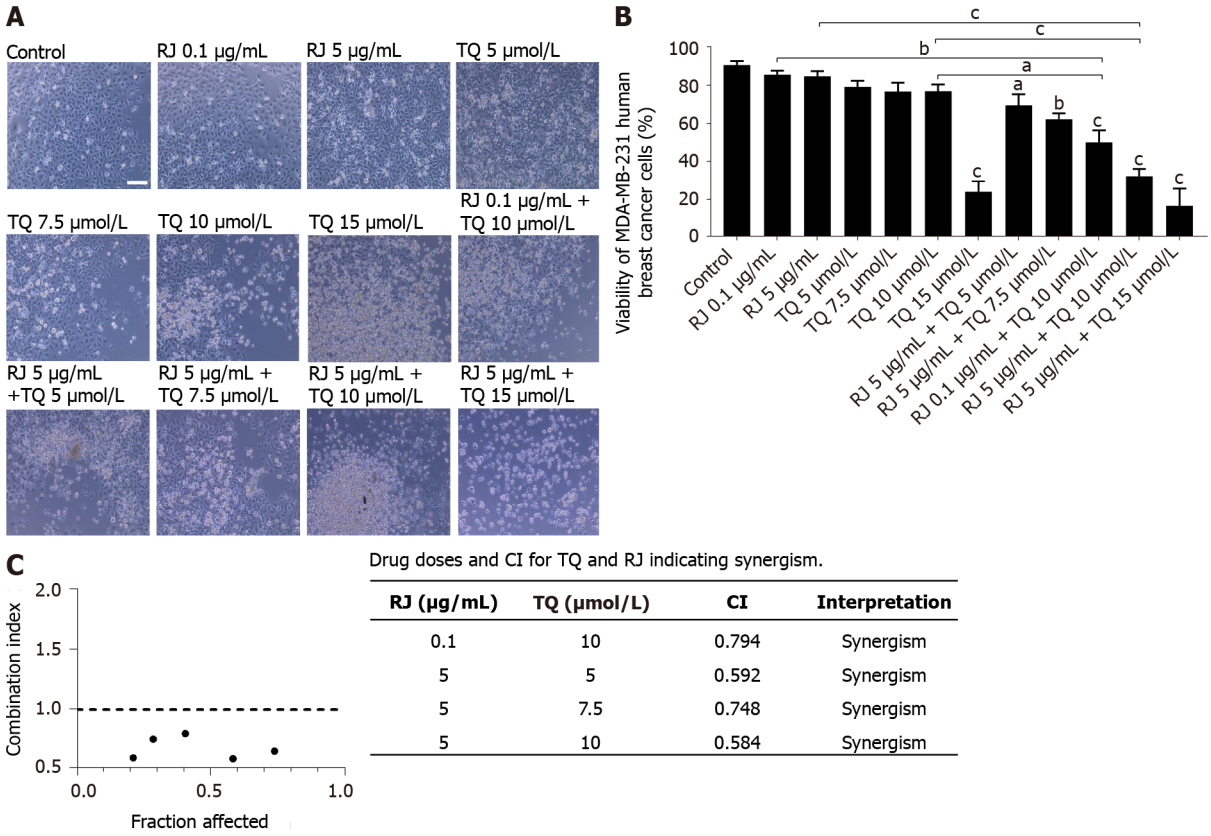


Figure 1 The inhibitory effect of thymoquinone and royal jelly on the viability of MDA-MB-231 and FHs74 Int cell line. A: 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showing the percentage viability of MDA-MB-231 cell line and the half-maximal inhibitory concentration of thymoquinone (TQ) on MDA-MB-231 human breast cancer cell line after 24 h of treatment with different TQ concentrations. Cell viability was estimated by measuring the absorbance of the cell suspension after incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; B: Trypan blue exclusion assay showing the percentage cell viability after 24 h of treatment with different royal jelly concentrations on FHs74 Int; C: MDA-MB-231 cell lines. Data shown are an average of 3 independent experiments for panels A and B, and 2 independent experiments for panel C, respectively, expressed as mean ± standard error of the mean. Asterisks represent statistically significant results compared to the control, ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). RJ: Royal jelly; TQ: Thymoquinone.



Drug doses and CI for TQ and RJ indicating synergism.

RJ (μg/mL)	TQ (μmol/L)	CI	Interpretation
0.1	10	0.794	Synergism
5	5	0.592	Synergism
5	7.5	0.748	Synergism
5	10	0.584	Synergism

Figure 2 Royal jelly and thymoquinone combinations enhanced the inhibition of MDA-MB-231 human breast cancer cell viability. A: Representative light microscopy images of MDA-MB-231 viability in response to different treatments. Cells were visualized by Axiovert inverted microscope from Zeiss at 10 × magnification with scale bar = 10 μmol/L; B: Trypan blue exclusion assay showing the percentage viability of MDA-MB-231 cell line after 24 h of treatment with different concentrations of royal jelly (RJ), thymoquinone, and combinations. Data shown are an average of three independent experiments expressed as mean ± standard error of the mean. Asterisks represent statistically significant results compared to the control and treatment conditions, ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$); C: Fraction affected-combination index plot showing combination index (CI) values plotted as a function of fraction affected values corresponding to the % cell death of five different combinations of thymoquinone (5 μmol/L, 7.5 μmol/L, 10 μmol/L and 15 μmol/L) and royal jelly (0.1 and 5 μg/mL) in MDA-MB-231 cells. The dotted line is the reference line, where CI value is equal to 1; circles in black represent CI values at different Fa. CI > 1, CI = 1, and CI < 1 indicate antagonistic, additive, and synergistic effects, respectively. CI: Combination index; RJ: Royal jelly; TQ: Thymoquinone.

μmol/L and 15 μmol/L TQ. Cell proliferation was then evaluated after 24 h of treatment by measuring the intensity of Ki67 fluorescence, a sensitive and specific proliferation biomarker. A minimal non-significant decrease in Ki67 expression was observed in response to all doses of RJ and TQ combinations as compared to the control (data not shown). This effect was confirmed by confocal imaging showing the

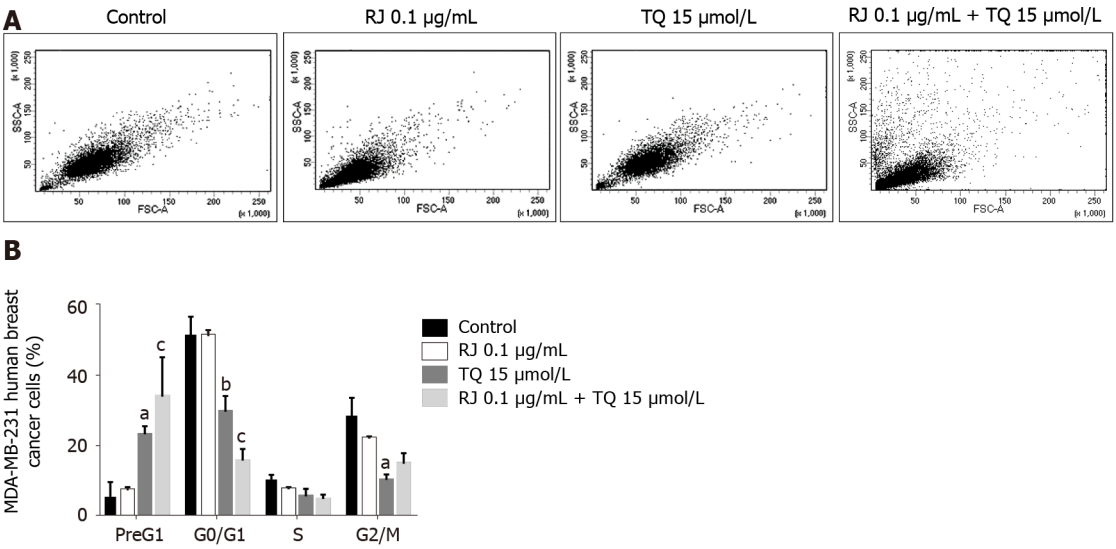


Figure 3 Cell death is enhanced by thymoquinone alone and by the combination thymoquinone and royal jelly. A: Representative density plots showing MDA-MB-231 cell distribution as a function of side scatter area and forward scatter area in the control and post-treatment with 15 µmol/L thymoquinone (TQ) and 0.1 µg/mL royal jelly alone and in combination for 24 h; B: Propidium iodide staining with flow cytometry showing the increase in Pre G1 upon treatment with TQ alone and the combination of TQ and royal jelly. Data shown are an average of three independent experiments expressed as mean ± standard error of the mean and analyzed by a two-way analysis of variance test followed by multiple comparisons test. Asterisks represent statistically significant results compared to the control, ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$). RJ: Royal jelly; TQ: Thymoquinone.

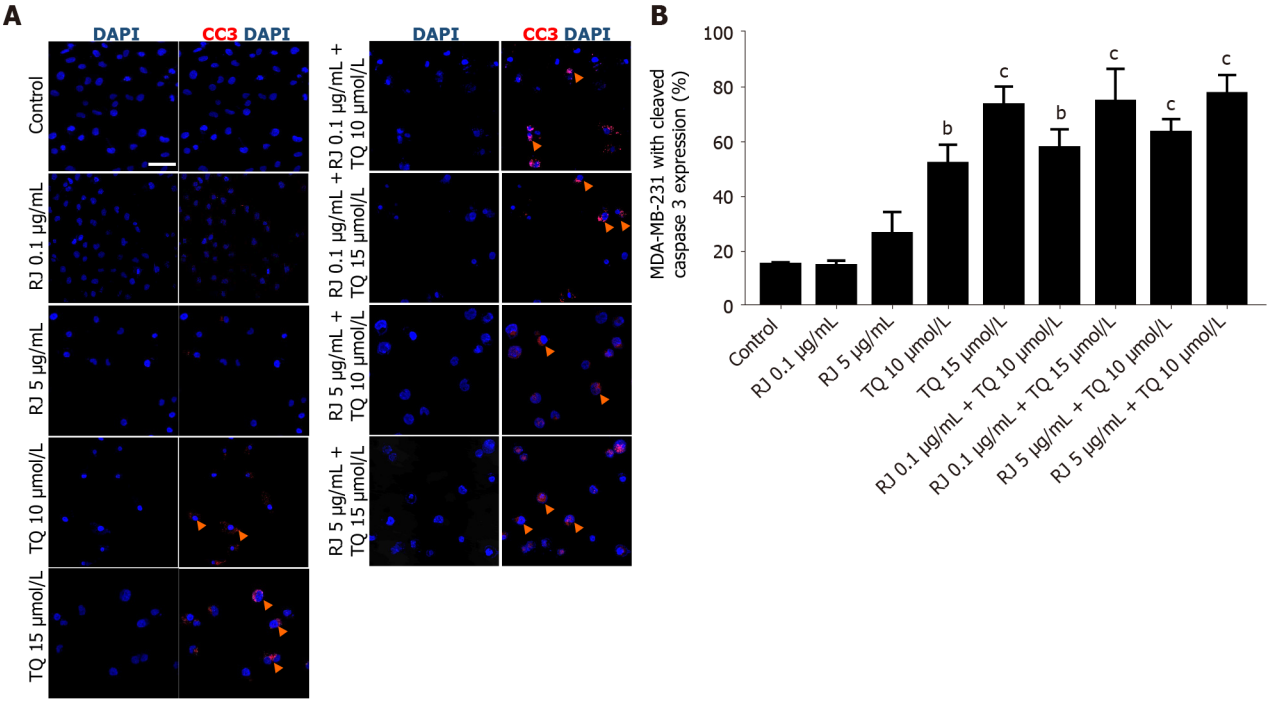


Figure 4 Effect of royal jelly, thymoquinone and combinations on caspase 3 cleavage in MDA-MB-231 human breast cancer cells. A: Immunofluorescence micrographs of cleaved caspase 3 expression at 24 h after treatment. Red indicates cleaved caspase 3 expression and blue indicates nuclei counter stained by 4',6-diamidino-2-phenylindole. Arrows indicate apoptotic nuclei. Nuclei were visualized by confocal Zeiss Axio microscope, 40 × oil immersion with scale bar = 50 µmol/L; B: Quantification of cleaved caspase 3 in MDA-MB-231 cells at 24 h of treatment with different concentrations of royal jelly, thymoquinone, and their combinations. Data shown are an average of 3 independent experiments expressed as mean ± standard error of the mean. Asterisks represent statistically significant results, ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$). RJ: Royal jelly; TQ: Thymoquinone.

modest change in the nuclear expression of Ki67 in response to the different treatments in MDA-MB-231 cells (data not shown). Therefore, RJ alone and TQ alone or their combinations did not modulate the expression of Ki67 in MDA-MB-231 human TNBC.

DISCUSSION

Breast cancer, the most common cancer among women, is identified as a heterogeneous disease arising from the differential expression of hormonal receptors along with genomic and intratumoral heterogeneity[5]. Despite the tremendous improvement in the therapeutic approaches, conventional treatments of breast cancer, including systemic therapy, exert organ-specific toxicity along with various side effects [49]. The interest in alternative treatments relying on relatively non-toxic and cost-effective natural resources has surged over the past decades, particularly from medicinal plants and honeybee products. TQ, the main constituent of *Nigella sativa* essential oil, and the nutritious honeybee secretions of RJ were shown to have potent anticancer activities against many types of cancers, including breast cancer[50,51].

Our study is the first to investigate the anticancer activity of both TQ and RJ alone and in combination against the triple-negative MDA-MB-231 human metastatic breast cancer cell line. TQ has been shown to possess potent anticancer activities against various cancer types, including colon cancer, with minimal cytotoxic effects on normal intestinal cells[48]. In the context of breast cancer, previous studies reported TQ-mediated induction of apoptosis, growth inhibition, in addition to suppression of viability and invasion of MDA-MB-231 and MCF7 cell lines mainly through the inhibition of Akt phosphorylation[23,25]. In line with these studies, we further confirmed TQ's anticancer activity in MDA-MB-231 cell line as evidenced by the dose-dependent cell death effects at concentrations below and above the IC_{50} value of 20 μmol/L. Our results showed that RJ exhibited minimal toxicity on FHs74 Int cell line at doses below 5 μg/mL, while a more pronounced inhibitory effect was observed at higher doses with a clear saturation effect obtained at doses equal or greater than 100 μg/mL. This indicates that RJ is relatively nontoxic to the non-tumorigenic human small intestinal cell line at doses equal or below 5 μg/mL. In line with the previously published studies[38,39], our findings demonstrate that RJ inhibits the viability of breast cancer cells. RJ exerted low to mild dose-dependent inhibitory effects on the viability of MDA-MB-231cell line at doses below 5 μg/mL. Cell death was more pronounced in MDA-MB-231 at 200 μg/mL RJ, suggesting the greater toxicity of RJ to breast cancer cells with the IC_{50} estimated to be 1.4 fold greater in FHs 74 Int cell line compared to that of MDA-MB-231 cell line.

Combination therapy is usually used to enhance the therapeutic response and overcome any possible drug resistance in cancer patients[52]. To assess for any possible anticancer synergy (or additive effects), concentrations that are not highly cytotoxic to cells (*i.e.* less than 50% cell death) should be used. Therefore, the anticancer effect of TQ in combination with RJ was assessed using drug doses below the IC_{50} values (*i.e.* 15 μmol/L). We documented an enhanced anticancer activity of TQ when combined with RJ against MDA-MB-231 cell line. Cancer cell viability decreased significantly in response to different combinations as compared to the treatment with each drug alone. Cell death was amplified by 3- and 5-fold in response to the combination of 5 μg/mL of RJ with 10 μmol/L and 15 μmol/L of TQ, respectively, with the lowest combination index obtained upon the combination of 5 μg/mL RJ with 10 μmol/L TQ, suggesting synergistic interaction. These results are consistent with the previous studies that reported the synergism of TQ in combination with different agents including melatonin[53] and piperine[24] against breast cancer, diosgenin on squamous cell carcinoma[54], docetaxel against prostate cancer[55], in addition to arsenic and interferon-alpha against human T-cell leukemia/lymphoma[56].

Cell cycle analysis using propidium iodide staining was performed to confirm further cell death and to examine whether TQ and RJ alone or in combination affect the cell cycle progression of breast cancer cells. In accordance with our findings using trypan blue exclusion assay, RJ alone at a dose of 0.1 μg/mL did not exert significant changes in cell viability compared to the control. Consistent with previous studies reporting the inhibitory activity of TQ[48,57,58], our study reports 4-fold increase in the PreG1 cell population, which was associated with a significant decrease in G0/G1 and G2/M cell populations in response to 15 μmol/L of TQ, further confirming TQ-mediated cell death. Interestingly, combining 15 μmol/L of TQ with 0.1 μg/mL RJ yielded a more pronounced cell death effect evidenced by the 6-fold increase in the PreG1 population. These results indicate that the cell death effect is enhanced upon the combination of TQ with RJ compared to single treatments with each compound alone. This indicates that RJ enhances the cell death effects of TQ in metastatic breast cancer.

To understand the underlying mechanism of the observed reduction in the viability of metastatic breast cancer cells upon the different treatments, we investigated apoptosis induction as a possible mechanism of cell death. Enhanced induction of apoptosis was evidenced by the increase in caspase 3 cleavage in response to the

increasing TQ doses alone or in combination with RJ. On the other hand, treatment with RJ alone did not induce any significant apoptotic effect compared to the control. Apoptotic cell death was increased by 4-fold in response to the combination of 10 μmol/L of TQ with 0.1 μg/mL and 5 μg/mL RJ, while a 5-fold increase was obtained upon combining 15 μmol/L of TQ with both RJ doses. Our results indicate that TQ is the main inducer of apoptosis, although an augmented apoptotic response was observed upon the combination with RJ, indicating that RJ could modestly potentiate the anticancer activity of TQ in TNBC. Our findings are consistent with previous studies showing induction of apoptosis upon treatment with TQ alone and in combination with other agents against cancer cells[24,56,59,60]. In line with previously published data[59], our study shows minimal changes in Ki67 intensity in response to TQ. Therefore, cell death of MDA-MB-231 cells was not due to the inhibition of proliferation but rather to apoptosis induction, as evidenced by the enhanced caspase 3 cleavage.

CONCLUSION

In conclusion, RJ and TQ, each being relatively non-toxic to normal cells, exhibit enhanced anti-tumor activities against human metastatic breast cancer when combined. Although TQ and RJ combination enhances apoptotic cell death, TQ appears to act as the main inducer of apoptosis mediating cell death by inducing caspase 3 dependent apoptosis in a dose-dependent manner. The combination of these two natural compounds deserves further investigation to identify the key molecules responsible for this enhanced anticancer activity.

ARTICLE HIGHLIGHTS

Research background

Despite the tremendous improvement in therapeutic approaches, triple-negative breast cancer has poor prognosis. Thymoquinone (TQ), the main constituent of *Nigella sativa* seeds and royal jelly (RJ), the honeybee secretion fed to honeybee queens, are effective against cancer. However, the anticancer activity of the combination of TQ and RJ against aggressive human breast cancer cells is yet unknown.

Research motivation

To establish novel treatments for breast cancer using natural, relatively non-toxic compounds with significant therapeutic value. We focused on investigating the anticancer activity of TQ and RJ combinations against triple-negative breast cancer.

Research objectives

This study aimed to characterize the anticancer activity of TQ and RJ alone and their combination *in vitro* against human triple-negative breast cancer.

Research methods

The inhibitory effect of TQ on triple-negative breast cancer cells was assessed by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Trypan blue exclusion assay was used to evaluate cell viability in response to different treatment conditions. Propidium iodide deoxyribonucleic acid staining followed by flow cytometry was performed to evaluate possible cell cycle regulation and cell death effects. Apoptosis and cell proliferation were determined using immunofluorescence assays for cleaved caspase 3 and Ki67 expression, respectively. The interaction between TQ and RJ and combination indices were evaluated using CompuSyn software.

Research results

TQ inhibited MDA-MB-231 breast cancer cell viability in a dose-dependent manner. RJ at low doses was relatively nontoxic to non-tumorigenic FHs 74 Int small intestinal epithelial cells, while at high doses greater toxicity against MDA-MB-231 breast cancer cells was observed. Inhibition of cell viability and cell death effects were more pronounced in response to TQ and RJ combinations compared to each drug alone. The reduction in breast cancer cell viability was mainly due to TQ-mediated caspase 3-dependent apoptosis.

Research conclusions

RJ and TQ are relatively non-toxic to normal cells and exhibited pronounced anticancer effects against human metastatic breast cancer. Although our findings demonstrate the potent pro-apoptotic activity of TQ compared to that of RJ, this is the first report of a significant enhancement in TQ's anticancer activity when combined with RJ.

Research perspectives

The reduction in breast cancer cell viability and enhanced cell death effects upon TQ and RJ combinations highlights their potential therapy for human triple-negative breast cancer.

ACKNOWLEDGEMENTS

We are grateful to all members of the Gali-Muhtasib Laboratory and the staff of the core facilities in the DTS Building at the American University of Beirut for their help and support.

REFERENCES

1 **Sung H**, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021 [PMID: 33538338 DOI: 10.3322/caac.21660]

2 **World Health Organization**. WHO report on cancer: setting priorities, investing wisely and providing care for all [Publications]. Geneva: World Health Organization; 2020 Available from: https://apps.who.int/iris/handle/10665/330745

3 **Tao Z**, Shi A, Lu C, Song T, Zhang Z, Zhao J. Breast Cancer: Epidemiology and Etiology. *Cell Biochem Biophys* 2015; **72**: 333-338 [PMID: 25543329 DOI: 10.1007/s12013-014-0459-6]

4 **Grieco MP**, Simonacci F, Bertozzi N, Grignaffini E, Raposio E. Breast reconstruction with breast implants. *Acta Biomed* 2019; **89**: 457-462 [PMID: 30657111 DOI: 10.23750/abm.v89i4.5748]

5 **Bertos NR**, Park M. Breast cancer - one term, many entities? *J Clin Invest* 2011; **121**: 3789-3796 [PMID: 21965335 DOI: 10.1172/JCI57100]

6 **Li CI**, Uribe DJ, Daling JR. Clinical characteristics of different histologic types of breast cancer. *Br J Cancer* 2005; **93**: 1046-1052 [PMID: 16175185 DOI: 10.1038/sj.bjc.6602787]

7 **Weigelt B**, Geyer FC, Reis-Filho JS. Histological types of breast cancer: how special are they? *Mol Oncol* 2010; **4**: 192-208 [PMID: 20452298 DOI: 10.1016/j.molonc.2010.04.004]

8 **Peddi PF**, Ellis MJ, Ma C. Molecular basis of triple negative breast cancer and implications for therapy. *Int J Breast Cancer* 2012; **2012**: 217185 [PMID: 22295242 DOI: 10.1155/2012/217185]

9 **Wahba HA**, El-Hadaad HA. Current approaches in treatment of triple-negative breast cancer. *Cancer Biol Med* 2015; **12**: 106-116 [PMID: 26175926 DOI: 10.7497/j.issn.2095-3941.2015.0030]

10 **Cailleau R**, Olivé M, Cruciger QV. Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro* 1978; **14**: 911-915 [PMID: 730202 DOI: 10.1007/BF02616120]

11 **Chavez KJ**, Garimella SV, Lipkowitz S. Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. *Breast Dis* 2010; **32**: 35-48 [PMID: 21778573 DOI: 10.3233/BD-2010-0307]

12 **Kang Y**, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordón-Cardo C, Guise TA, Massagué J. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003; **3**: 537-549 [PMID: 12842083 DOI: 10.1016/s1535-6108(03)00132-6]

13 **Minn AJ**, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massagué J. Genes that mediate breast cancer metastasis to lung. *Nature* 2005; **436**: 518-524 [PMID: 16049480 DOI: 10.1038/nature03799]

14 **Palmieri D**, Smith QR, Lockman PR, Bronder J, Gril B, Chambers AF, Weil RJ, Steeg PS. Brain metastases of breast cancer. *Breast Dis* 2006-2007; **26**: 139-147 [PMID: 17473372 DOI: 10.3233/bd-2007-26112]

15 **Palumbo MO**, Kavan P, Miller WH Jr, Panasci L, Assouline S, Johnson N, Cohen V, Patenaude F, Pollak M, Jagoe RT, Batist G. Systemic cancer therapy: achievements and challenges that lie ahead. *Front Pharmacol* 2013; **4**: 57 [PMID: 23675348 DOI: 10.3389/fphar.2013.00057]

16 **Schneider-Stock R**, Fakhoury IH, Zaki AM, El-Baba CO, Gali-Muhtasib HU. Thymoquinone: fifty years of success in the battle against cancer models. *Drug Discov Today* 2014; **19**: 18-30 [PMID: 24001594 DOI: 10.1016/j.drudis.2013.08.021]

17 **Aycan IÖ**, Tüfek A, Tokgöz O, Evliyaoğlu O, Fırat U, Kavak GÖ, Turgut H, Yüksel MU. Thymoquinone treatment against acetaminophen-induced hepatotoxicity in rats. *Int J Surg* 2014; **12**: 213-218 [PMID: 24389315 DOI: 10.1016/j.ijsu.2013.12.013]

18 **Abulfadl YS**, El-Maraghy NN, Ahmed AAE, Nofal S, Badary OA. Protective effects of

thymoquinone on D-galactose and aluminum chloride induced neurotoxicity in rats: biochemical, histological and behavioral changes. *Neurol Res* 2018; **40**: 324-333 [PMID: 29464986 DOI: 10.1080/01616412.2018.1441776]

19 **Asaduzzaman Khan M**, Tania M, Fu S, Fu J. Thymoquinone, as an anticancer molecule: from basic research to clinical investigation. *Oncotarget* 2017; **8**: 51907-51919 [PMID: 28881699 DOI: 10.18632/oncotarget.17206]

20 **Shaterzadeh-Yazdi H**, Noorbakhsh MF, Hayati F, Samarghandian S, Farkhondeh T. Immunomodulatory and Anti-inflammatory Effects of Thymoquinone. *Cardiovasc Hematol Disord Drug Targets* 2018; **18**: 52-60 [PMID: 29437018 DOI: 10.2174/1871529X18666180212114816]

21 **Alobaedi OH**, Talib WH, Basheti IA. Antitumor effect of thymoquinone combined with resveratrol on mice transplanted with breast cancer. *Asian Pac J Trop Med* 2017; **10**: 400-408 [PMID: 28552110 DOI: 10.1016/j.apjtm.2017.03.026]

22 **Bashmail HA**, Alamoudi AA, Noorwali A, Hegazy GA, AJabnoor G, Choudhry H, Al-Abd AM. Thymoquinone synergizes gemcitabine anti-breast cancer activity *via* modulating its apoptotic and autophagic activities. *Sci Rep* 2018; **8**: 11674 [PMID: 30076320 DOI: 10.1038/s41598-018-30046-z]

23 **Ganji-Harsini S**, Khazaei M, Rashidi Z, Ghanbari A. Thymoquinone Could Increase The Efficacy of Tamoxifen Induced Apoptosis in Human Breast Cancer Cells: An In Vitro Study. *Cell J* 2016; **18**: 245-254 [PMID: 27540530 DOI: 10.22074/cellj.2016.4320]

24 **Talib WH**. Regressions of Breast Carcinoma Syngraft Following Treatment with Piperine in Combination with Thymoquinone. *Sci Pharm* 2017; **85** [PMID: 28671634 DOI: 10.3390/scipharm85030027]

25 **Attoub S**, Sperandio O, Raza H, Arafat K, Al-Salam S, Al Sultan MA, Al Safi M, Takahashi T, Adem A. Thymoquinone as an anticancer agent: evidence from inhibition of cancer cells viability and invasion in vitro and tumor growth in vivo. *Fundam Clin Pharmacol* 2013; **27**: 557-569 [PMID: 22788741 DOI: 10.1111/j.1472-8206.2012.01056.x]

26 **Kocot J**, Kielczykowska M, Luchowska-Kocot D, Kurzepa J, Musik I. Antioxidant Potential of Propolis, Bee Pollen, and Royal Jelly: Possible Medical Application. *Oxid Med Cell Longev* 2018; **2018**: 7074209 [PMID: 29854089 DOI: 10.1155/2018/7074209]

27 **Rajput S**, Kumar BN, Dey KK, Pal I, Parekh A, Mandal M. Molecular targeting of Akt by thymoquinone promotes G(1) arrest through translation inhibition of cyclin D1 and induces apoptosis in breast cancer cells. *Life Sci* 2013; **93**: 783-790 [PMID: 24044882 DOI: 10.1016/j.lfs.2013.09.009]

28 **Kamakura M**. [Royalactin induces queen differentiation in honeybees]. *Seikagaku* 2012; **84**: 994-1003 [PMID: 23350509 DOI: 10.1038/nature10093]

29 **Melliou E**, Chinou I. Chemistry and bioactivity of royal jelly from Greece. *J Agric Food Chem* 2005; **53**: 8987-8992 [PMID: 16277392 DOI: 10.1021/jf051550p]

30 **Taniguchi Y**, Kohno K, Inoue S, Koya-Miyata S, Okamoto I, Arai N, Iwaki K, Ikeda M, Kurimoto M. Oral administration of royal jelly inhibits the development of atopic dermatitis-like skin lesions in NC/Nga mice. *Int Immunopharmacol* 2003; **3**: 1313-1324 [PMID: 12890429 DOI: 10.1016/s1567-5769(03)00132-2]

31 **Liu JR**, Yang YC, Shi LS, Peng CC. Antioxidant properties of royal jelly associated with larval age and time of harvest. *J Agric Food Chem* 2008; **56**: 11447-11452 [PMID: 19007163 DOI: 10.1021/jf802494e]

32 **Kohno K**, Okamoto I, Sano O, Arai N, Iwaki K, Ikeda M, Kurimoto M. Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages. *Biosci Biotechnol Biochem* 2004; **68**: 138-145 [PMID: 14745176 DOI: 10.1271/bbb.68.138]

33 **Kamakura M**, Moriyama T, Sakaki T. Changes in hepatic gene expression associated with the hypocholesterolaemic activity of royal jelly. *J Pharm Pharmacol* 2006; **58**: 1683-1689 [PMID: 17331334 DOI: 10.1211/jpp.58.12.0017]

34 **Chiu HF**, Chen BK, Lu YY, Han YC, Shen YC, Venkatakrishnan K, Golovinskaia O, Wang CK. Hypocholesterolemic efficacy of royal jelly in healthy mild hypercholesterolemic adults. *Pharm Biol* 2017; **55**: 497-502 [PMID: 27937077 DOI: 10.1080/13880209.2016.1253110]

35 **Fan P**, Han B, Feng M, Fang Y, Zhang L, Hu H, Hao Y, Qi Y, Zhang X, Li J. Functional and Proteomic Investigations Reveal Major Royal Jelly Protein 1 Associated with Anti-hypertension Activity in Mouse Vascular Smooth Muscle Cells. *Sci Rep* 2016; **6**: 30230 [PMID: 27444336 DOI: 10.1038/srep30230]

36 **Zargar HR**, Hemmati AA, Ghafourian M, Arzi A, Rezaie A, Javad-Moosavi SA. Long-term treatment with royal jelly improves bleomycin-induced pulmonary fibrosis in rats. *Can J Physiol Pharmacol* 2017; **95**: 23-31 [PMID: 27841941 DOI: 10.1139/cjpp-2015-0451]

37 **Pyrzanowska J**, Piechal A, Blecharz-Klin K, Joniec-Maciejak I, Graikou K, Chinou I, Widy-Tyszkiewicz E. Long-term administration of Greek Royal Jelly improves spatial memory and influences the concentration of brain neurotransmitters in naturally aged Wistar male rats. *J Ethnopharmacol* 2014; **155**: 343-351 [PMID: 24882731 DOI: 10.1016/j.jep.2014.05.032]

38 **Nakaya M**, Onda H, Sasaki K, Yukiyoshi A, Tachibana H, Yamada K. Effect of royal jelly on bisphenol A-induced proliferation of human breast cancer cells. *Biosci Biotechnol Biochem* 2007; **71**: 253-255 [PMID: 17213647 DOI: 10.1271/bbb.60453]

39 **Zhang S**, Shao Q, Geng H, Su S. The effect of royal jelly on the growth of breast cancer in mice. *Oncol Lett* 2017; **14**: 7615-7621 [PMID: 29344209 DOI: 10.3892/ol.2017.7078]

40 **Filipič B**, Gradišnik L, Rihar K, Šooš E, Pereyra A, Potokar J. The influence of royal jelly and human interferon-alpha (HuIFN-αN3) on proliferation, glutathione level and lipid peroxidation in human

colorectal adenocarcinoma cells in vitro. *Arh Hig Rada Toksikol* 2015; **66**: 269-274 [PMID: 26751858 DOI: 10.1515/aiht-2015-66-2632]

41 **Gismondi A**, Trionfera E, Canuti L, Di Marco G, Canini A. Royal jelly lipophilic fraction induces antiproliferative effects on SH-SY5Y human neuroblastoma cells. *Oncol Rep* 2017; **38**: 1833-1844 [PMID: 28737831 DOI: 10.3892/or.2017.5851]

42 **Izuta H**, Chikaraishi Y, Shimazawa M, Mishima S, Hara H. 10-Hydroxy-2-decenoic acid, a major fatty acid from royal jelly, inhibits VEGF-induced angiogenesis in human umbilical vein endothelial cells. *Evid Based Complement Alternat Med* 2009; **6**: 489-494 [PMID: 18955252 DOI: 10.1093/ecam/nem152]

43 **Nedeljković M**, Damjanović A. Mechanisms of Chemotherapy Resistance in Triple-Negative Breast Cancer-How We Can Rise to the Challenge. *Cells* 2019; **8** [PMID: 31443516 DOI: 10.3390/cells8090957]

44 **Yao Y**, Chu Y, Xu B, Hu Q, Song Q. Risk factors for distant metastasis of patients with primary triple-negative breast cancer. *Biosci Rep* 2019; **39** [PMID: 31113872 DOI: 10.1042/BSR20190288]

45 **Galvao J**, Davis B, Tilley M, Normando E, Duchen MR, Cordeiro MF. Unexpected low-dose toxicity of the universal solvent DMSO. *FASEB J* 2014; **28**: 1317-1330 [PMID: 24327606 DOI: 10.1096/fj.13-235440]

46 **Cevallos AM**, Herrera J, López-Villaseñor I, Hernández R. Differential Effects of Two Widely Used Solvents, DMSO and Ethanol, on the Growth and Recovery of *Trypanosoma cruzi* Epimastigotes in Culture. *Korean J Parasitol* 2017; **55**: 81-84 [PMID: 28285511 DOI: 10.3347/kjp.2017.55.1.81]

47 **Chou TC**, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**: 27-55 [PMID: 6382953 DOI: 10.1016/0065-2571(84)90007-4]

48 **El-Najjar N**, Chatila M, Moukadem H, Vuorela H, Ocker M, Gandesiri M, Schneider-Stock R, Gali-Muhtasib H. Reactive oxygen species mediate thymoquinone-induced apoptosis and activate ERK and JNK signaling. *Apoptosis* 2010; **15**: 183-195 [PMID: 19882352 DOI: 10.1007/s10495-009-0421-z]

49 **Bodai BI**, Tuso P. Breast cancer survivorship: a comprehensive review of long-term medical issues and lifestyle recommendations. *Perm J* 2015; **19**: 48-79 [PMID: 25902343 DOI: 10.7812/TPP/14-241]

50 **Ahmad S**, Campos MG, Fratini F, Altaye SZ, Li J. New Insights into the Biological and Pharmaceutical Properties of Royal Jelly. *Int J Mol Sci* 2020; **21** [PMID: 31936187 DOI: 10.3390/ijms21020382]

51 **Imran M**, Rauf A, Khan IA, Shahbaz M, Qaisrani TB, Fatmawati S, Abu-Izneid T, Imran A, Rahman KU, Gondal TA. Thymoquinone: A novel strategy to combat cancer: A review. *Biomed Pharmacother* 2018; **106**: 390-402 [PMID: 29966985 DOI: 10.1016/j.biopha.2018.06.159]

52 **Palmer AC**, Sorger PK. Combination Cancer Therapy Can Confer Benefit *via* Patient-to-Patient Variability without Drug Additivity or Synergy. *Cell* 2017; **171**: 1678-1691.e13 [PMID: 29245013 DOI: 10.1016/j.cell.2017.11.009]

53 **Odeh LH**, Talib WH, Basheti IA. Synergistic effect of thymoquinone and melatonin against breast cancer implanted in mice. *J Cancer Res Ther* 2018; **14**: S324-S330 [PMID: 29970684 DOI: 10.4103/0973-1482.235349]

54 **Das S**, Dey KK, Dey G, Pal I, Majumder A, MaitiChoudhury S, Kundu SC, Mandal M. Antineoplastic and apoptotic potential of traditional medicines thymoquinone and diosgenin in squamous cell carcinoma. *PLoS One* 2012; **7**: e46641 [PMID: 23077516 DOI: 10.1371/journal.pone.0046641]

55 **Dirican A**, Atmaca H, Bozkurt E, Erten C, Karaca B, Uslu R. Novel combination of docetaxel and thymoquinone induces synergistic cytotoxicity and apoptosis in DU-145 human prostate cancer cells by modulating PI3K-AKT pathway. *Clin Transl Oncol* 2015; **17**: 145-151 [PMID: 25060568 DOI: 10.1007/s12094-014-1206-6]

56 **Houssein M**, Fatfat M, Habli Z, Ghazal N, Moodad S, Khalife H, Khalil M, Gali-Muhtasib H. Thymoquinone synergizes with arsenic and interferon alpha to target human T-cell leukemia/lymphoma. *Life Sci* 2020; **251**: 117639 [PMID: 32272181 DOI: 10.1016/j.lfs.2020.117639]

57 **Gali-Muhtasib H**, Kuester D, Mawrin C, Bajbouj K, Diestel A, Ocker M, Habold C, Foltzer-Jourdainne C, Schoenfeld P, Peters B, Diab-Assaf M, Pommrich U, Itani W, Lippert H, Roessner A, Schneider-Stock R. Thymoquinone triggers inactivation of the stress response pathway sensor CHEK1 and contributes to apoptosis in colorectal cancer cells. *Cancer Res* 2008; **68**: 5609-5618 [PMID: 18632613 DOI: 10.1158/0008-5472.CAN-08-0884]

58 **Khalife R**, Hodroj MH, Fakhoury R, Rizk S. Thymoquinone from Nigella sativa Seeds Promotes the Antitumor Activity of Noncytotoxic Doses of Topotecan in Human Colorectal Cancer Cells in Vitro. *Planta Med* 2016; **82**: 312-321 [PMID: 26848703 DOI: 10.1055/s-0035-1558289]

59 **Gali-Muhtasib H**, Ocker M, Kuester D, Krueger S, El-Hajj Z, Diestel A, Evert M, El-Najjar N, Peters B, Jurjus A, Roessner A, Schneider-Stock R. Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. *J Cell Mol Med* 2008; **12**: 330-342 [PMID: 18366456 DOI: 10.1111/j.1582-4934.2007.00095.x]

60 **Roepke M**, Diestel A, Bajbouj K, Walluscheck D, Schonfeld P, Roessner A, Schneider-Stock R, Gali-Muhtasib H. Lack of p53 augments thymoquinone-induced apoptosis and caspase activation in human osteosarcoma cells. *Cancer Biol Ther* 2007; **6**: 160-169 [PMID: 17218778 DOI: 10.4161/cbt.6.2.3575]

World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2021 May 24; 12(5): 355-366

DOI: 10.5306/wjco.v12.i5.355

ISSN 2218-4333 (online)

ORIGINAL ARTICLE

Retrospective Study

Prognostic role of sarcopenia in metastatic colorectal cancer patients during first-line chemotherapy: A retrospective study

Chiara Maddalena, Andrea Ponsiglione, Luigi Camera, Lidia Santarpia, Fabrizio Pasanisi, Dario Bruzzese, Camilla Panico, Giovanni Fiore, Simona Camardella, Tolomeo Caramia, Alessia Farinaro, Sabino De Placido, Chiara Carlomagno

ORCID number: Chiara Maddalena 0000-0003-1755-6729; Andrea Ponsiglione 0000-0002-0105-935X; Luigi Camera 0000-0002-4250-5176; Lidia Santarpia 0000-0003-2332-4836; Fabrizio Pasanisi 0000-0003-4224-7821; Dario Bruzzese 0000-0001-9911-4646; Camilla Panico 0000-0002-0472-1118; Giovanni Fiore 0000-0003-4535-1480; Simona Camardella 0000-0001-7106-8780; Tolomeo Caramia 0000-0003-2971-3218; Alessia Farinaro 0000-0003-2336-2955; Sabino De Placido 0000-0001-7903-2088; Chiara Carlomagno 0000-0001-5295-3004.

Author contributions: Maddalena C contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and writing the original draft; Ponsiglione A and Panico C contributed to the analysis and interpretation of data; Camera L, Santarpia L and Pasanisi F contributed to the conception and design of the study; Bruzzese D contributed to the biostatistical analysis; Fiore G, Camardella S, Caramia T and Farinaro A contributed to the acquisition of data; De Placido S contributed to the supervision; Carlomagno C contributed to the conception and design of the study, analysis and interpretation of data, supervision,

Chiara Maddalena, Lidia Santarpia, Fabrizio Pasanisi, Giovanni Fiore, Simona Camardella, Tolomeo Caramia, Alessia Farinaro, Sabino De Placido, Chiara Carlomagno, Department of Clinical Medicine and Surgery, University of Naples Federico II, Naples 80131, Italy

Andrea Ponsiglione, Luigi Camera, Camilla Panico, Department of Advanced Biomedical Sciences, University of Naples Federico II, Naples 80131, Italy

Dario Bruzzese, Department of Public Health, University of Naples Federico II, Naples 80131, Italy

Corresponding author: Chiara Maddalena, MD, Academic Fellow, Department of Clinical Medicine and Surgery, University of Naples Federico II, Via Pansini 5, Naples 80131, Italy. chiara.maddalena@yahoo.it

Abstract

BACKGROUND

Sarcopenia is a condition characterized by decreased skeletal muscle mass due to physiological ageing or to a concomitant disease such as neoplasia. In cancer patients, a low lean body mass is suggested to be a negative prognostic factor for survival and for the development of dose-limiting chemotherapy toxicities irrespective of disease stage.

AIM

To evaluate the prognostic role of sarcopenia in patients with metastatic colorectal cancer (mCRC) undergoing first-line chemotherapy.

METHODS

Our retrospective analysis included 56 mCRC patients who received first-line chemotherapy from 2014 to 2017 at the Medical Oncology Unit of our hospital. Computerized scans were performed before starting chemotherapy and at the first disease reassessment. Sarcopenia was assessed using the skeletal mass index = muscle area in cm^2/(height in m^2) calculated at the L3 vertebra. Overall survival and objective response rate were evaluated. Toxicities were analyzed during the first four cycles of therapy and graded according to Common Terminology Criteria for Adverse Events version 4.0. A loss of skeletal muscle mass ≥ 5% was

review and editing, and final approval of the version of the article to be published.

Institutional review board statement: The study was approved by the panel of scientists proposing the research and by all the collaborators who participated in the research.

Informed consent statement: Informed written consent was obtained from the patients for publication of this report.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

Specialty type: Oncology

Country/Territory of origin: Italy

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: December 26, 2020
Peer-review started: December 26, 2020
First decision: January 18, 2021
Revised: January 31, 2021
Accepted: March 18, 2021
Article in press: March 18, 2021

considered indicative of deterioration in muscle condition.

RESULTS

Median age was 67 years and 35.7% of patients were ≥ 70 years old. Fourteen patients (25%) were sarcopenic at baseline computed tomography (CT) scan (7/33 men; 7/23 women); 5/14 sarcopenic patients were ≥ 70 years old. Median follow-up was 26.8 mo (3.8-66.8 mo) and median overall survival was 27.2 mo (95%CI: 23.3-37.3). Sarcopenia was not correlated to overall survival (P = 0.362), to higher toxicities reported during the first 4 cycles of chemotherapy (P = 1.0) or to response to treatment (P = 0.221). At the first disease reassessment, a skeletal muscle loss (SML) ≥ 5% was found in 17 patients (30.3%) 3 of whom were already sarcopenic at baseline CT scan, while 7 patients became sarcopenic. SML was not correlated to overall survival (P = 0.961). No statistically significant correlation was found between baseline sarcopenia and age (P = 1.0), body mass index (P = 0.728), stage at diagnosis (P = 0.355) or neutrophil/lymphocyte ratio (P = 0.751).

CONCLUSION

Neither baseline sarcopenia nor SML affected survival. In addition, baseline sarcopenia was not related to worse treatment toxicity. However, these results must be interpreted with caution due to the limited sample size.

Key Words: Sarcopenia; Lean body mass; Skeletal muscle mass; Metastatic colorectal cancer

Core Tip: According to previous studies, sarcopenia is associated with a poorer prognosis in metastatic colorectal cancer (mCRC) patients. We analyzed the prognostic role of sarcopenia in 56 mCRC patients treated with first-line chemotherapy. Neither sarcopenia nor muscle mass loss was significantly associated with survival. Other prospective studies are needed to clarify the role of sarcopenia in mCRC patients. Moreover, greater efforts should be made to diagnose sarcopenia earlier to correct strength and muscle mass, and thus improve patient tolerability to treatment and survival.

Citation: Maddalena C, Ponsiglione A, Camera L, Santarpia L, Pasanisi F, Bruzzese D, Panico C, Fiore G, Camardella S, Caramia T, Farinaro A, De Placido S, Carlomagno C. Prognostic role of sarcopenia in metastatic colorectal cancer patients during first-line chemotherapy: A retrospective study. *World J Clin Oncol* 2021; 12(5): 355-366
URL: https://www.wjgnet.com/2218-4333/full/v12/i5/355.htm
DOI: https://dx.doi.org/10.5306/wjco.v12.i5.355

INTRODUCTION

In medical oncology, the dose of cytotoxic drugs is calculated according to the patient's body surface area (BSA) using formulae devised in the early twentieth century and validated on a limited number of subjects[1,2]. For instance, the formula proposed by Du Bois *et al*[2], which is routinely used in adult cancer patients, was based on data of only nine patients. Furthermore, individualized dosage of antineoplastic agents based on BSA does not necessarily equate to a patient's drug exposure because the quantity of active drug circulating in the body and the duration of circulation may vary due to various factors[3,4]. In fact, pharmacokinetic parameters of a particular agent, such as area under the curve and clearance, may differ substantially among patients not only because of genetic factors, pharmacological interactions and the physiological characteristics of patients, but also because of body composition variations that are typical of the natural history of cancer patients[1,5].

The total body mass consists of two major compartments, fat and lean, which are the major sites of distribution of lipophilic and non-lipophilic drugs, respectively[6]. Therefore, the ratio of fat and lean tissue masses could be a better parameter than BSA

Published online: May 24, 2021

P-Reviewer: Bordonaro M, Wang YH
S-Editor: Zhang H
L-Editor: Webster JR
P-Editor: Wu YXJ

with which to determine the dose of cytotoxic agents, as it affects metabolism, plasma concentration and the toxicity of chemotherapy drugs[6,7]. Moreover, patients with a similar or identical body weight, BSA or body mass index (BMI) may have a different lean body mass (LBM)[8,9]. Skeletal muscle tissue accounts for most of the LBM and is the predominant source of proteins which are essential for all cell processes[4,10]. People with a low skeletal muscle mass may have a lower volume of drug distribution and reduced protein binding compared to people with a normal muscle mass thereby resulting in a higher plasma drug concentration and worse treatment toxicity[8,11]. The skeletal muscle mass decrease due to physiological ageing or concomitant disease such as neoplasia is defined as "sarcopenia"[12]. In cancer patients, a low LBM and sarcopenia are negative prognostic factors for survival[8,9,13] and for the development of dose-limiting chemotherapy toxicities[6,14] irrespective of disease stage. The aim of the present study was to retrospectively analyze the prevalence of sarcopenia in patients with metastatic colorectal cancer (mCRC) and its prognostic role.

In 2018, the European Working Group on Sarcopenia in Older People 2 published an updated definition that uses low muscle strength as the primary parameter for recognizing sarcopenia, together with additional items of low muscle quantity or quality[15]. However, due to the retrospective nature of our analysis, we used the computed tomography (CT) scans performed at the time of first diagnosis of metastatic disease to evaluate the muscle area, and therefore the muscle quantity, at the level of the third lumbar vertebra.

MATERIALS AND METHODS

The primary end-point of this study was to assess the association between baseline sarcopenia, estimated before starting first-line chemotherapy, and overall survival (OS) in mCRC patients. The secondary end-points were: (1) to evaluate the potential correlation of baseline sarcopenia with the objective response rate (ORR) to first-line chemotherapy and with the development of side effects to antineoplastic therapy during the first four cycles of treatment; (2) to investigate the association between skeletal muscle loss (SML) at first disease reassessment and OS; and (3) to examine the relationship between sarcopenia and age, BMI, disease stage at the time of first diagnosis and the neutrophil/lymphocyte ratio (NLR) as an inflammation index.

Our retrospective analysis included 56 mCRC patients who received first-line chemotherapy for metastatic disease from 2014 to 2017 at the Medical Oncology Unit of the Federico II University Hospital. All patients had signed the informed consent document for the use of personal data in the medical record according to the Italian privacy legislation. The study was approved by the panel of scientists proposing the research and by all the collaborators who participated in the research and it was conducted in accordance with the 2013 Declaration of Helsinki.

Computerized scans were performed before starting chemotherapy (baseline) and at first disease reassessment (2-3 mo after starting therapy). The images were analyzed by a subspecialty trained abdominal radiologist. Sarcopenia was assessed using the skeletal mass index [SMI = muscle area in cm^2/(height in m^2)][16]. The cross-sectional area of all skeletal muscles was calculated at the third lumbar vertebra on pre-contrast axial CT images with a slice thickness of 5 mm, using the open-source Horos software (version 3.3.6)[16,17]. An attenuation threshold ranging from -29 to 150 Hounsfield units was set for muscle tissue[16].

Sarcopenia was defined by Martin SMI cut-offs[8], that combined both sex-specific and BMI cut-offs: 43 cm^2/m^2 for men with BMI < 25 kg/m^2, 53 cm^2/m^2 for men with BMI ≥ 25 kg/m^2 and 41 cm^2/m^2 for women regardless of BMI. A loss of skeletal muscle mass ≥ 5% from baseline CT to first disease reassessment was considered indicative of a deterioration in muscle condition[18]. Patients' characteristics were categorized as follows: age (< 70 years *vs* ≥ 70 years), BMI (underweight < 18.5 kg/m^2; normal weight 18.5-24.9 kg/m^2; overweight 25-30 kg/m^2 and obese > 30 kg/m^2) and disease stage at the time of first diagnosis (limited *vs* metastatic). A NLR ≥ 3 was considered as an inflammation index[19]. Toxicities were analyzed during the first four cycles of therapy and graded according to the Common Terminology Criteria for Adverse Events version 4.0. Survival was calculated from the date of baseline CT, at the time of metastatic disease diagnosis, to death or until the last outpatient visit. Disease status was assessed using the Response Evaluation Criteria in Solid Tumors version 1.1.

The statistical review of the study was performed by a biomedical statistician. Univariate and multivariate analysis and the calculation of the hazard ratio (95%CI) were carried out according to the Cox regression. Survival curves were estimated

using the Kaplan–Meier method. The Chi-squared test was used to correlate sarcopenia and ORR, toxicities, age, BMI, disease stage at the time of first diagnosis and NLR. The statistical analysis was performed using the SPSS version 20.0 software (SPSS Inc.).

RESULTS

We examined 56 consecutive mCRC patients who had received first-line chemotherapy and whose CT-scans were available in our archive. Fourteen patients (25%) were sarcopenic at baseline CT scan (7/33 men; 7/23 women). The median age of patients was 67 years (37-85 years) and 20 of the 56 patients (35.7%) were 70 years or older. Five of the 14 sarcopenic patients were 70 years or older. BMI distribution was 0% underweight, 37.5% normal weight, 39.3% overweight and 23.2% obese. SMI varied within each BMI category: 6/21 normal weight patients, 6/22 overweight patients and 2/13 obese patients were sarcopenic at baseline CT scan. Eighteen patients (32.1%) had a NLR ≥ 3. At the time of first diagnosis, 23 patients (41.1%) had II or III stage disease according to the pTNM classification and they subsequently developed metastases; 33 patients (58.9%) received the diagnosis at the metastatic stage. Of the 14 sarcopenic patients at the time of first diagnosis of metastatic disease, 4 had metachronous metastases and 10 had synchronous metastases.

The median follow-up was 26.8 mo (3.8-66.8 mo) and the median OS was 27.2 mo (95%CI: 23.3-37.3) (Figure 1). Sarcopenia was not correlated to either OS (HR, 0.72 95%CI: 0.35-1.47, P = 0.362) (Figure 2) or higher toxicity during the first 4 cycles of chemotherapy (P = 1.0) (Table 1). Four of the 14 (28.6%) sarcopenic patients and 13 of the 42 (31%) non-sarcopenic patients had at least one reduction in drug dosage due to toxicity during the first four cycles of therapy (P = 1.0). Twenty-seven patients (48.2%) had a partial or complete response, the disease was stable in 24 patients (42.8%), and 5 patients (8.9%) had disease progression as best response to first-line treatment. Response rate was not correlated to baseline sarcopenia (P = 0.221) (Table 1).

At first disease reassessment, 17 patients had an SML ≥ 5% (30.3%); 3 of these patients were already sarcopenic at baseline CT scan, while 7 patients became sarcopenic. Of these 6 men and 1 woman, 4 were under the age of 70 years; at baseline, 3/7 patients were normal weight, 3/7 were overweight and 1/7 was obese. One normal weight patient became overweight, while one overweight patient became normal weight at first disease reassessment. The median OS of these 7 patients was 27.93 mo, similar to that of the entire study population. Muscle mass loss was not correlated to OS (P = 0.961) (Figure 3).

No statistically significant correlation was found between baseline sarcopenia and age (P = 1.0), BMI (P = 0.728), stage at diagnosis (P = 0.355) and NLR (P = 0.751) (Table 1).

DISCUSSION

Skeletal muscle decrease is generally associated with physiological ageing. The probable mechanism of sarcopenia is an imbalance in muscle protein turnover due to endocrine changes (*e.g.*, reduction of sex hormones and growth factors), age-related cell damage and mitochondrial dysfunction, oxidative stress, low-grade systemic inflammation, physical inactivity and malnutrition[20-23]. In cancer patients the loss of muscle mass can occur earlier than in healthy people due to the synergy between physiologic and tumor factors (*e.g.*, production of inflammatory cytokines that induces a catabolic state)[24]. Depending on histology and disease stage, the prevalence of sarcopenia varies greatly among patients affected by neoplasia; for example, sarcopenia has been diagnosed in 30%-65% of patients with pancreatic neoplasia[25,26]; in 15.9%-66.9% of women with breast cancer[27,28]; in 47.9%-89% of gastric cancer patients[29-31]; in 27.5% of patients with advanced hepatocellular carcinoma[32]; in 52.5%-54.5% of patients with metastatic renal cell carcinoma[33,34]; in 19.4%-39% of patients with colorectal cancer[35,36].

In our study population, the prevalence of sarcopenia was 25%: 14 of the 56 patients were sarcopenic at baseline CT and most of them (9/14, 64%) were under the age of 70 years, which indicates that it is not uncommon to find a low skeletal mass in young adults. In this context, it is notable that Miyamoto *et al*[37] found that young CRC patients (< 65 years) with sarcopenia had a significantly shorter OS than those without sarcopenia, while the prognostic role of sarcopenia was lost in patients above 65 years

Table 1 Correlation between sarcopenia, age, body mass index, stage at diagnosis, neutrophil/lymphocyte ratio, toxicity and response to treatment, *n* (%)

	Sarcopenia			*P* value
	Total	No	Yes	
Age				1
≥ 70 yr	20 (35.7)	15 (35.7)	5 (35.7)	
BMI (kg/m^2)				0.728
18.5-24.9	21 (37.5)	15 (35.7)	6 (42.9)	
25-30	22 (39.3)	16 (38.1)	6 (42.9)	
> 30	13 (23.2)	11 (26.2)	2 (14.3)	
Stage at diagnosis				0.355
TNM II/III3	23 (41.1)	19 (45.2)	4 (28.6)	
TNM IV	33 (58.9)	23 (54.8)	10 (71.4)	
NLR				0.751
≥ 3	18 (32.1)	13 (31)	5 (35.7)	
Toxicity during the first 4 chemotherapy cycles				
At least one dose reduction	17 (30.4)	13 (31)	4 (28.6)	1
Diarrhea G ≥ 2	10 (17.9)	8 (19)	2 (14.3)	1
Neutropenia G ≥ 3/4	11 (19.6)	8 (19)	3 (21.4)	1
Response to treatment				0.221
Partial/complete response	27 (48.2)	18 (42.9)	9 (64.3)	

BMI: Body mass index; NLR: Neutrophil/lymphocyte ratio.

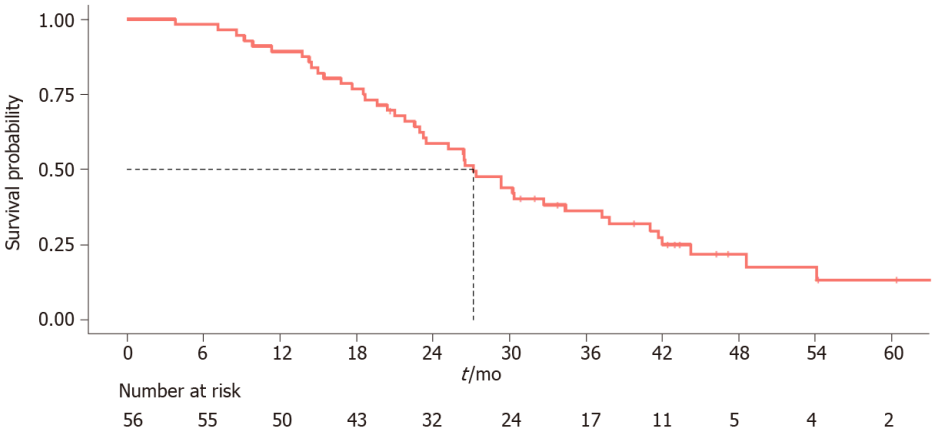


Figure 1 Overall survival.

of age. Consequently, it is also important to assess muscle mass in young CRC patients upon diagnosis, to better define the prognosis of each patient, and possibly to tailor anticancer treatment and improve the correction of sarcopenia. Indeed, various strategies have been reported to improve muscle mass and strength, namely exercise [38,39], dietary supplementation of proteins[40] and long-term intake of omega-3 fatty acids, which have anti-inflammatory and anabolic activities[41].

Clinicians should determine whether patients have sarcopenia not only regardless of age, but also regardless of BMI. In fact, a feature of sarcopenia that differentiates it from cachexia, is that it can occur without a concomitant loss of adipose tissue. In our study, no patient was underweight, and 8 of the 14 (57%) sarcopenic patients were overweight or obese (Figure 4). Notably, not all sarcopenic patients have a low BMI:

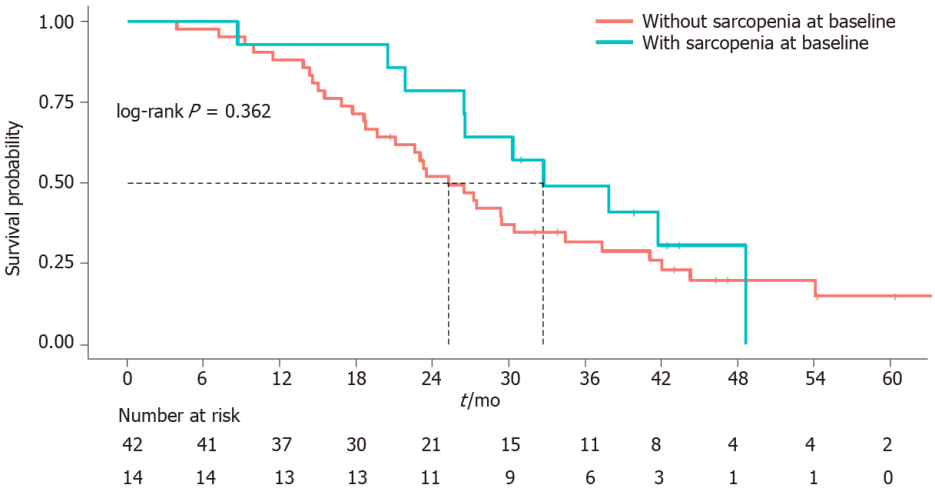


Figure 2 Overall survival according to baseline sarcopenia.

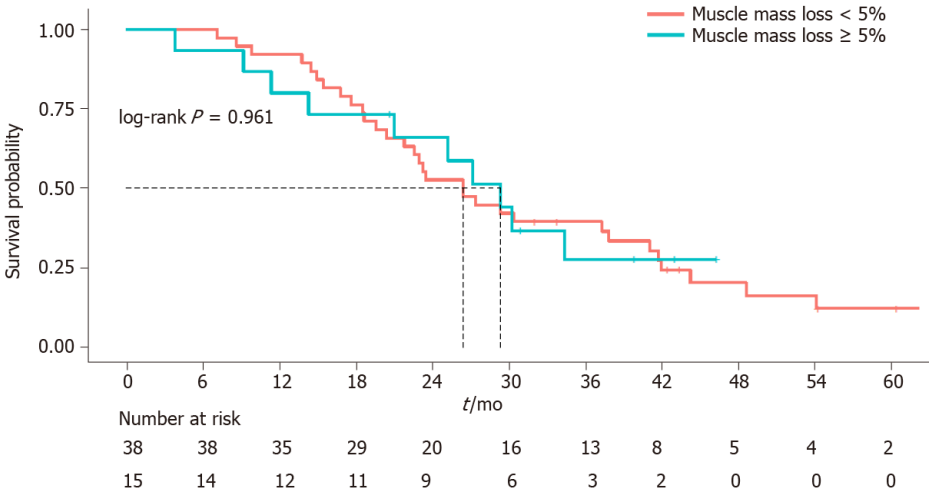


Figure 3 Overall survival according to muscle mass loss.

unlike other causes of muscle loss, sarcopenia can be associated with normal or even excessive body weight, *i.e.*, the so-called "sarcopenic obesity"[8,9]. The loss of muscle tissue can be associated with increased intramuscular fat, which results in a reduction in strength and muscle mass[40]. Martin *et al*[8] found that high weight loss, a low muscle index and low muscle attenuation due to fat infiltration, independently worsened survival in 1473 patients with lung or gastrointestinal cancer. Patients with all three of these poor prognostic variables survived 8.4 mo (95%CI: 6.5 to 10.3) regardless of BMI, in contrast to patients who had none of these features, who survived 28.4 mo (95%CI: 24.2 to 32.6; $P < 0.001$)[8]. In addition, BMI was predictive of survival, with the heaviest patients showing the longest survival. However, obese patients without any risk factorS survived 35.6 mo, which is twice longer than the median survival of the entire population (16.7 mo), while obese patients with three poor prognostic variables survived only 8.5 mo[8].

A low LBM and sarcopenia have been correlated to a worse prognosis and a worse quality of life in patients with solid tumors[6,7,13]. A meta-analysis of 38 studies, involving a total of 7843 patients, showed that subjects with a reduced SMI had a shorter OS, cancer-specific survival and disease-free survival than subjects with a normal SMI[13]. However, it included studies of various tumor types (*e.g.*, pancreaticobiliary cancer, hepatocellular carcinoma and esophageal cancer that have worse outcomes than other malignancies, such as colorectal cancer), disease stages (limited and advanced), therapeutic strategies, imaging techniques and sarcopenia cut-off values.

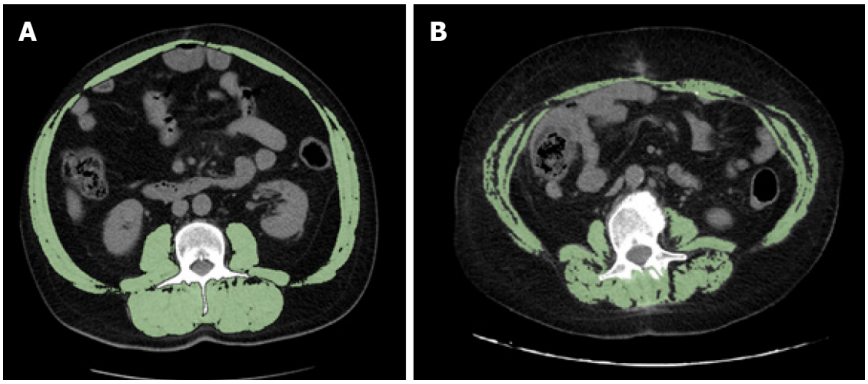


Figure 4 Representative examples of two obese patients with different skeletal mass index values. A: The patient is a 56-year-old man with body mass index (BMI) = 32.91 kg/m^2 and normal skeletal mass index (SMI) value = 62.70 cm^2/m^2; B: The patient is a 61-year-old woman showing BMI = 32.54 kg/m^2 and reduced SMI value = 39.45 cm^2/m^2.

In our patient cohort undergoing first-line chemotherapy for mCRC, sarcopenia was not related to either survival or response rate. Previous studies reported that low muscle mass was a negative prognostic factor both in resectable[37,42] and in advanced[43-45] colorectal cancer. However, those studies included patients with clinical and disease-related characteristics different to our patients. For example, Vashi *et al*[43] studied patients younger than ours (median age 53.3 years) who were at different disease stages (early and metastatic disease), some of whom had already been treated for metastatic disease. Moreover, they used cut-off values that did not consider gender or BMI in their definition of sarcopenia. Also the reports by Kurk *et al*[44] and Charette *et al*[45] were based on data derived from clinical trials designed for different endpoints, and included patients undergoing maintenance chemotherapy after the first therapeutic line or heavily pretreated patients. The latter two groups of patients have a better and worse prognosis, respectively, than our patients.

Differently, other studies did not find a correlation between basal sarcopenia and survival, but they suggested that muscle mass loss during treatment plays a negative prognostic role. For example, Miyamoto *et al*[18] analyzed 182 Asian patients with unresectable CRC. Female gender (P < 0.001) and BMI < 25 kg/m^2 (P < 0.001) were significantly associated with a lower SMI. There were no significant associations between baseline skeletal muscle mass, progression-free survival (PFS) and OS[18]. However, 22 patients with SML > 5% after first-line chemotherapy had significantly shorter PFS and OS *vs* those without SML (PFS, log-rank P = 0.029; OS, log-rank P = 0.009)[18]. Sasaki *et al*[46] found sarcopenia in 135 of 219 Asian mCRC patients (mostly male, older, with a lower BMI, lower visceral and subcutaneous fat content and a lower waist circumference than patients without sarcopenia). Baseline sarcopenia was not associated with prognosis, but SML ≥ 9% at 3 mo was associated with a high incidence of adverse events (P = 0.01), poor ORR (P < 0.01) and poor PFS (P = 0.03)[46]. Also Blauwhoff-Buskermolen *et al*[47] observed that the muscle area of 67 patients with mCRC (78% at first-line treatment and 22% at second-line treatment) decreased by 6.1% (95%CI: 28.4% to 23.8%; P < 0.001) during 3 mo of chemotherapy. Changes in muscle area were not associated with any treatment dosage modifications (dose reduction, delay or discontinuation), but patients with a muscle loss of 9% or more during treatment had significantly lower survival rates (at 6 mo, 33% *vs* 69% of patients alive; at 1 year, 17% *vs* 49% of patients alive; log-rank P = 0.001)[47].

We found no association between muscle loss during first-line treatment and survival, but it is interesting to note that a SML ≥ 5% occurred in 17 patients (30.3%); only 3 of whom were already sarcopenic at baseline CT scan, while 7 patients became sarcopenic during therapy. Chemotherapy probably induces progressive muscle damage both directly *via* a cytotoxic mechanism, and indirectly consequent to a more sedentary lifestyle because of the development of toxicity and asthenia.

In our analysis, sarcopenia was not related to higher toxicity reported during the first four cycles of chemotherapy, but 30.4% of all patients had at least one reduction in drug dosage due to toxicity, which indicates that approximately one-third of our patients did not receive an adequate drug dosage as calculated based on BSA. In this context, it is interesting to refer to the data reported by Prado *et al*[14], who examined 62 patients with stage II/III colorectal cancer receiving adjuvant treatment with 5-fluorouracil (5-FU). Exposure to 5-FU was then normalized per kilogram of LBM. In women, the 5-FU dose/kg LBM varied from 12.8 to 23 mg/kg LBM and, in men, from

12 to 20.1 mg/kg LBM[14]. Levels greater than or equal to 20 mg of 5-FU/kg of LBM were associated with an increased risk of developing dose-limiting toxicities (any grade 3/4 toxicity, dose delay or reduction) at first therapy cycle, especially in women [14]. The population analyzed in the latter study differed greatly from our patients as it included only early-stage colon cancer patients who had undergone surgery on the primary tumor and they were treated with a single adjuvant drug, administered with an obsolete schedule (5-FU and leucovorin by i.v. bolus for 5 d every 28 d); however, these data illustrate how drug exposure varies widely among patients and how this variation affects treatment tolerability.

Other studies investigated the correlation between low muscle mass and worse toxicity during chemotherapy. For instance, Ali *et al*[6] assessed data from one prospective (n = 80 patients) and one retrospective study (n = 58 patients) that included patients at different stages of CRC, treated with different therapeutic regimens with one or more drugs. They observed that a low LBM was an independent determinant of toxicity and neuropathy in patients administered a FOLFOX-based regimen (5-FU + oxaliplatin) using conventional BSA dosing[6]. Gökyer *et al*[48] evaluated 36 patients with mCRC who received regorafenib. Dose-limiting toxicity (DLT), defined as toxicity requiring dose reduction or drug withdrawal, occurred in 13 of the 23 patients (56.5%) with basal sarcopenia, whereas only 1 of the 13 patients (7.6%) without sarcopenia experienced DLT (P = 0.005)[48]. Kurk *et al*[44,49], using data of the randomized phase 3 CAIRO3 study[50], found that sarcopenia at the start of maintenance capecitabine + bevacizumab was not associated with DLT, whereas patients with > 2% SMI loss had a significantly higher risk of DLT. When capecitabine + oxaliplatin + bevacizumab was reintroduced due to disease progression, 25% of patients started the treatment at a reduced dose and most of them were patients with previous SMI loss[49]. Interestingly, after drug dose adjustment, no further DLT was observed in the subgroup of patients with SMI loss[49].

Currently, data on the prognostic and predictive role of sarcopenia are based mostly on retrospective studies or on clinical trials designed for other endpoints. Conflicting results highlight the need to investigate further the role of low muscle mass in cancer patients. Indeed, there is a need for prospective studies of more homogeneous populations in terms of age, sex, tumor histology, stage of disease, treatment setting, and mono- or polychemotherapy regimens. In the future, clinicians might evaluate the body composition of cancer patients before starting chemotherapy in order to select the drug (*e.g.* lipophilic, hydrophilic, immunotherapy or biological) with the shortest regime (for example, shortening induction therapy in favor of a weakened therapy in sarcopenic patients), the most adequate dosage, and ancillary support strategies (*e.g.* exercise, specific nutrition supplements, drugs, *etc.*).

CONCLUSION

In our study, neither baseline sarcopenia nor muscle mass loss during first-line chemotherapy influenced survival in mCRC patients. Moreover, baseline sarcopenia did not worsen treatment toxicities during first-line chemotherapy. However, these results must be interpreted with caution given the limited sample size. Further prospective studies are needed to investigate the actual role of sarcopenia in prognosis and therapeutic decision-making. Greater efforts should be made to diagnose sarcopenia upon cancer diagnosis to correct strength and muscle mass as early as possible and thus improve the patient's tolerability to treatment and survival.

ARTICLE HIGHLIGHTS

Research background

People with a low skeletal muscle mass, defined as "sarcopenia", may have a lower volume of drug distribution and reduced protein binding compared to people with a normal muscle mass thereby resulting in a higher plasma drug concentration and worse treatment toxicity. In cancer patients, sarcopenia is considered a negative prognostic factor for survival and for the development of dose-limiting chemotherapy toxicities.

Research motivation

Pharmacokinetic parameters of a given drug, such as area under the curve and

clearance, may differ substantially among patients depending on body composition. The ratio of fat to lean tissue mass could be a better tool than body surface area with which to determine the dose of cytotoxic agents as it affects metabolism, plasma concentration and the toxicity of drugs.

Research objectives

The primary end-point of this study was to assess the association between baseline sarcopenia, evaluated before starting first-line chemotherapy, and overall survival in metastatic colorectal cancer patients. The secondary end-points were to investigate: (1) the potential correlation of baseline sarcopenia with the objective response rate to first-line chemotherapy and with the development of side effects during the first four cycles of treatment; (2) the association between skeletal muscle loss (SML) at first disease reassessment and overall survival (OS); and (3) the relationship between sarcopenia and age, body mass index (BMI), disease stage at the time of first diagnosis and the neutrophil/lymphocyte ratio as an inflammation index.

Research methods

Computed tomography (CT)-scans were performed before starting chemotherapy and at the first disease reassessment. Sarcopenia was assessed using the skeletal mass index [SMI = muscle area in cm^2/(height in m^2)] calculated at the L3 vertebra. Sarcopenia was defined by Martin SMI cut-offs that combined both sex-specific and BMI cut-offs: 43 cm^2/m^2 for men with BMI < 25 kg/m^2, 53 cm^2/m^2 for men with BMI ≥ 25 kg/m^2, and 41 cm^2/m^2 for women regardless of BMI. OS and objective response rate were evaluated. Toxicities were analyzed during the first four cycles of therapy and graded according to Common Terminology Criteria for Adverse Events version 4.0. A loss of skeletal muscle mass ≥ 5% was considered indicative of deterioration in muscle condition.

Research results

The prevalence of sarcopenia was 25%: 14 of the 56 patients were sarcopenic at baseline CT and most of them (9/14, 64%) were under the age of 70 years, which indicates that it is not uncommon to find a low skeletal mass in young adults. No patient was underweight, and 8 of the 14 (57%) sarcopenic patients were overweight or obese. Sarcopenia was not correlated to overall survival (P = 0.362), to higher toxicities reported during the first 4 cycles of chemotherapy (P = 1) or to response to treatment (P = 0.221). At the first disease reassessment, a SML ≥ 5% was found in 17 patients (30.3%) 3 of whom were already sarcopenic at baseline CT scan, while 7 became sarcopenic. SML was not correlated to overall survival (P = 0.961).

Research conclusions

Although this is a negative study, our results must be interpreted with caution given the limited sample size. Moreover, the body composition of cancer patients should be evaluated before starting chemotherapy to better select the drug (*e.g.* lipophilic, hydrophilic, immunotherapy or biological) with the shortest regime (for example, shortening induction therapy in favor of a weakened therapy in sarcopenic patients), the most adequate dosage, and ancillary support strategies (*e.g.* exercise, specific nutrition supplements, drugs, *etc.*).

Research perspectives

There is a need for prospective studies of more homogeneous populations in terms of age, sex, tumor histology, stage of disease, treatment setting, and mono- or polychemotherapy regimens, to investigate the actual role of sarcopenia in prognosis and therapeutic decisions. Greater efforts should be made to diagnose sarcopenia upon cancer diagnosis in order to correct strength and muscle mass as early as possible and thus improve the patient's treatment tolerability and survival.

ACKNOWLEDGEMENTS

We thank Jean Ann Gilder (Scientific Communication Srl., Naples, Italy) for language assistance.

REFERENCES

1 **Mathijssen RH**, de Jong FA, Loos WJ, van der Bol JM, Verweij J, Sparreboom A. Flat-fixed dosing versus body surface area based dosing of anticancer drugs in adults: does it make a difference? *Oncologist* 2007; **12**: 913-923 [PMID: 17766650 DOI: 10.1634/theoncologist.12-8-913]

2 **Du Bois D**, Du Bois EF. A formula to estimate the approximate surface area if height and weight be known. 1916. *Nutrition* 1989; **5**: 303-11; discussion 312 [PMID: 2520314]

3 **Baker SD**, Verweij J, Rowinsky EK, Donehower RC, Schellens JH, Grochow LB, Sparreboom A. Role of body surface area in dosing of investigational anticancer agents in adults, 1991-2001. *J Natl Cancer Inst* 2002; **94**: 1883-1888 [PMID: 12488482 DOI: 10.1093/jnci/94.24.1883]

4 **Sawyer M**, Ratain MJ. Body surface area as a determinant of pharmacokinetics and drug dosing. *Invest New Drugs* 2001; **19**: 171-177 [PMID: 11392451 DOI: 10.1023/a:1010639201787]

5 **Gusella M**, Toso S, Ferrazzi E, Ferrari M, Padrini R. Relationships between body composition parameters and fluorouracil pharmacokinetics. *Br J Clin Pharmacol* 2002; **54**: 131-139 [PMID: 12207632 DOI: 10.1046/j.1365-2125.2002.01598.x]

6 **Ali R**, Baracos VE, Sawyer MB, Bianchi L, Roberts S, Assenat E, Mollevi C, Senesse P. Lean body mass as an independent determinant of dose-limiting toxicity and neuropathy in patients with colon cancer treated with FOLFOX regimens. *Cancer Med* 2016; **5**: 607-616 [PMID: 26814378 DOI: 10.1002/cam4.621]

7 **Prado CM**, Lima IS, Baracos VE, Bies RR, McCargar LJ, Reiman T, Mackey JR, Kuzma M, Damaraju VL, Sawyer MB. An exploratory study of body composition as a determinant of epirubicin pharmacokinetics and toxicity. *Cancer Chemother Pharmacol* 2011; **67**: 93-101 [PMID: 20204364 DOI: 10.1007/s00280-010-1288-y]

8 **Martin L**, Birdsell L, Macdonald N, Reiman T, Clandinin MT, McCargar LJ, Murphy R, Ghosh S, Sawyer MB, Baracos VE. Cancer cachexia in the age of obesity: skeletal muscle depletion is a powerful prognostic factor, independent of body mass index. *J Clin Oncol* 2013; **31**: 1539-1547 [PMID: 23530101 DOI: 10.1200/JCO.2012.45.2722]

9 **Prado CM**, Lieffers JR, McCargar LJ, Reiman T, Sawyer MB, Martin L, Baracos VE. Prevalence and clinical implications of sarcopenic obesity in patients with solid tumours of the respiratory and gastrointestinal tracts: a population-based study. *Lancet Oncol* 2008; **9**: 629-635 [PMID: 18539529 DOI: 10.1016/S1470-2045(08)70153-0]

10 **Tsai S**. Importance of lean body mass in the oncologic patient. *Nutr Clin Pract* 2012; **27**: 593-598 [PMID: 22898746 DOI: 10.1177/0884533612457949]

11 **Hopkins JJ**, Sawyer MB. A review of body composition and pharmacokinetics in oncology. *Expert Rev Clin Pharmacol* 2017; **10**: 947-956 [PMID: 28649898 DOI: 10.1080/17512433.2017.1347503]

12 **Cruz-Jentoft AJ**, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, Martin FC, Michel JP, Rolland Y, Schneider SM, Topinková E, Vandewoude M, Zamboni M; European Working Group on Sarcopenia in Older People. Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. *Age Ageing* 2010; **39**: 412-423 [PMID: 20392703 DOI: 10.1093/ageing/afq034]

13 **Shachar SS**, Williams GR, Muss HB, Nishijima TF. Prognostic value of sarcopenia in adults with solid tumours: A meta-analysis and systematic review. *Eur J Cancer* 2016; **57**: 58-67 [PMID: 26882087 DOI: 10.1016/j.ejca.2015.12.030]

14 **Prado CM**, Baracos VE, McCargar LJ, Mourtzakis M, Mulder KE, Reiman T, Butts CA, Scarfe AG, Sawyer MB. Body composition as an independent determinant of 5-fluorouracil-based chemotherapy toxicity. *Clin Cancer Res* 2007; **13**: 3264-3268 [PMID: 17545532 DOI: 10.1158/1078-0432.CCR-06-3067]

15 **Cruz-Jentoft AJ**, Bahat G, Bauer J, Boirie Y, Bruyère O, Cederholm T, Cooper C, Landi F, Rolland Y, Sayer AA, Schneider SM, Sieber CC, Topinkova E, Vandewoude M, Visser M, Zamboni M; Writing Group for the European Working Group on Sarcopenia in Older People 2 (EWGSOP2); and the Extended Group for EWGSOP2. Sarcopenia: revised European consensus on definition and diagnosis. *Age Ageing* 2019; **48**: 16-31 [PMID: 30312372 DOI: 10.1093/ageing/afy169]

16 **Mourtzakis M**, Prado CM, Lieffers JR, Reiman T, McCargar LJ, Baracos VE. A practical and precise approach to quantification of body composition in cancer patients using computed tomography images acquired during routine care. *Appl Physiol Nutr Metab* 2008; **33**: 997-1006 [PMID: 18923576 DOI: 10.1139/H08-075]

17 **Shen W**, Punyanitya M, Wang Z, Gallagher D, St-Onge MP, Albu J, Heymsfield SB, Heshka S. Total body skeletal muscle and adipose tissue volumes: estimation from a single abdominal cross-sectional image. *J Appl Physiol (1985)* 2004; **97**: 2333-2338 [PMID: 15310748 DOI: 10.1152/japplphysiol.00744.2004]

18 **Miyamoto Y**, Baba Y, Sakamoto Y, Ohuchi M, Tokunaga R, Kurashige J, Hiyoshi Y, Iwagami S, Yoshida N, Watanabe M, Baba H. Negative Impact of Skeletal Muscle Loss after Systemic Chemotherapy in Patients with Unresectable Colorectal Cancer. *PLoS One* 2015; **10**: e0129742 [PMID: 26069972 DOI: 10.1371/journal.pone.0129742]

19 **Feliciano EMC**, Kroenke CH, Meyerhardt JA, Prado CM, Bradshaw PT, Kwan ML, Xiao J, Alexeeff S, Corley D, Weltzien E, Castillo AL, Caan BJ. Association of Systemic Inflammation and Sarcopenia With Survival in Nonmetastatic Colorectal Cancer: Results From the C SCANS Study. *JAMA Oncol* 2017; **3**: e172319 [PMID: 28796857 DOI: 10.1001/jamaoncol.2017.2319]

20 **Rier HN**, Jager A, Sleijfer S, Maier AB, Levin MD. The Prevalence and Prognostic Value of Low

Muscle Mass in Cancer Patients: A Review of the Literature. *Oncologist* 2016; **21**: 1396-1409 [PMID: 27412391 DOI: 10.1634/theoncologist.2016-0066]

21 **Argilés JM**, Busquets S, Felipe A, López-Soriano FJ. Molecular mechanisms involved in muscle wasting in cancer and ageing: cachexia versus sarcopenia. *Int J Biochem Cell Biol* 2005; **37**: 1084-1104 [PMID: 15743680 DOI: 10.1016/j.biocel.2004.10.003]

22 **Muscaritoli M**, Lucia S, Molfino A, Cederholm T, Rossi Fanelli F. Muscle atrophy in aging and chronic diseases: is it sarcopenia or cachexia? *Intern Emerg Med* 2013; **8**: 553-560 [PMID: 22773188 DOI: 10.1007/s11739-012-0807-8]

23 **Jeejeebhoy KN**. Malnutrition, fatigue, frailty, vulnerability, sarcopenia and cachexia: overlap of clinical features. *Curr Opin Clin Nutr Metab Care* 2012; **15**: 213-219 [PMID: 22450775 DOI: 10.1097/MCO.0b013e328352694f]

24 **Bennani-Baiti N**, Davis MP. Cytokines and cancer anorexia cachexia syndrome. *Am J Hosp Palliat Care* 2008; **25**: 407-411 [PMID: 18403577 DOI: 10.1177/1049909108315518]

25 **Chan MY**, Chok KSH. Sarcopenia in pancreatic cancer - effects on surgical outcomes and chemotherapy. *World J Gastrointest Oncol* 2019; **11**: 527-537 [PMID: 31367272 DOI: 10.4251/wjgo.v11.i7.527]

26 **Tan BH**, Birdsell LA, Martin L, Baracos VE, Fearon KC. Sarcopenia in an overweight or obese patient is an adverse prognostic factor in pancreatic cancer. *Clin Cancer Res* 2009; **15**: 6973-6979 [PMID: 19887488 DOI: 10.1158/1078-0432.CCR-09-1525]

27 **Zhang XM**, Dou QL, Zeng Y, Yang Y, Cheng ASK, Zhang WW. Sarcopenia as a predictor of mortality in women with breast cancer: a meta-analysis and systematic review. *BMC Cancer* 2020; **20**: 172 [PMID: 32131764 DOI: 10.1186/s12885-020-6645-6]

28 **Prado CM**, Baracos VE, McCargar LJ, Reiman T, Mourtzakis M, Tonkin K, Mackey JR, Koski S, Pituskin E, Sawyer MB. Sarcopenia as a determinant of chemotherapy toxicity and time to tumor progression in metastatic breast cancer patients receiving capecitabine treatment. *Clin Cancer Res* 2009; **15**: 2920-2926 [PMID: 19351764 DOI: 10.1158/1078-0432.CCR-08-2242]

29 **Rinninella E**, Cintoni M, Raoul P, Pozzo C, Strippoli A, Bria E, Tortora G, Gasbarrini A, Mele MC. Muscle mass, assessed at diagnosis by L3-CT scan as a prognostic marker of clinical outcomes in patients with gastric cancer: A systematic review and meta-analysis. *Clin Nutr* 2020; **39**: 2045-2054 [PMID: 31718876 DOI: 10.1016/j.clnu.2019.10.021]

30 **Lee JS**, Kim YS, Kim EY, Jin W. Prognostic significance of CT-determined sarcopenia in patients with advanced gastric cancer. *PLoS One* 2018; **13**: e0202700 [PMID: 30125312 DOI: 10.1371/journal.pone.0202700]

31 **Sugiyama K**, Narita Y, Mitani S, Honda K, Masuishi T, Taniguchi H, Kadowaki S, Ura T, Ando M, Tajika M, Muro K. Baseline Sarcopenia and Skeletal Muscle Loss During Chemotherapy Affect Survival Outcomes in Metastatic Gastric Cancer. *Anticancer Res* 2018; **38**: 5859-5866 [PMID: 30275211 DOI: 10.21873/anticanres.12928]

32 **Mir O**, Coriat R, Blanchet B, Durand JP, Boudou-Rouquette P, Michels J, Ropert S, Vidal M, Pol S, Chaussade S, Goldwasser F. Sarcopenia predicts early dose-limiting toxicities and pharmacokinetics of sorafenib in patients with hepatocellular carcinoma. *PLoS One* 2012; **7**: e37563 [PMID: 22666367 DOI: 10.1371/journal.pone.0037563]

33 **Antoun S**, Baracos VE, Birdsell L, Escudier B, Sawyer MB. Low body mass index and sarcopenia associated with dose-limiting toxicity of sorafenib in patients with renal cell carcinoma. *Ann Oncol* 2010; **21**: 1594-1598 [PMID: 20089558 DOI: 10.1093/annonc/mdp605]

34 **Huillard O**, Mir O, Peyromaure M, Tlemsani C, Giroux J, Boudou-Rouquette P, Ropert S, Delongchamps NB, Zerbib M, Goldwasser F. Sarcopenia and body mass index predict sunitinib-induced early dose-limiting toxicities in renal cancer patients. *Br J Cancer* 2013; **108**: 1034-1041 [PMID: 23462722 DOI: 10.1038/bjc.2013.58]

35 **van Vledder MG**, Levolger S, Ayez N, Verhoef C, Tran TC, Ijzermans JN. Body composition and outcome in patients undergoing resection of colorectal liver metastases. *Br J Surg* 2012; **99**: 550-557 [PMID: 22246799 DOI: 10.1002/bjs.7823]

36 **Thoresen L**, Frykholm G, Lydersen S, Ulveland H, Baracos V, Prado CM, Birdsell L, Falkmer U. Nutritional status, cachexia and survival in patients with advanced colorectal carcinoma. Different assessment criteria for nutritional status provide unequal results. *Clin Nutr* 2013; **32**: 65-72 [PMID: 22695408 DOI: 10.1016/j.clnu.2012.05.009]

37 **Miyamoto Y**, Baba Y, Sakamoto Y, Ohuchi M, Tokunaga R, Kurashige J, Hiyoshi Y, Iwagami S, Yoshida N, Yoshida M, Watanabe M, Baba H. Sarcopenia is a Negative Prognostic Factor After Curative Resection of Colorectal Cancer. *Ann Surg Oncol* 2015; **22**: 2663-2668 [PMID: 25564158 DOI: 10.1245/s10434-014-4281-6]

38 **Maddocks M**, Murton AJ, Wilcock A. Improving muscle mass and function in cachexia: non-drug approaches. *Curr Opin Support Palliat Care* 2011; **5**: 361-364 [PMID: 21934503 DOI: 10.1097/SPC.0b013e32834bdde3]

39 **Lenk K**, Schuler G, Adams V. Skeletal muscle wasting in cachexia and sarcopenia: molecular pathophysiology and impact of exercise training. *J Cachexia Sarcopenia Muscle* 2010; **1**: 9-21 [PMID: 21475693 DOI: 10.1007/s13539-010-0007-1]

40 **Cruz-Jentoft AJ**, Dawson Hughes B, Scott D, Sanders KM, Rizzoli R. Nutritional strategies for maintaining muscle mass and strength from middle age to later life: A narrative review. *Maturitas* 2020; **132**: 57-64 [PMID: 31883664 DOI: 10.1016/j.maturitas.2019.11.007]

41 **Di Girolamo FG**, Situlin R, Mazzucco S, Valentini R, Toigo G, Biolo G. Omega-3 fatty acids and

protein metabolism: enhancement of anabolic interventions for sarcopenia. *Curr Opin Clin Nutr Metab Care* 2014; **17**: 145-150 [PMID: 24500439 DOI: 10.1097/MCO.0000000000000032]

42 **van Vugt JLA**, Coebergh van den Braak RRJ, Lalmahomed ZS, Vrijland WW, Dekker JWT, Zimmerman DDE, Vles WJ, Coene PLO, IJzermans JNM. Impact of low skeletal muscle mass and density on short and long-term outcome after resection of stage I-III colorectal cancer. *Eur J Surg Oncol* 2018; **44**: 1354-1360 [PMID: 29914788 DOI: 10.1016/j.ejso.2018.05.029]

43 **Vashi PG**, Gorsuch K, Wan L, Hill D, Block C, Gupta D. Sarcopenia supersedes subjective global assessment as a predictor of survival in colorectal cancer. *PLoS One* 2019; **14**: e0218761 [PMID: 31220163 DOI: 10.1371/journal.pone.0218761]

44 **Kurk SA**, Peeters PHM, Dorresteijn B, de Jong PA, Jourdan M, Creemers GM, Erdkamp FLG, de Jongh FE, Kint PAM, Poppema BJ, Radema SA, Simkens LHJ, Tanis BC, Tjin-A-Ton MLR, Van Der Velden A, Punt CJA, Koopman M, May AM. Loss of skeletal muscle index and survival in patients with metastatic colorectal cancer: Secondary analysis of the phase 3 CAIRO3 trial. *Cancer Med* 2020; **9**: 1033-1043 [PMID: 31850687 DOI: 10.1002/cam4.2787]

45 **Charette N**, Vandeputte C, Ameye L, Bogaert CV, Krygier J, Guiot T, Deleporte A, Delaunoit T, Geboes K, Van Laethem JL, Peeters M, Demolin G, Holbrechts S, Flamen P, Paesmans M, Hendlisz A. Prognostic value of adipose tissue and muscle mass in advanced colorectal cancer: a post hoc analysis of two non-randomized phase II trials. *BMC Cancer* 2019; **19**: 134 [PMID: 30744591 DOI: 10.1186/s12885-019-5319-8]

46 **Sasaki S**, Oki E, Saeki H, Shimose T, Sakamoto S, Hu Q, Kudo K, Tsuda Y, Nakashima Y, Ando K, Akagi Y, Kakeji Y, Baba H, Maehara Y. Skeletal muscle loss during systemic chemotherapy for colorectal cancer indicates treatment response: a pooled analysis of a multicenter clinical trial (KSCC 1605-A). *Int J Clin Oncol* 2019; **24**: 1204-1213 [PMID: 31062115 DOI: 10.1007/s10147-019-01460-8]

47 **Blauwhoff-Buskermolen S**, Versteeg KS, de van der Schueren MA, den Braver NR, Berkhof J, Langius JA, Verheul HM. Loss of Muscle Mass During Chemotherapy Is Predictive for Poor Survival of Patients With Metastatic Colorectal Cancer. *J Clin Oncol* 2016; **34**: 1339-1344 [PMID: 26903572 DOI: 10.1200/JCO.2015.63.6043]

48 **Gökyer A**, Küçükarda A, Köstek O, Hacıoğlu MB, Sunal BS, Demircan NC, Uzunoğlu S, Solak S, İşsever K, Çiçin I, Erdoğan B. Relation between sarcopenia and dose-limiting toxicity in patients with metastatic colorectal cancer who received regorafenib. *Clin Transl Oncol* 2019; **21**: 1518-1523 [PMID: 30868388 DOI: 10.1007/s12094-019-02080-4]

49 **Kurk S**, Peeters P, Stellato R, Dorresteijn B, de Jong P, Jourdan M, Creemers GJ, Erdkamp F, de Jongh F, Kint P, Simkens L, Tanis B, Tjin-A-Ton M, Van Der Velden A, Punt C, Koopman M, May A. Skeletal muscle mass loss and dose-limiting toxicities in metastatic colorectal cancer patients. *J Cachexia Sarcopenia Muscle* 2019; **10**: 803-813 [PMID: 31094083 DOI: 10.1002/jcsm.12436]

50 **Simkens LH**, van Tinteren H, May A, ten Tije AJ, Creemers GJ, Loosveld OJ, de Jongh FE, Erdkamp FL, Erjavec Z, van der Torren AM, Tol J, Braun HJ, Nieboer P, van der Hoeven JJ, Haasjes JG, Jansen RL, Wals J, Cats A, Derleyn VA, Honkoop AH, Mol L, Punt CJ, Koopman M. Maintenance treatment with capecitabine and bevacizumab in metastatic colorectal cancer (CAIRO3): a phase 3 randomised controlled trial of the Dutch Colorectal Cancer Group. *Lancet* 2015; **385**: 1843-1852 [PMID: 25862517 DOI: 10.1016/S0140-6736(14)62004-3]

World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2021 May 24; 12(5): 367-381

DOI: 10.5306/wjco.v12.i5.367

ISSN 2218-4333 (online)

SYSTEMATIC REVIEWS

Reduction of muscle contraction and pain in electroporation-based treatments: An overview

Roberta Fusco, Elio Di Bernardo, Valeria D'Alessio, Simona Salati, Matteo Cadossi

ORCID number: Roberta Fusco 0000-0002-0469-9969; Elio Di Bernardo 0000-0001-5850-1391; Valeria D'Alessio 0000-0003-2950-4168; Simona Salati 0000-0002-8072-1128; Matteo Cadossi 0000-0002-0671-4165.

Author contributions: Fusco R, Di Bernardo E, D'Alessio V wrote the manuscript; Salati S and Cadossi M revised the manuscript.

Conflict-of-interest statement: The authors declare that they have no competing interests.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Roberta Fusco, Elio Di Bernardo, Valeria D'Alessio, Simona Salati, Matteo Cadossi, Department of Medical Oncology, IGEA SpA, Carpi 41012, Modena, Italy

Corresponding author: Valeria D'Alessio, Department of Medical Oncology, IGEA SpA, Via Parmenide 10A, Carpi 41012, Modena, Italy. v.dalessio@igeamedical.com

Abstract

BACKGROUND

In the first studies of electrochemotherapy (ECT), small cutaneous metastases were treated and only mild or moderate pain was observed; therefore, pain was not considered a significant issue. As the procedure began to be applied to larger cutaneous metastases, pain was reported more frequently. For that reason, reduction of both muscle contractions and pain have been investigated over the years.

AIM

To present an overview of different protocols described in literature that aim to reduce muscle contractions and pain caused by the electroporation (EP) effect in both ECT and irreversible EP treatments.

METHODS

Thirty-three studies published between January 1999 and November 2020 were included. Different protocol designs and electrode geometries that reduce patient pain and the number of muscle contractions and their intensity were analysed.

RESULTS

The analysis showed that both high frequency and bipolar/biphasic pulses can be used to reduce pain and muscle contractions in patients who undergo EP treatments. Moreover, adequate electrode design can decrease EP-related morbidity. Particularly, needle length, diameter and configuration of the distance between the needles can be optimised so that the muscle volume crossed by the current is reduced as much as possible. Bipolar/biphasic pulses with an inadequate pulse length seem to have a less evident effect on the membrane permeability compared with the standard pulse protocol. For that reason, the number of pulses and the voltage amplitude, as well as the pulse duration and frequency, must be chosen so that the dose of delivered energy guarantees EP efficacy.

CONCLUSION

Pain reduction in EP-based treatments can be achieved by appropriately defining

Manuscript source: Invited manuscript

Specialty type: Oncology

Country/Territory of origin: Italy

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Received: December 23, 2020
Peer-review started: December 24, 2020
First decision: March 17, 2021
Revised: March 17, 2021
Accepted: April 22, 2021
Article in press: April 22, 2021
Published online: May 24, 2021

P-Reviewer: Aureliano M, Gadzijev EM
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Yuan YY

the protocol parameters and electrode design. Most results can be achieved with high frequency and/or bipolar/biphasic pulses. However, the efficacy of these alternative protocols remains a crucial point to be assessed further.

Key Words: Electrochemotherapy; Irreversible electroporation; Pain; Muscle contraction; Monopolar or monophasic pulses; Bipolar or biphasic pulses

Core Tip: This is an overview of different published protocols that aim to reduce muscle contractions and pain due to the electroporation (EP) effect. The analysis showed that both high frequency and bipolar/biphasic pulses can be used to reduce pain and muscle contractions. Moreover, appropriate electrode design can lower EP-related morbidity.

Citation: Fusco R, Di Bernardo E, D'Alessio V, Salati S, Cadossi M. Reduction of muscle contraction and pain in electroporation-based treatments: An overview. *World J Clin Oncol* 2021; 12(5): 367-381
URL: https://www.wjgnet.com/2218-4333/full/v12/i5/367.htm
DOI: https://dx.doi.org/10.5306/wjco.v12.i5.367

INTRODUCTION

Electrochemotherapy (ECT) is a locoregional anti-tumour therapy that combines a low dose of a chemotherapy drug with high-intensity electric pulses to induce cell membrane electroporation (EP). Consequently, the drugs enter the tumour cells and exert their cytotoxicity[1-4]. Unlike other antitumour treatments based on physical phenomena, ECT is able to exert a specific effect at the cellular level, causing the death of the treated tumour cells. Because it is highly effective in treating cutaneous and subcutaneous tumours regardless of histology[5-7], ECT treatment has been extended to more deeply located tumours[8-13].

To increase the efficacy of EP treatment, the voltage amplitude and the duration or the number of electric pulses are often increased, as long as the required current does not exceed the limit set by the pulse generator. The standard operating procedures[6] for ECT define the electric protocol that, combined with intra-tumour or intravenous delivery of bleomycin or cisplatin[14-19], guarantees an adequate efficacy of the therapy: A train of eight high voltage 100 μs monopolar electric pulses with a repetition frequency of either 1 Hz or 5 kHz is often used. However, the application of high voltage monopolar pulses may cause pain and muscle contractions[20]. For that reason, the use of muscle relaxants and general anaesthesia[21-23] are often required.

In the first studies on ECT, small cutaneous metastases were treated with the observation of only mild or moderate pain was[24-26]; therefore, pain was not considered a significant issue[6,7]. Subsequently, as the procedure started to be applied to larger cutaneous metastases, pain was reported more frequently[27,28]. For that reason, reduction of both muscle contractions and pain have been investigated over the years. The main improvements were achieved by applying pulses at a higher frequency[29-34] or by using special electrode designs.

Repetition frequency of electric pulses has a close relationship with muscle contraction, which leads to a painful burning sensation and patient complaints[19,20]. An increase in repetition frequency by reducing the pulse-to-pulse pause, seems to reduce unpleasant sensations that occur during ECT[24,35-42]. Moreover, many authors reported that electric pulses lasting microseconds at a high repetition frequency do not decrease ECT antitumour efficacy[35,38]. However, although the pulse frequency is related to muscle contraction, the pain sensation also depends on other pulse characteristics such as voltage amplitude and pulse number, duration and shape[36]. The electrodes used for ECT treatment can also affect the onset of pain. Particularly, needle length, diameter and configuration that changes the distance between needles can be optimised so that the muscle volume crossed by the current is reduced as much as possible. Electrodes with a smaller distance between needles are

less painful because they require lower voltages. However, they only treat small portions of tissue, and thus must be applied multiple times to cover the entire lesion.

More recently, it has been demonstrated that the use of high frequency irreversible electroporation (H-FIRE)[28-33], namely bursts of short high frequency bipolar pulses, can further reduce muscle contraction and the subsequent pain caused by the electric pulses. Treatment with H-FIRE pulses, however, may require an electric field intensity higher than the standard electric protocol, both for ECT and for irreversible electroporation (IRE), to reach an equivalent treatment efficacy. An additional disadvantage is delivering pulses at considerably higher voltage amplitudes[34].

The aim of this review is to present an overview of the different protocols proposed in the literature to reduce muscle contractions and pain caused by the EP effect in both ECT and IRE treatments. The main findings of a number of researchers are reported in the results section. The impact of different electrode designs is also considered, as the reduction of muscle contraction and patient morbidity can also be obtained with an appropriate electrode design.

MATERIALS AND METHODS

This review is the result of a self-study without protocol or a registration number. In order to ensure an adequate variety of the assessed studies, several electronic databases were searched: PubMed (United States National Library of Medicine, http://www.ncbi.nlm.nih.gov/PubMed); Scopus (Elsevier, http://www.scopus.com/); the Web of Science (Thomson Reuters, http://apps.webofknowledge.com/); and Google Scholar (https://scholar.goo-gle.it/). Only studies published between January 1999 and November 2020 were analysed because that time window is consistent with recent developments in the fields of ECT and IRE. Papers not indexed in the electronic databases were evaluated through the references of included studies. The systematic search for papers of interest is shown in the flow chart in Figure 1. The inclusion criteria evaluated the article title, abstract and contents and included pre-clinical and clinical studies that examined pain or muscle contractions caused by reversible or IRE treatments. Only articles written in English were included. Studies with insufficient reported data, case reports, reviews or letters to the editor were excluded Four investigators carried out data extraction from the included papers, focusing on the type of study (*i.e.* numerical analysis, *in vitro*, *in vivo* or *ex vivo*), the type of EP (ECT or IRE), the pulse characteristics and the main results regarding reduction of muscle contraction and pain.

RESULTS

A considerable number of protocols that aim at reducing muscle contractions and pain, caused by the EP effect were found in the literature. The research was conducted with the aim of identifying the parameters that are most responsible for the perception of pain and the stimulation of muscle contraction in patients undergoing EP treatment. Thirty-three studies published between January 1999 and November 2020 were retrieved and papers not indexed in the electronic databases were identified in their reference lists. As *per* the approach described in Figure 1, 15 studies did not meet the abstract inclusion criteria and were therefore rejected. Five papers were found to be case reports, reviews or editorial letters, did not satisfy the inclusion criteria and were not included in the analysis. The remaining thirteen articles[29,30,33,34,37-45] were included in this manuscript, as they met all the required criteria (Figure 2). Four papers described treatment of cutaneous and subcutaneous tumours[37,40,41,44], two papers included sarcomas[38,42] and pancreatic tumours[43,44] and six were conducted in healthy subjects or phantoms[29,30,33,34,39,45].

Reduction of muscle contraction and pain: ECT protocols

In a study published in 1999, Daskalov *et al*[36] compared monophasic and biphasic pulses *in vivo*. The monophasic pulse protocol consisted of eight exponentially or rectangular-shaped pulses of 100 μs with a frequency of 1 Hz. In the biphasic pulse protocol, a rectangular pulse of 50 + 50 μs without intra-pulse delay was used in two different ways: (1) Eight pulses with a 1 s interval; and (2) A single burst of eight pulses spaced at 1 ms, with a total duration of 7.1 ms. In both protocols, the selected pulse amplitude ranged from 750 V to 1250 V, depending on the tumour size, with a

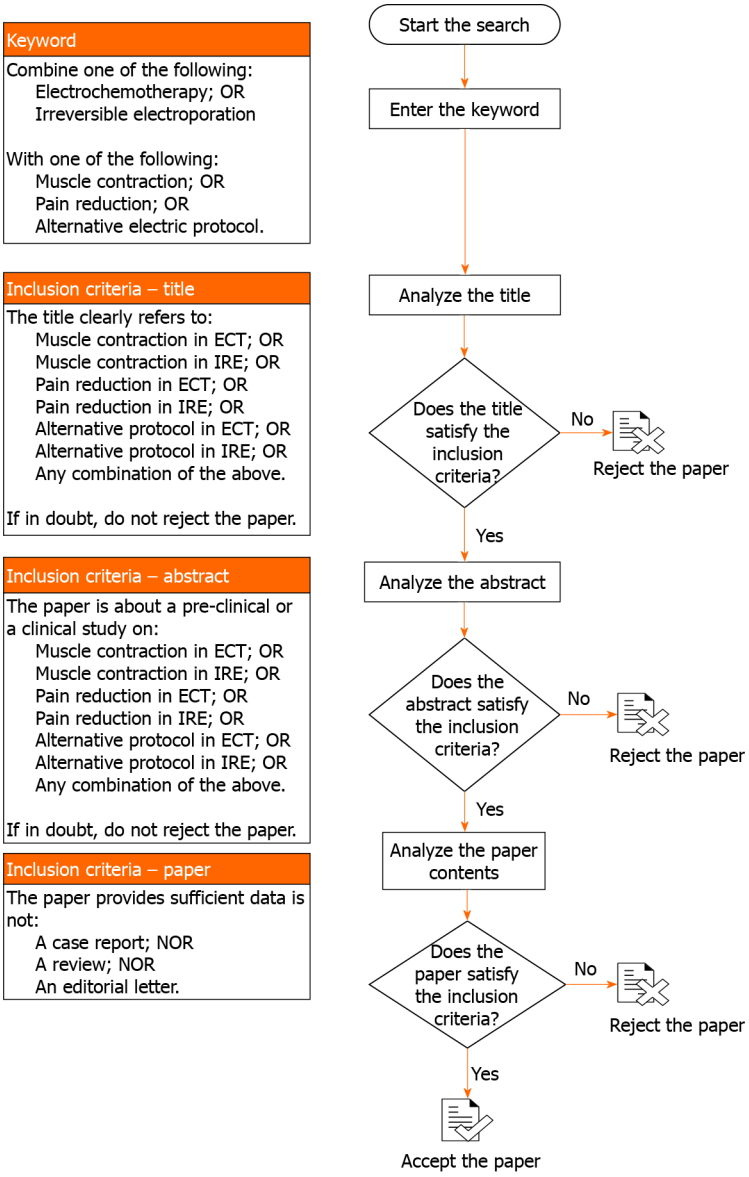


Figure 1 Systematic search of relevant literature. ECT: Electrochemotherapy; IRE: Irreversible electroporation.

resulting electrical field strength varying between 330 V/cm and 1250 V/cm. The study showed that the two pulse protocols (monophasic or biphasic) provided the same effect in terms of treatment result. However, the biphasic pulses were better tolerated by the patients. Particularly, the second biphasic mode, a single burst of eight pulses, was considered more acceptable than the first, which comprised eight separate stimuli. Thus, applying the pulses in a rapid sequence was as effective as the use of a larger inter-pulse interval and was better tolerated by the patients. The results of this study[36] were also confirmed by Melzack[46] who previously noted that increasing the number, (*N*) of applied pulses led to a better effect compared with increasing the pulse duration (*T*), provided that $N \times T$ was constant. In a later study, Miklavcic *et al* [37] demonstrated that pulse frequencies above the frequency of tetanic contraction (100 Hz) gradually reduced the number of individual muscle contractions. They identified muscle contractions associated with high voltage pulses as the main source of pain for patients undergoing ECT. When the pulse frequency was relatively low, the patient experienced separate muscle contractions associated with each delivered pulse. For that reason, the authors investigated the relationship between muscle contraction and pulse characteristics; particularly, repetition frequency and pulse amplitude. A train of eight 100 μs rectangular pulses at either low or high voltage amplitudes was used. At a low voltage (70 V), the measurements were performed at pulse repetition frequencies of 1, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 Hz. At a high voltage (250 V), the measurements were performed at 1, 100, 500, 1000 and 5000 Hz. To investigate the effect of both frequency and amplitude on muscle contraction, they measured the

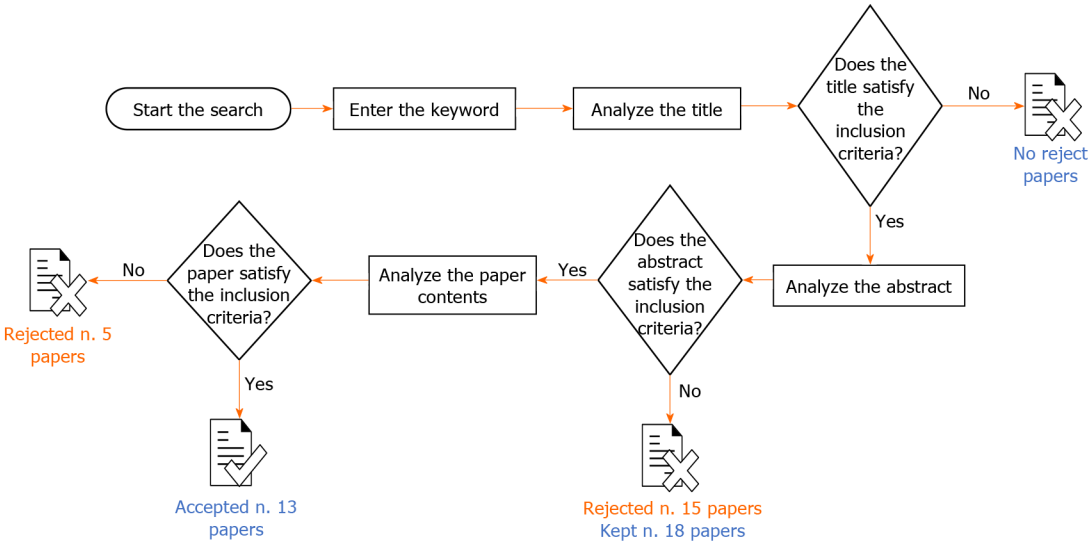


Figure 2 Included and excluded studies in the systematic review.

muscle torque in response to electric pulses. They also studied the antitumour efficacy of ECT at different pulse repetition frequencies to be sure that the pain reduction did not lead to a loss of treatment efficacy. Measurements of muscle torque confirmed that high frequency pulses reduced the number of individual contractions to a single muscle contraction. More precisely, with increasing pulse frequency, muscle torque increased up to the frequency of 100 or 200 Hz, reaching a maximum value of 0.16-0.24 nm; however, a further increase of frequency above 200 Hz reduced the muscle torque regardless of the pulse amplitude, with a mean value of about 0.07 nm at 5 kHz, a value similar to that observed during the application of 1 Hz pulse trains. Moreover, by increasing the frequency of electric pulses above the frequency of tetanic contraction (*i.e.* at pulse frequencies higher than 2000 Hz), the authors reported that, even if the muscle torque was similar to that observed in a typical ECT protocol (1 Hz pulse trains), the patients perceived only one muscle contraction instead of eight. Finally, *in vivo* experiments also demonstrated that ECT achieved similar efficacies regardless of the pulse frequency that was used (1 Hz-5 kHz), which suggest that there is a considerable potential for the clinical use of high frequency pulses in ECT.

The relationship of pulse frequency and muscle contraction and subsequent patient pain, was successively studied by Zupanic *et al*[38]. A train of eight electric pulses, of 1 Hz and 5 kHz repetition frequencies, was delivered to 40 healthy patients. After the conclusion of each protocol, the subjects completed the short-form McGill Pain Questionnaire[47] with separate visual analogue scales for pain intensity and unpleasantness. Their results confirmed what Miklavcic *et al*[37] had previously demonstrated, by finding that muscle contractions, which contribute to the discomfort felt by the subjects during the delivery of electric pulses, are strictly related to pulse frequency. When evaluating the sensorial and affective quality of pain (in the short-form McGill Pain Questionnaire, the most frequently selected pain descriptors were stabbing (80%), cramping (57.5%), throbbing (60%), shooting (60%) and hot-burning (53.8%). However, while both protocols of EP received similar average intensity scores for most descriptors (1.4 for stabbing, 1.0 for cramping, 1.1 for throbbing, 1.1-0.9 for shooting and 1.0-0.7 for hot-burning), treatment with 5 kHz electric pulses was less unpleasant. Therefore, the latter (P = 0.017) was preferred over the standard 1 Hz pulses, even though the perceived pain intensity, ranging from 6 mm to 94 mm, with similar visual analogic scores, was almost the same regardless of the frequency.

In a 2014 study, Spugnini *et al*[39] analysed the effects of biphasic pulse length on both treatment efficacy and morbidity. The authors investigated two different protocols of trains of eight biphasic pulses, at a voltage of 1300 V/cm. The standard protocol consisted of pulses lasting 50 + 50 ms each, with a frequency of 1 Hz and with 1 ms intra- and inter-pulse intervals. The investigational protocol consisted of pulses lasting 50 + 50 μs each, with a frequency of 1 Hz and with 10 μs intra- and inter-pulse intervals. The protocols were tested both *in vitro* (human lung cancer cell line A549) and *in vivo* (mice xenografts; privately owned rabbits with spontaneous tumours). Three of the mice treated with the standard protocol had a strong (grade 4) muscular contraction and the other four had a moderate (grade 3) muscular contraction. Mice

treated with the investigational protocol had muscular contractions reported as flicker (grade 1, two mice), weak (grade 2, four mice) and moderate (one mouse). The rabbits treated with the investigational protocol had muscular contractions graded as flicker (two rabbits), weak (three rabbits) and moderate (one rabbit). Given the results obtained from the study, it was concluded that the investigational protocol substantially reduced the morbidity associated with the delivery of electric pulses and achieved a significantly higher efficacy compared with the standard protocol.

In a more recent *in vitro* study in mouse skin melanoma (B16-F1) cells, Scuderi *et al* [40] delivered the electric pulses after adding 1–330 μM cisplatin. Two pulse protocols were evaluated: (1) Eight 100 μs monopolar pulses, 0.4–1.2 kV/cm, 1 Hz (standard ECT protocol); and (2) Eight bursts at 1 Hz, consisting of 50 bipolar pulses with 1 + 1 μs width, 0.5–5 kV/cm, 1 μs intra-pulse delay [high frequency EP (HF-EP)]. The analysis of the results was conducted by evaluating the difference between the two protocols (monopolar or bipolar pulses), focusing on their effect on both the efficacy of the treatment and the associated cytotoxicity. First, the results showed that both monopolar and bipolar pulse protocols, in combination with cisplatin, achieved the desired efficacy in killing cells. However, as the onset of membrane permeabilisation was higher in the HF-EP (2 kV/cm) protocol than in the ECT (0.8 kV/cm, $P = 0.036$) protocol, the bipolar pulse protocol needed a higher electric field (2 kV/cm, $P < 0.001$ *vs* 1.2 kV/cm, $P < 0.001$). Second, the results obtained suggest that HF-EP could be used in ECT with potential alleviation of muscle contractions and pain. In fact, even if the pain was not evaluated, it has been previously demonstrated that a short negative pulse delivered after a positive pulse accelerates passive repolarisation that abolishes the action potential. That means that fewer muscle contractions, and thus less pain, can be expected with HF-EP than with the classic 100 μs pulses. As the authors themselves concluded, although it is still at the *in vitro* testing stage, the clinical use of HF-EP pulses for ECT could potentially decrease the discomfort associated with muscle contractions and pain, and simplify the treatment procedure by lowering the dose of muscle relaxants and anaesthesia and avoiding synchronisation with the electrocardiogram.

Finally, in 2020, García-Sánchez *et al*[41] assessed the ability of sine waves to perform ECT. They compared the classic ECT protocol (eight squared unipolar pulses of 100 μs and 1 Hz repetition frequency, electric field of 1300 V/cm) with both bipolar square pulses and sinusoidal bursts. The analysed protocols (bipolar and sinusoidal) were made for pulses with no intra- nor inter-pulse pauses. The bursts were delivered at various frequencies between 10 and 100 kHz and with electric fields of at least 1300 V/cm, and the duration and number of pulses varied depending on the experiment. The authors also carried out a computer simulation to calculate the electric field distribution and the temperature increase during the delivery of the treatment. Furthermore, verification of the effectiveness of the treatment was essential in the comparison between the different protocols, which was taken into account by considering the tumour response. Specifically, the efficacy of the treatment was assessed by comparing sinusoidal bursts at three frequencies (10, 50 and 100 kHz) and two electric field intensities (1300 and 1600 V/cm). Their results showed that sinusoidal pulses reduced both the extent of muscle contractions and skin damage. The effects were significantly lower when a high frequency wave was applied and when the square bipolar pulse was used. However, there was a clear loss of efficacy with the increase in frequency, confirming that the external electric field should be increased to 1600 V/cm in to achieve an equivalent EP effect, thus allowing for a tumour volume growth of less than 200 mm^2 within a 25 d follow-up period.

Reduction of muscle contraction and pain: Numerical analysis

Golberg and Rubinsky[28] performed a numerical analysis to evaluate the influence of the electrode geometry in the reduction of pain and muscle contractions. The numerical analysis considered various electrode configurations. For each experimental setup, a single pulse of 400 V and 100 μs was delivered. The results showed that conventional EP protocols and electrode design could generate muscle contraction, inducing electric fields in surprisingly large volumes of non-target tissue surrounding the EP-treated tissue. They also found that electrode placement in a structure referred to as a "current cage" substantially reduced the volume of non-target tissue exposed to electric fields above the threshold of muscle contraction. Furthermore, in an experimental study using a tissue phantom, they compared a commercial two parallel needle EP system with the current cage design. They found that a certain arrangement of needle electrodes limited the amount of tissue exposed to electric fields that above the muscle contraction threshold, while having a minimal impact on the extent of EP. The design consisted of a central, energised electrode surrounded by an array of grounded

electrodes. Similar geometries have been used successfully for cardiac defibrillation and ECT. Interestingly, by having 16 or more grounded electrodes and by reducing the insertion depth of the central energised electrode relative to the grounded electrodes, the predicted amount of tissue experiencing muscle contractions fell dramatically. In fact, the analysis revealed that the ratio of the volume affected by the muscle contraction (V_{mc}) and that affected by the EP phenomenon (V_{ep}) using a commercial parallel eight-electrode array, was 135 and was 410, with an electric field of than 600 V/cm and 1120 V/cm. The corresponding ratios were 73 and 26 when the 26-electrode current cage was used. Moreover, the total V_{mc} was 15.09 mm^2 when the commercial parallel eight-electrode array was used, compared with 2.90 mm^2 when using the 26-electrode current cage.

Reduction of muscle contraction and pain: IRE protocols

In 2011, Arena *et al*[32] used a combination of analytical, numerical and experimental techniques to investigate H-FIRE. In their *in vivo* protocols, they compared a standard IRE pulse protocol to H-FIRE. In both protocols, 180 bursts were delivered, with each burst lasting 200 μs and being delivered at a frequency of 1 Hz. In the IRE protocol, each burst consisted of a single pulse of 200 μs width. In the H-FIRE protocol, each burst consisted of (1) 50 bipolar pulses at 250 kHz and a single polarity duration of 2 μs; and (2) 100 bipolar pulses at 500 kHz with single polarity duration of 1 μs. No visual or tactile evidence of muscle contraction was seen during H-FIRE, but all IRE protocols resulted in detectable muscle contractions. The mean peak accelerations (0.8 *g*, 0.4 *g* and 0.1 *g*) during IRE treatments at the cervicothoracic junction for each applied voltage (200 V, 100 V and 50 V) were significantly different from each other. On the other hand, H-FIRE resulted in no detectable acceleration at the cervicothoracic junction. The *in vivo* experiments also showed that H-FIRE produced an ablative effect on brain tissue comparable to that obtained in non-thermal IRE treatments. Specifically, there was complete uniformity of tissue death within the targeted areas. A sharp transition zone was present between lesions and normal brain tissue.

In 2014, Sano *et al*[42] studied the effects of bipolar pulses on both muscle contractions and cell viability using an IRE protocol. Each monopolar waveform typical of the standard protocol was replaced with a burst of alternating polarity pulses; the total energised burst time was the same as that used in the standard protocol (100 μs). The bipolar protocol consisted of 80 bursts at a frequency of 1 Hz; in each burst, the positive/negative wavelength varied from 250 ns to 50 μs, with an intra-pulse delay fixed at 2 μs. The authors showed that, at 1500 V/cm, only treatments with bursts containing 50 + 50 μs pulses (Table 1) resulted in an interesting compromise between low viability (below 10%) and muscle contraction reduction that less undesirable than those associated with longer monopolar pulses. Sano *et al*[43]. analysed muscle contraction in a murine model when using different pulse protocols [43]. Treatment efficacy was also tested in an *in vitro* tumour model using PPT8182 murine primary pancreatic tumour cells. To facilitate comparison between groups, the authors applied the following simplified electrical dose formula:

$$Dose = V^2 \times T_p \times n \times N$$

Where V is the applied voltage, T_p is the pulse width, n is the number of pulses *per* burst, and N is the number of bursts *per* treatment, which was typically 80. Thanks to the use of a custom pulse generation system, bursts of bipolar pulses with constitutive pulse widths of 250 ns, 500 ns, 1 μs, 2 μs, 5 μs, 10 μs and 50 μs were delivered. They also used custom-made electrodes with 1.27 mm diameter dispensing needles and a 2.0 mm edge-to-edge separation distance. Given the formula above, the lethal electric field thresholds were found to be 2022, 1687, 1070, 755, 640, 629 and 531 V/cm for bursts containing 0.25, 0.5, 1, 2, 5, 10 and 50 μs pulses, respectively. Qualitatively, the results showed that muscle contractions occurred to a lesser extent in treatments with bipolar bursts of pulses between 1μs and 5 μs, compared with those in treatments with standard IRE protocol (100 μs monopolar pulses at 200 V). At 400 V, the 100-μs pulses induced such strong muscle contractions that complete anaesthesia was necessary to carry out the procedure. In contrast, 1000 V treatments with bursts of 5 μs pulses were well tolerated with light sedation and local anaesthesia.

Similarly, in 2016, Sweeney *et al*[33] carried out a quantitative comparison between different pulsing schemes. They compared trains of 100 μs monopolar pulses conventionally used in IRE and ECT, with pulse trains containing bursts or evenly spaced 1 μs bipolar pulses. They assessed both the reduction of muscle contractions and the cell permeability obtained with the different pulsed electric field protocols. Cell permeability was evaluated by real-time microscopic imaging of propidium iodide transport at the single cell level during and after each treatment. The protocols under investigation were: (1) A train of 200 monopolar pulses of 300 V amplitude, each

Table 1 Summary of the studies analysed in this review

Ref.		Electroporation protocol						Muscle contraction/pain reduction
			Type of pulse	Number of pulses	Pulse duration	Pulse frequency	Electric field	
Daskalov *et al* [36], 1999	*In vivo*	ECT	Monophasic	1 burst of 8 pulses	100 μs	1 Hz	0.33-1.25 kV/cm	Achievable with biphasic pulses
			Biphasic	1 burst of 8 pulses	50-0-50 μs	1 Hz		
			Biphasic	1 burst of 8 pulses	50-0-50 μs	Approximately 909 Hz		
Miklavcic *et al*[37], 2005	*In vivo*	ECT	Monopolar	1 burst of 8 pulses	100 μs	1 to 5000 Hz (ten or five steps)	88 or 313 V/cm	Achievable with high frequency pulses
Zupanic *et al* [38], 2007	*In vivo*	ECT	Monopolar	1 burst of 8 pulses	100 μs	1 Hz	600 V/cm	Achievable with high frequency pulses
			Monopolar	1 burst of 8 pulses	100 μs	5000 Hz		
Spugnini *et al* [39], 2014	*In vitro* *In vivo*	ECT	Biphasic	1 burst of 8 pulses	50-10-50 μs	9 kHz	1.3 kV/cm	Achievable with biphasic pulses
Scuderi *et al* [40], 2019	*In vitro*	ECT	Monopolar	1 burst of 8 pulses	100 μs	1 Hz	1.2 kV/cm	Achievable with bipolar HF-EP
			Bipolar	8 bursts of 50 pulses	1-1-1 μs	250 kHz	3 kV/cm	
García-Sánchez *et al* [41], 2020	*In vivo*	ECT	Unipolar	Bursts of 8 pulses	100 μs	1 Hz	1.3 kV/cm	Achievable with sinusoidal pulses
			Bipolar	Number of bursts and pulses depend on experiments and frequency	100 μs-5 ms	10-100 kHz	> 1.3 kV/cm	
			Sinusoidal					
Golberg and Rubinsky [28], 2012	*Numerical*	-	Monopolar	1 pulse	100 μs	-	> 800 V/cm	Achievable with an appropriate electrode design and arrangement
Arena *et al* [32], 2011	*In vivo*	IRE	Monopolar	90-180 pulses	200 μs	1 Hz	0.5-2 kV/cm	Achievable with H-FIRE
			Bipolar	180 bursts of 50 pulses	2-0-2 μs	250 kHz	1-4 kV/cm	
			Bipolar	180 bursts of 100 pulses	1-0-1 μs	500 kHz	4 kV/cm	
Sano *et al* [42], 2014	*In vitro*	IRE	Bipolar	80 bursts of 1 pulse	50-2-50 μs	1 Hz	1.5 kV/cm	Achievable with biphasic pulses
Sano *et al* [43], 2015	*In vitro*	IRE	Monopolar	1 pulse	100 μs	1 Hz	1.5 kV/cm	Achievable with bipolar pulses
	In vivo		Bipolar	8-120 bursts of 1-200 pulses	Pulse width: 250 ns-50 μs	20 to 20000 kHz (seven steps)	Approximately 0.5-2 kV/cm	
Sweeney *et al* [33], 2016	*In vitro*	IRE	Monopolar	1 burst of 200 pulses	100 μs	2 kHz	750-1250 V/cm	Achievable with high-frequency bipolar pulses
			Bipolar	200 bursts of 50 pulses	1 -1-1 μs	250 kHz	1250 V/cm	
			Bipolar	200 bursts of 50 pulses	1-4-1 μs	100 kHz	1250 V/cm	
Yao *et al*[44], 2017	*In vivo*	IRE	Bipolar	90 bursts of 50 pulses	2-2-2 μs	250 kHz	1-2 kV/cm	Achievable with H-FIRE and insulated needle electrodes
			Bipolar	90 bursts of 20 pulses	5-2-5 μs	Approximately 143 kHz		
			Bipolar	90 bursts of 10 pulses	10-2-10 μs	Approximately 83 kHz		
			Bipolar	90 bursts of 4 pulses	25-2-25 μs	Approximately 37 kHz	1–1.75 kV/cm	
			Bipolar	90 bursts of 2 pulses	50-2-50 μs	Approximately 20 kHz	1–1.5 kV/cm	
			Monopolar	90 bursts of 1 pulse	100 μs	1 Hz	0.8–1.5 kV/cm	
Sano *et al* [29], 2018	*Ex vivo*	IRE	Monopolar	5 pulses	100 μs	1 Hz	1 kV/cm	Achievable with (symmetric) H-FIRE pulses
			Bipolar	5 bursts of 1-200 pulses	2-2-2 μs	Not available	0.17-1.7 kV/cm	

ECT: Electrochemotherapy; H-FIRE: High-frequency irreversible electroporation; HF-EP: High frequency electroporation; IRE: Irreversible electroporation.

lasting 100 μs and repeated at a rate of 2 kHz for 500 μs; (2) A train with the same characteristics but with a pulse amplitude of 500 V; and (3) 200 bursts of 25 bipolar pulses with a 1 + 1 μs duration and 500 V, separated by a 4 μs intra- and inter-pulse delay. Each treatment consisted of 200 periods (bursts) lasting 500 μs each, for a total treatment time of exactly 100 ms for each pulsing scheme. Even though bipolar pulses at a high frequency were able to mitigate undesirable muscle contraction during IRE, the bipolar pulses induced less evident membrane permeabilisation than equivalent monopolar pulses. In fact, intracellular detection of propidium iodide was observed at electric field intensities of approximately 500 V/cm, which was lower than that observed in bipolar pulse treatments (900-1250 V/cm). That was attributed to the inability of the short-duration bipolar pulses to complete the membrane charging despite the higher applied voltages. However, bipolar pulse protocols can be designed to obtain more efficient, symmetric and homogeneous uptake of small molecules into cells than conventional monopolar pulses.

In 2017, Yao *et al*[44] explored the effect of IRE ablation on muscle contractions. The authors studied how to reduce muscle contractions by acting both on the frequency of monopolar pulses and on the nature of the electrodes used. The study was conducted with rabbit liver tissue. The H-FIRE protocol consisted of a series of 90 bursts. Each burst had a repetition frequency of 1 Hz and comprised 50, 20, 10, 4 or 2 monopolar pulses with individual pulse widths of 2, 5, 10, 20 or 50 μs. The total energised time was 100 μs. The experiments were conducted with both traditional and insulated needle electrodes with the aim of investigating how the electrode design influenced the muscle contractions. Each pair of electrodes were separated by a fixed distance of 10 mm. A finite element model was also used to establish the lethal thresholds of H-FIRE protocols; consequently, the pulse voltage amplitude range was set from 800 V to 2000 V. An accelerometer was used to measure muscle contractions. The authors observed that the H-FIRE protocol reduced muscle contractions. The muscle contraction strength increased with the increase in voltage amplitude and pulse width. A quite linear increase in acceleration occurred when the voltage was increased, regardless of the pulse duration. For example, a 10 μs pulse produced an acceleration of about 1.5 *g* at 1000 V, and about 4 *g* at 2000 V Conversely, at a fixed voltage, a consistent increase in the acceleration value was observed when the pulse length was also increased (*e.g.*, less than 1 g of acceleration for 2 μs pulses at 1500 V *vs* more than 7 *g* of acceleration for 100 μs pulses at 1500 V). Moreover, fewer muscle contractions were detected when using insulated needle electrodes and the ablation area was smaller than that obtained with traditional needle electrodes (*e.g.*, about 5 *g* of acceleration with 50 μs pulses at 1500 V using insulated needles *vs* about 6.5 *g* of acceleration with 50 μs pulses at 1500 V using non-insulated needles).

Sano *et al*[29] compared the effect on muscle contraction associated with IRE to those associated with different H-FIRE protocols. The experiments were conducted *ex vivo* and muscle contractions were measured with an accelerometer. In order to make the comparison consistent, the total energised time in H-FIRE protocols was ensured to be equal to one of the standard IRE protocols. The traditional IRE protocols consisted of five monopolar pulses lasting 25, 50, 75 and 100 μs, with a repetition frequency of 0.5 or 1 Hz and with an amplitude of 3000 V. To examine alternative strategies, high-energy bipolar bursts with energised times between 100 μs and 200 μs and voltages between 3000 V and 4500 V were delivered. The investigated H-FIRE protocols were split into three subgroups: (1) Symmetric 2 + 2 μs high frequency pulses with an intra-pulse delay of 2 μs, voltages of from 500 to 5000 V and total energised times of 100 or 200 μs; (2) Symmetric 2 + 2 μs high frequency pulses with an intra-pulse delay of 5 μs or 10 μs, a voltage of 5000 V and a total energised time of 100 μs; and (3) Asymmetric high frequency pulses with a 2 μs positive wave, an intra-pulse of 2 μs and negative waves of 0.25, 0.5 or 1 μs (voltage of 3000 V). An energised time of 100 μs with 2–2–2 H-FIRE pulses produced muscle contractions that increased with the voltage (accelerations of 0.005 *g* and 0.210 *g* for voltages of 500 and 5000 V, respectively). When the voltage was set at 3000 V, the acceleration peak obtained in the symmetrical H-FIRE protocol was 9-12 times smaller than that seen with traditional IRE pulses (0.72 *g* with a 75 μs pulse length). Moreover, symmetrical high frequency pulses enabled the delivery of substantially higher voltages and energised times while producing smaller accelerations than traditional IRE pulses. In fact, the acceleration values remained relatively constant when the total energised time was increased from 100 μs to 200 μs, and even when the applied voltage was increased to 4500 V. Conversely, both

symmetric pulses with a variable intra-pulse delay and asymmetric pulses produced significantly greater muscle contractions. However, asymmetrical H-FIRE produced significantly greater ($\alpha < 0.001$) muscle contractions at 3000 V compared with the symmetrical waveforms. The maximum peak acceleration (0.80 *g*) comparable to that achieved with the traditional IRE pulses, was achieved with the 2–2–0.25 waveform. The authors concluded that muscle contractions can be reduced with H-FIRE pulses when the voltage and energised time are held constant (3000 V, 100 μs). Additionally, high voltage and high-energy H-FIRE treatments produced less intense muscle contractions. However, since the experiments were conducted *ex vivo*, it is reasonable to consider that muscle contractions *in vivo* may be greater than those observed in this study. Ablation efficacy should also be assessed.

Summary table

Table 1 summarises the outcomes of the literature analysis. The type of study (numerical analysis, *in vitro*, *in vivo* or *ex vivo*), the type of electroporation protocol (ECT or IRE), the pulse characteristics and the main results are reported. With reference to the pulse characteristics, it was considered of particular interest to report: (1) The type of pulse; (2) The number of pulses (*i.e.* the number of bursts and the number of pulses *per* burst); (3) The pulse duration (when bipolar/biphasic, as positive pulse width–intra-pulse delay–negative pulse width); (4) The pulse frequency (*i.e.* the inverse of a single monopolar/monophasic or bipolar/biphasic pulse period); and (5) The electric field applied (Figure 3).

DISCUSSION

Muscle contractions and pain are the main undesirable effects associated with EP treatments, both ECT and IRE. Many authors have investigated different protocol designs and electrode geometries in order to reduce patient pain, the number of muscle contractions and their intensity. As this review shows, particular importance was given to the length, frequency and type of the delivered pulses. Less attention was paid to the influence of the electrode design even though it does affect the portion of the muscle through which the current flows. As reported by Miklavcic *et al*[37] the reduction of voltage amplitude, does not result in an appreciable reduction of patient discomfort. Moreover, even if it is widely accepted that a decrease in the pulse amplitude can be balanced by an increase in the pulse duration, there is still disagreement as to how to determine the correct increase in the number of pulses to be applied in order not to alter treatment efficacy.

Pain reduction and pulse frequency

The reduction of pain due to muscle contractions can be obtained by increasing the pulse repetition frequency above that of tetanic contraction (100 Hz)[38]. When the frequency was higher than 2 kHz, patients experienced a single muscle contraction rather than multiple muscle contractions after every single pulse[38,39]. Moreover, treatment efficacy was not altered by an increase in frequency of up to 5 kHz[38,39]. That was confirmed by Yang *et al*[47], who observed that steep pulsed electric fields with a given frequency and appropriate electric field intensity achieved a cytotoxicity of close to 100%. However, even though the total number of muscle contractions *per* treatment was reduced, the intensity of the contractions remained similar to that observed in standard protocols.

When altering the pulse repetition frequency, attention should be paid to the choice of pulse numbers and amplitude[48,49]. In fact, the relationship between the pulse parameters for ECT and treatment efficacy, assessed by the cell cytotoxicity rate, can display a highly linear behaviour up to a certain number of pulses and/or field intensity. Thereafter, an exponential model is more appropriate. That is consistent with a recent study by García-Sánchez *et al*[41], which found that convenient, reversible EP and efficient ECT of subcutaneous tumours and a remarkable reduction of muscle contraction could be achieved by applying sinusoidal fields. However, the frequency of sine waves has been shown to significantly affect ECT effectiveness. At 100 kHz, a clear loss of efficacy was observed[41]. In order to achieve a tumour regression similar to that obtained at 10 kHz, the electric field intensity should be theoretically increased 1.56 times. Those results highlight the charge-dependent nature of the EP phenomenon, where the cell membrane must be charged at the minimum induced transmembrane voltage in order to achieve effective electro-permeabilisa-tion [49,50].

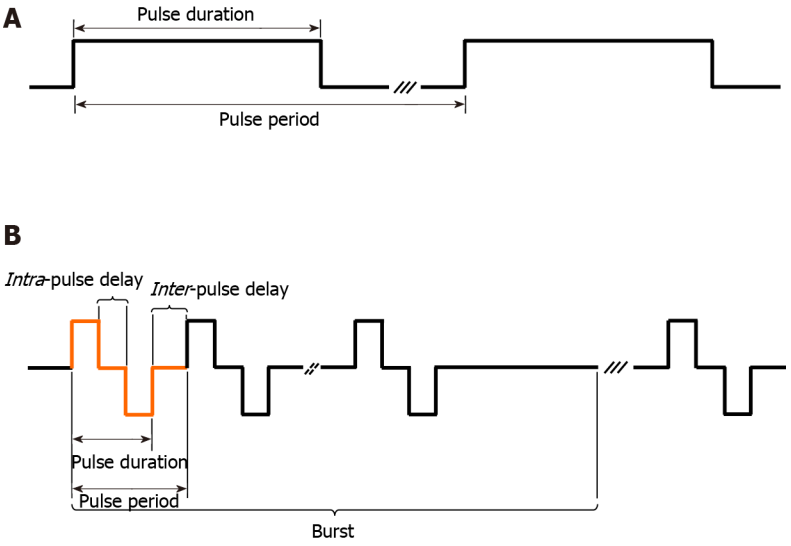


Figure 3 Pulse structure. A: Monopolar/monophasic pulse; B: Bipolar/biphasic pulse.

Pain reduction and bipolar/biphasic pulses

Several authors have investigated reduction in morbidity achieved with bipolar/biphasic pulses. They all reported that altering the pulse polarity not only reduced the occurrence but also the intensity of muscle contractions. In fact, when a μs-pulse was applied, there was a latency period between the end of the pulse and the rising phase of the action potential. A rapid reversal of polarity within this latency period can accelerate passive repolarisation and inhibit the action potential generation. Therefore, with proper tuning of the bipolar/biphasic pulse parameters, it is possible to achieve a drop in muscle force that can be attributed to the termination of action potentials in part of the motor unit population[33,34,40,43,44,48,51], without losing in EP efficacy. With that in mind, interesting results were reported by Spugnini *et al*[39]. They reported that trains of eight biphasic electric pulses lasting 50 + 50 μs each, at a frequency of 1 Hz, and 10 μs interpulse intervals at 1300 V/cm, achieved a significantly higher response in mice (70%-90% tumour necrosis) compared with that achieved with trains of eight biphasic electric pulses lasting 50 + 50 ms each, at a frequency of 1 Hz and 1-ms interpulse intervals (40%-55% tumour necrosis)[40].

Sano *et al*[43] reported that bursts of bipolar pulses resulted in both instantaneous and delayed cell death and that an inverse relationship existed between pulse width and toxicity, despite the delivery of equal quantities of energy. However, 1500 V/cm bursts containing 50 + 50 μs pulses resulted in a viability below 10% and low muscle contractions, which was less undesirable than those induced by longer monopolar/monophasic pulses. This result is comparable with the standard IRE protocol, as reported by Arena *et al*[51], who showed that after eighty 100 μs monopolar pulses at 1500 V/cm, cell viability was approximately 8%. Bipolar/biphasic pulses in the same electric field seem to have appreciable efficacy when biphasic pulses of 50 + 50 μs are used; however, in order to obtain a comparable cytotoxicity rate with bipolar/biphasic and monopolar/monophasic protocols, the bipolar/biphasic pulses generally need a stronger electric field[41].

Finally, the protocols described by Scuderi *et al*[40] and Sweeney *et al*[33] achieved the results obtained with high frequency pulses together with those achieved with bipolar pulses in ECT and IRE. They found that bipolar pulses at a high frequency were able to mitigate both undesirable muscle contraction and patient pain in EP therapies. Additional reduction was achieved when the bipolar pulse had a symmetrical structure[30]. However, short bipolar pulses may result in less pronounced membrane permeabilisation, suggesting that pulse duration is a critical parameter that must be carefully chosen[34,50].

Pain reduction and electrode design

Reductions of muscle contraction and morbidity can be achieved with an appropriate electrode design. Fewer and less intense muscle contractions were reported by Yao *et al*[44] when using insulated needle electrodes. A more sophisticated electrode design was proposed by Golberg and Rubinsky[28] where a central energised electrode was surrounded by at least 16 grounded electrodes, obtaining significant pain reduction.

However, the impact of the new electrode designs on treatment efficacy remains to be more deeply evaluated.

CONCLUSION

This review found that both pulse frequency and shape can be modified to reduce pain and muscle contractions in patients who undergo EP treatments. Furthermore, a combination of high frequency pulses with bipolar/biphasic ones were shown to enhance this capability. However, simply ensuring that equivalent energy is administered by standard and new protocols (high frequency, bipolar/biphasic) is not sufficient to guarantee clinical efficacy. Preclinical *in vitro* and *in vivo* studies together with clinical data are necessary to evaluate the clinical relevance of alternative pulse protocols. In addition, sinusoidal pulses with the appropriate frequency, as well as electrode design (*e.g.*, insulated needles), may successfully mitigate these drawbacks. Further study is required to evaluate how these aspects influence the efficacy of the therapy. A main limitation of this systematic review is the absence of a risk of bias analysis both in individual studies and across studies. Moreover, the authors did not investigate principal summary measures (*e.g.*, risk ratio, difference in means, and others), as most studies did not report numerical results. Those deficiencies should be addressed in subsequent investigations. To summarise, pain reduction in EP-based treatments can be achieved by appropriately defining the protocol parameters and the electrode design. The desired results can be achieved with high frequency and/or bipolar/biphasic pulses. However, the efficacy of these alternative protocols remains a crucial point to be assessed further.

ARTICLE HIGHLIGHTS

Research background

In electrochemotherapy (ECT), pain and muscular contractions were reported as the most frequent drawbacks.

Research motivation

To review aimed to assess the literature describing technical advances intended to reduce muscle contraction and pain associated with electroporation (EP) effects.

Research objectives

The objective was to present an overview of different protocols proposed in the literature that aim to reduce muscle contraction in both ECT and irreversible EP treatments.

Research methods

Thirty-three published studies reporting different protocol designs and electrode geometries were selected for analysis.

Research results

Both high frequency and bipolar/biphasic pulses can be used to reduce pain and muscle contractions in patients who undergo EP treatments. Moreover, adequate electrode design can lower EP-related morbidity.

Research conclusions

Pain reduction in EP-based treatments can be achieved by appropriately defining the protocol parameters and the electrode design.

Research perspectives

The desired results can be achieved with high frequency and/or bipolar/biphasic pulses.

REFERENCES

1 **Tozon N**, Sersa G, Cemazar M. Electrochemotherapy: potentiation of local antitumour effectiveness of cisplatin in dogs and cats. *Anticancer Res* 2001; **21**: 2483-2488 [PMID: 11724311]
2 **Jaroszeski MJ**, Dang V, Pottinger C, Hickey J, Gilbert R, Heller R. Toxicity of anticancer agents mediated by electroporation in vitro. *Anticancer Drugs* 2000; **11**: 201-208 [PMID: 10831279 DOI: 10.1097/00001813-200003000-00008]
3 **Orlowski S**, Belehradek J Jr, Paoletti C, Mir LM. Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs. *Biochem Pharmacol* 1988; **37**: 4727-4733 [PMID: 2462423 DOI: 10.1016/0006-2952(88)90344-9]
4 **Sersa G**, Cemazar M, Miklavcic D. Antitumor effectiveness of electrochemotherapy with cis-diamminedichloroplatinum(II) in mice. *Cancer Res* 1995; **55**: 3450-3455 [PMID: 7614485]
5 **Mali B**, Jarm T, Snoj M, Sersa G, Miklavcic D. Antitumor effectiveness of electrochemotherapy: a systematic review and meta-analysis. *Eur J Surg Oncol* 2013; **39**: 4-16 [PMID: 22980492 DOI: 10.1016/j.ejso.2012.08.016]
6 **Marty M**, Sersa G, Garbay JR, Gehl J, Collins CG, Snoj M, Billard V, Geertsen PF, Larkin JO, Miklavcic D, Pavlovic I, Paulin-Kosir SM, Cemazar M, Morsli N, Soden DM, Rudolf Z, Robert C, O'Sullivan GC, Mir LM: Electrochemotherapy – an easy, highly effective and safe treatment of cutaneous and subcutaneous metastases. Results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. *Eur J Cancer* 2006; **S4**: 3-13 [DOI: 10.1016/j.ejcsup.2006.08.002]
7 **Campana LG**, Mocellin S, Basso M, Puccetti O, De Salvo GL, Chiarion-Sileni V, Vecchiato A, Corti L, Rossi CR, Nitti D. Bleomycin-based electrochemotherapy: clinical outcome from a single institution's experience with 52 patients. *Ann Surg Oncol* 2009; **16**: 191-199 [PMID: 18987914 DOI: 10.1245/s10434-008-0204-8]
8 **Miklavčič D**, Serša G, Brecelj E, Gehl J, Soden D, Bianchi G, Ruggieri P, Rossi CR, Campana LG, Jarm T. Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors. *Med Biol Eng Comput* 2012; **50**: 1213-1225 [PMID: 23179413 DOI: 10.1007/s11517-012-0991-8]
9 **Edhemovic I**, Brecelj E, Gasljevic G, Marolt Music M, Gorjup V, Mali B, Jarm T, Kos B, Pavliha D, Grcar Kuzmanov B, Cemazar M, Snoj M, Miklavcic D, Gadzijev EM, Sersa G. Intraoperative electrochemotherapy of colorectal liver metastases. *J Surg Oncol* 2014; **110**: 320-327 [PMID: 24782355 DOI: 10.1002/jso.23625]
10 **Tarantino L**, Busto G, Nasto A, Fristachi R, Cacace L, Talamo M, Accardo C, Bortone S, Gallo P, Tarantino P, Nasto RA, Di Minno MN, Ambrosino P. Percutaneous electrochemotherapy in the treatment of portal vein tumor thrombosis at hepatic hilum in patients with hepatocellular carcinoma in cirrhosis: A feasibility study. *World J Gastroenterol* 2017; **23**: 906-918 [PMID: 28223736 DOI: 10.3748/wjg.v23.i5.906]
11 **Tarantino L**, Busto G, Nasto A, Nasto RA, Tarantino P, Fristachi R, Cacace L, Bortone S. Electrochemotherapy of cholangiocellular carcinoma at hepatic hilum: A feasibility study. *Eur J Surg Oncol* 2018; **44**: 1603-1609 [PMID: 30017329 DOI: 10.1016/j.ejso.2018.06.025]
12 **Granata V**, Fusco R, Piccirillo M, Palaia R, Petrillo A, Lastoria S, Izzo F. Electrochemotherapy in locally advanced pancreatic cancer: Preliminary results. *Int J Surg* 2015; **18**: 230-236 [PMID: 25917204 DOI: 10.1016/j.ijsu.2015.04.055]
13 **Miklavčič D**, Mali B, Kos B, Heller R, Serša G. Electrochemotherapy: from the drawing board into medical practice. *Biomed Eng Online* 2014; **13**: 29 [PMID: 24621079 DOI: 10.1186/1475-925X-13-29]
14 **Mir LM**, Tounekti O, Orlowski S. Bleomycin: revival of an old drug. *Gen Pharmacol* 1996; **27**: 745-748 [PMID: 8842674 DOI: 10.1016/0306-3623(95)02101-9]
15 **Tounekti O**, Pron G, Belehradek J Jr, Mir LM. Bleomycin, an apoptosis-mimetic drug that induces two types of cell death depending on the number of molecules internalized. *Cancer Res* 1993; **53**: 5462-5469 [PMID: 7693342]
16 **Spreckelmeyer S**, Orvig C, Casini A. Cellular transport mechanisms of cytotoxic metallodrugs: an overview beyond cisplatin. *Molecules* 2014; **19**: 15584-15610 [PMID: 25268716 DOI: 10.3390/molecules191015584]
17 **Mir LM**, Gehl J, Serša G, Collins CG, Garbay J-R, Billard V, Geertsend PF, Rudolf 17. Gerald C. O'Sullivan GC, Marty M. Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invasive or non-invasive electrodes. *Eur J Cancer Suppl* 2006; **4**: 14-25 [DOI: 10.1016/j.ejcsup.2006.08.003]
18 **Gehl J**, Sersa G, Matthiessen LW, Muir T, Soden D, Occhini A, Quaglino P, Curatolo P, Campana LG, Kunte C, Clover AJP, Bertino G, Farricha V, Odili J, Dahlstrom K, Benazzo M, Mir LM. Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. *Acta Oncol* 2018; **57**: 874-882 [PMID: 29577784 DOI: 10.1080/0284186X.2018.1454602]
19 **Arena CB**, Davalos RV. Advances in therapeutic electroporation to mitigate muscle contractions. *J Membr Sci Technol* 2012; **2**: 1-3 [DOI: 10.4172/2155-9589.1000e102]
20 **Ball C**, Thomson KR, Kavnoudias H. Irreversible electroporation: a new challenge in "out of operating theatre" anesthesia. *Anesth Analg* 2010; **110**: 1305-1309 [PMID: 20142349 DOI: 10.1213/ANE.0b013e3181d27b30]

21 **Mali B**, Jarm T, Corovic S, Paulin-Kosir MS, Cemazar M, Sersa G, Miklavcic D. The effect of electroporation pulses on functioning of the heart. *Med Biol Eng Comput* 2008; **46**: 745-757 [PMID: 18415132 DOI: 10.1007/s11517-008-0346-7]

22 **Deodhar A**, Dickfeld T, Single GW, Hamilton WC Jr, Thornton RH, Sofocleous CT, Maybody M, Gónen M, Rubinsky B, Solomon SB. Irreversible electroporation near the heart: ventricular arrhythmias can be prevented with ECG synchronization. *AJR Am J Roentgenol* 2011; **196**: W330-W335 [PMID: 21343484 DOI: 10.2214/AJR.10.4490]

23 **Larkin JO**, Collins CG, Aarons S, Tangney M, Whelan M, O'Reily S, Breathnach O, Soden DM, O'Sullivan GC. Electrochemotherapy: aspects of preclinical development and early clinical experience. *Ann Surg* 2007; **245**: 469-479 [PMID: 17435555 DOI: 10.1097/01.sla.0000250419.36053.33]

24 **Landström FJ**, Nilsson CO, Crafoord S, Reizenstein JA, Adamsson GB, Löfgren LA. Electroporation therapy of skin cancer in the head and neck area. *Dermatol Surg* 2010; **36**: 1245-1250 [PMID: 20666812 DOI: 10.1111/j.1524-4725.2010.01617.x]

25 **Campana LG**, Valpione S, Falci C, Mocellin S, Basso M, Corti L, Balestrieri N, Marchet A, Rossi CR. The activity and safety of electrochemotherapy in persistent chest wall recurrence from breast cancer after mastectomy: a phase-II study. *Breast Cancer Res Treat* 2012; **134**: 1169-1178 [PMID: 22821399 DOI: 10.1007/s10549-012-2095-4]

26 **Matthiessen LW**, Johannesen HH, Hendel HW, Moss T, Kamby C, Gehl J. Electrochemotherapy for large cutaneous recurrence of breast cancer: a phase II clinical trial. *Acta Oncol* 2012; **51**: 713-721 [PMID: 22731832 DOI: 10.3109/0284186X.2012.685524]

27 **Cabula C**, Campana LG, Grilz G, Galuppo S, Bussone R, De Meo L, Bonadies A, Curatolo P, De Laurentiis M, Renne M, Valpione S, Fabrizio T, Solari N, Guida M, Santoriello A, D'Aiuto M, Agresti R. Electrochemotherapy in the Treatment of Cutaneous Metastases from Breast Cancer: A Multicenter Cohort Analysis. *Ann Surg Oncol* 2015; **22** Suppl 3: S442-S450 [PMID: 26242370 DOI: 10.1245/s10434-015-4779-6]

28 **Golberg A**, Rubinsky B. Towards electroporation based treatment planning considering electric field induced muscle contractions. *Technol Cancer Res Treat* 2012; **11**: 189-201 [PMID: 22335414 DOI: 10.7785/tcrt.2012.500249]

29 **Sano MB**, Fan RE, Cheng K, Saenz Y, Sonn GA, Hwang GL, Xing L. Reduction of Muscle Contractions during Irreversible Electroporation Therapy Using High-Frequency Bursts of Alternating Polarity Pulses: A Laboratory Investigation in an Ex Vivo Swine Model. *J Vasc Interv Radiol* 2018; **29**: 893-898.e3 [PMID: 29628296 DOI: 10.1016/j.jvir.2017.12.019]

30 **Latouche EL**, Arena CB, Ivey JW, Garcia PA, Pancotto TE, Pavlisko N, Verbridge SS, Davalos RV, Rossmeisl JH. High-Frequency Irreversible Electroporation for Intracranial Meningioma: A Feasibility Study in a Spontaneous Canine Tumor Model. *Technol Cancer Res Treat* 2018; **17**: 1533033818785285 [PMID: 30071778 DOI: 10.1177/1533033818785285]

31 **Dong S**, Wang H, Zhao Y, Sun Y, Yao C. First Human Trial of High-Frequency Irreversible Electroporation Therapy for Prostate Cancer. *Technol Cancer Res Treat* 2018; **17**: 1533033818789692 [PMID: 30045668 DOI: 10.1177/1533033818789692]

32 **Arena CB**, Sano MB, Rossmeisl JH Jr, Caldwell JL, Garcia PA, Rylander MN, Davalos RV. High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction. *Biomed Eng Online* 2011; **10**: 102 [PMID: 22104372 DOI: 10.1186/1475-925X-10-102]

33 **Sweeney DC**, Reberšek M, Dermol J, Rems L, Miklavčič D, Davalos RV. Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses. *Biochim Biophys Acta* 2016; **1858**: 2689-2698 [PMID: 27372268 DOI: 10.1016/j.bbamem.2016.06.024]

34 **Pucihar G**, Mir LM, Miklavcic D. The effect of pulse repetition frequency on the uptake into electropermeabilized cells in vitro with possible applications in electrochemotherapy. *Bioelectrochemistry* 2002; **57**: 167-172 [PMID: 12160614 DOI: 10.1016/s1567-5394(02)00116-0]

35 **Zhang L**, Rabussay DP. Clinical evaluation of safety and human tolerance of electrical sensation induced by electric fields with non-invasive electrodes. *Bioelectrochemistry* 2002; **56**: 233-236 [PMID: 12009482 DOI: 10.1016/s1567-5394(02)00057-9]

36 **Daskalov I**, Mudrov N, Peycheva E. Exploring new instrumentation parameters for electrochemotherapy. Attacking tumors with bursts of biphasic pulses instead of single pulses. *IEEE Eng Med Biol Mag* 1999; **18**: 62-66 [PMID: 9934602 DOI: 10.1109/51.740982]

37 **Miklavcic D**, Pucihar G, Pavlovec M, Ribaric S, Mali M, Macek-Lebar A, Petkovsek M, Nastran J, Kranjc S, Cemazar M, Sersa G. The effect of high frequency electric pulses on muscle contractions and antitumor efficiency in vivo for a potential use in clinical electrochemotherapy. *Bioelectrochemistry* 2005; **65**: 121-128 [PMID: 15713562 DOI: 10.1016/j.bioelechem.2004.07.004]

38 **Zupanic A**, Ribaric S, Miklavcic D. Increasing the repetition frequency of electric pulse delivery reduces unpleasant sensations that occur in electrochemotherapy. *Neoplasma* 2007; **54**: 246-250 [PMID: 17447858]

39 **Spugnini EP**, Melillo A, Quagliuolo L, Boccellino M, Vincenzi B, Pasquali P, Baldi A. Definition of novel electrochemotherapy parameters and validation of their in vitro and in vivo effectiveness. *J Cell Physiol* 2014; **229**: 1177-1181 [PMID: 24403005 DOI: 10.1002/jcp.24548]

40 **Scuderi M**, Rebersek M, Miklavcic D, Dermol-Cerne J. The use of high-frequency short bipolar pulses in cisplatin electrochemotherapy in vitro. *Radiol Oncol* 2019; **53**: 194-205 [PMID: 31194692 DOI: 10.2478/raon-2019-0025]

41 **García-Sánchez T**, Mercadal B, Polrot M, Muscat A, Sarnago H, Lucia O, Mir LM. Successful Tumor Electrochemotherapy Using Sine Waves. *IEEE Trans Biomed Eng* 2020; **67**: 1040-1049 [PMID: 31329545 DOI: 10.1109/TBME.2019.2928645]

42 **Sano MB**, Arena CB, DeWitt MR, Saur D, Davalos RV. In-vitro bipolar nano- and microsecond electro-pulse bursts for irreversible electroporation therapies. *Bioelectrochemistry* 2014; **100**: 69-79 [PMID: 25131187 DOI: 10.1016/j.bioelechem.2014.07.010]

43 **Sano MB**, Arena CB, Bittleman KR, DeWitt MR, Cho HJ, Szot CS, Saur D, Cissell JM, Robertson J, Lee YW, Davalos RV. Bursts of Bipolar Microsecond Pulses Inhibit Tumor Growth. *Sci Rep* 2015; **5**: 14999 [PMID: 26459930 DOI: 10.1038/srep14999]

44 **Yao C**, Dong S, Zhao Y, Lv Y, Liu H, Gong L, Ma J, Wang H, Sun Y. Bipolar Microsecond Pulses and Insulated Needle Electrodes for Reducing Muscle Contractions During Irreversible Electroporation. *IEEE Trans Biomed Eng* 2017; **64**: 2924-2937 [PMID: 28391185 DOI: 10.1109/TBME.2017.2690624]

45 **Orlowski S**, Mir LM. Cell electropermeabilization: a new tool for biochemical and pharmacological studies. *Biochim Biophys Acta* 1993; **1154**: 51-63 [PMID: 8507646 DOI: 10.1016/0304-4157(93)90016-h]

46 **Melzack R**. The McGill Pain Questionnaire: major properties and scoring methods. *Pain* 1975; **1**: 277-299 [PMID: 1235985 DOI: 10.1016/0304-3959(75)90044-5]

47 **Yang XJ**, Li J, Sun CX, Zheng FY, Hu LN. The effect of high frequency steep pulsed electric fields on in vitro and in vivo antitumor efficiency of ovarian cancer cell line skov3 and potential use in electrochemotherapy. *J Exp Clin Cancer Res* 2009; **28**: 53 [PMID: 19386105 DOI: 10.1186/1756-9966-28-53]

48 **García-Sánchez T**, Leray I, Ronchetti M, Cadossi R, Mir LM. Impact of the number of electric pulses on cell electrochemotherapy in vitro: Limits of linearity and saturation. *Bioelectrochemistry* 2019; **129**: 218-227 [PMID: 31200252 DOI: 10.1016/j.bioelechem.2019.05.021]

49 **Silve A**, Leray I, Leguèbe M, Poignard C, Mir LM. Cell membrane permeabilization by 12-ns electric pulses: Not a purely dielectric, but a charge-dependent phenomenon. *Bioelectrochemistry* 2015; **106**: 369-378 [PMID: 26138342 DOI: 10.1016/j.bioelechem.2015.06.002]

50 **Kotnik T**, Pucihar G, Miklavcic D. Induced transmembrane voltage and its correlation with electroporation-mediated molecular transport. *J Membr Biol* 2010; **236**: 3-13 [PMID: 20617432 DOI: 10.1007/s00232-010-9279-9]

51 **Arena CB**, Szot CS, Garcia PA, Rylander MN, Davalos RV. A three-dimensional in vitro tumor platform for modeling therapeutic irreversible electroporation. *Biophys J* 2012; **103**: 2033-2042 [PMID: 23199931 DOI: 10.1016/j.bpj.2012.09.017]

World Journal of Clinical Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Clin Oncol 2021 May 24; 12(5): 382-392

DOI: 10.5306/wjco.v12.i5.382

ISSN 2218-4333 (online)

SYSTEMATIC REVIEWS

Intestinal metastasis from breast cancer: Presentation, treatment and survival from a systematic literature review

Elena Bolzacchini, Olga Nigro, Davide Inversini, Monica Giordano, Giovanni Maconi

ORCID number: Elena Bolzacchini 0000-0003-1677-8477; Olga Nigro 0000-0002-1030-2809; Davide Inversini 0000-0002-4071-7706; Monica Giordano 0000-0002-8652-3907; Giovanni Maconi 0000-0003-0810-4026.

Author contributions: All authors contributed to the study conception and design; material preparation, data collection and analysis were performed by Bolzacchini E, Nigro O, Inversini D and Maconi G; the first draft of the manuscript was written by Bolzacchini E and Maconi G; Giordano M revised it carefully; all authors read and approved the final manuscript.

Conflict-of-interest statement: The authors have no conflict of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Elena Bolzacchini, Department of Oncology, Ospedale Sant' Anna, ASST Lariana, Como 22100, Italy, Department of Medicine and Surgery, University of Insubria, Varese 21100, Italy

Olga Nigro, Department of Oncology, Ospedale di Circolo ASST-Sette Laghi, Varese 21100, Italy

Davide Inversini, Department of General Surgery, Ospedale Sant' Antonio Abate, ASST Lariana, Cantu' 22100, Italy.

Monica Giordano, Department of Oncology, Ospedale Sant' Anna, ASST Lariana, Como 22100, Italy

Giovanni Maconi, Department of Biomedical and Clinical Sciences, Gastroenterology Unit, "Luigi Sacco" University Hospital, Milano 20157, Italy

Corresponding author: Elena Bolzacchini, MD, Doctor, Department of Oncology, Ospedale Sant' Anna, ASST Lariana, Via Ravona 20, San Fermo della Battaglia, Como 22100, Italy. elena.bolzacchini@hotmail.it

Abstract

BACKGROUND

Intestinal metastases from breast cancer (BC) arerare; available data depend mainly on case reports and case series.

AIM

To conduct a review of the literature regarding presentation, diagnosis, treatment and survival of patients with intestinal metastasis from BC.

METHODS

We identified all articles that described patients with intestinal metastasis (from duodenum to anum) from BC using MEDLINE (1975 to 2020) and EMBASE (1975 to 2020) electronic databases.

RESULTS

We found 96 cases of intestinal metastasis of BC. Metastasization involved large bowel (cecum, colon, sigmoid, rectum) (51%), small bowel (duodenum, jejunum, ileum) (49%), and anum (< 1%). Median age of patients was 61-years. The most frequent histology was infiltrating lobular carcinoma followed by infiltrating ductal carcinoma. In more than half of patients, the diagnosis was made after the diagnosis of BC (median: 7.2 years) and in many cases of emergency, for bowel

Manuscript source: Invited manuscript

Specialty type: Oncology

Country/Territory of origin: Italy

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 5, 2021
Peer-review started: January 5, 2021
First decision: January 18, 2021
Revised: January 23, 2021
Accepted: April 13, 2021
Article in press: April 13, 2021
Published online: May 24, 2021

P-Reviewer: Riis M
S-Editor: Zhang H
L-Editor: A
P-Editor: Wang LL

obstruction, bleeding or perforation. Diagnosis was achieved through endoscopy, radiological examination or both. In most of the cases, patients underwent surgery with or without systemic therapies. Survival of patients included in this review was available in less than 50% of patients and showed an overall median of 12 mo since diagnosis of the intestinal metastasis.

CONCLUSION
Although, intestinal metastases of BC are considered a rare condition, clinicians should consider the possibility of intestinal involvement in case of abdominal symptoms even in acute setting and many years after the diagnosis of BC, especially in patients with a histology of lobular carcinoma.

Key Words: Breast cancer; Intestinal metastasis; Diagnosis; Treatment; Small bowel; Large bowel

Core Tip: We conducted a review of the literature regarding presentation, diagnosis, treatment and survival of patients with intestinal metastasis from breast cancer (BC). Although intestinal metastases of BC are considered a rare condition, several cases are reported from the available literature. Clinicians should consider the possibility of intestinal involvement in case of abdominal symptoms even in acute setting and many years after the diagnosis of BC.

Citation: Bolzacchini E, Nigro O, Inversini D, Giordano M, Maconi G. Intestinal metastasis from breast cancer: Presentation, treatment and survival from a systematic literature review. *World J Clin Oncol* 2021; 12(5): 382-392
URL: https://www.wjgnet.com/2218-4333/full/v12/i5/382.htm
DOI: https://dx.doi.org/10.5306/wjco.v12.i5.382

INTRODUCTION

Breast cancer (BC) is the most common malignancy among women and a leading cause of cancer-related deaths[1,2]. In case of early diagnosis and application of new therapies, approximately 30% of patients are still at risk of developing distant metastasis, while 5% of the cases are metastatic at diagnosis[3]. The most common metastatic sites of BC are bones, lungs, liver and brain[4]. Gastrointestinal (GI) tract metastases are uncommon, detected in less than 5% of all BC patients[5]. Distinguishing primary and secondary GI cancer tumors can be clinically challenging. In particular, intestinal metastases from BC are rare, and related symptoms are not specific and often attributed to oncologic treatment, the main problem is to recognize them promptly and discriminate peritoneal carcinomatosis from other GI diseases to avoid any diagnostic delay and establish an effective treatment as soon as possible to improve survival of patients[6].

Despite well-known potential of intestinal metastatization of the BC, available data rely mainly on case reports and case series. Therefore, we report a literature review on presentation, diagnosis, treatment and survival of patients with intestinal metastasis from BC.

MATERIALS AND METHODS

We identified all articles that described patients with intestinal metastasis (from duodenum to anum) from breast cancer using MEDLINE (1975 to 2020) and EMBASE (1975 to 2020) electronic databases.

The search strategy was developed with a language restriction (only English texts) and literature search performed by applying the words: "Breast cancer", "gastrointestinal metastasis", "gastrointestinal tract", "intestine, bowel", "duodenum", "jejunum, cecum", "ileum", "small bowel", "large bowel", "colon", "sigma", "sigmoid

tract", "rectum", "anus" using the following string [(breast) AND (cancer or tumor or neoplasm) AND [duodenal neoplasms (secondary) OR jejunal neoplasms (secondary) OR cecal neoplasms (secondary) OR ileal neoplasm (secondary), OR small bowel neoplasm (secondary), OR colorectal neoplasms (secondary) OR sigma OR sigmoid neoplasm (secondary) OR anal neoplasms (secondary) NOT review], and filtering them for English studies and for humans studies (Figure 1).

The research of the literature was performed independently by two investigators (Nigro O and Bolzacchini E).

Studies were included if they met the following criteria: (1) Patients ≥ 18 years; (2) Patients with intestinal metastasis from breast cancer; and (3) Diagnosis of intestinal metastasis was objectively confirmed (histology).

Two investigators (Nigro O and Bolzacchini E) independently extracted data on study (year of publication, study centre), patients' characteristics (number of subjects studied, age, gender), tumour's characteristics (histology, time and site of metastatization), clinical presentation (main symptoms reported) as well as treatment and survival from the diagnosis of intestinal metastasis. We tried to contact the authors of the articles with missing survival data.

RESULTS

We identified 96 cases (86 articles) of intestinal metastases from BC[5-91]. Metastases are described in all parts of the intestinal tract, from the duodenum to the anum. Site of metastasis, presentation symptoms, treatment and clinical data are reported in Table 1.

Metastatization arose in large bowel (cecum, colon, sigmoid, rectum) (50/96; 52%), small bowel (duodenum, jejunum, ileum) (47/96; 49%), and anum (4/96, < 1%). Four patients presented multiple sites of intestinal metastases (small and large bowel); in three patients, gastric metastasis was also found, while peritoneum was also involved in six patients. Median age of patients was 61-years (between 31 and 88-years-old); only two patients were males. Histology comprehended lobular carcinoma (56/96; 58%), ductal carcinoma (17/96, 18%), phyllodes tumor (3), tubular carcinoma (1), or mixed histology (6).; 13/96 histologies were unknown. Intestinal involvement was diagnosed after the diagnosis of BC in 59/96 patients (median time; 7.2 years; range: 3 mo-25 years); the diagnosis was concomitant in 20/96 patients, in one case, the diagnosis of BC was made months after the metastatic involvement of the intestine and in another case, BC remained occult. In many cases, the diagnosis was made in emergency, for bowel obstruction (39 patients, 40.6%), bleeding (10 patients, 10.4%) and perforation (2 patients, 2%). Other patients complained of symptoms such as pain, changes in bowel habits, and in few patients, the diagnosis was incidental.

Diagnosis was achieved through endoscopy (esophagogastroduodenoscopy, colonoscopy or video capsule enteroscopy) in 54/96 cases (56.2%), radiological examination [computed tomography (CT), magnetic resonance imaging, barium enema or positron emission tomography (PET)] in 82/96 cases (85.4%) or both endoscopy and radiological imaging in 44/96 cases (45%).

In most of the cases, patients underwent surgery (69/96; 72%), with or without systemic therapies. The other patients started or continued medical therapy (18/96, 18.7%) such as hormone therapy and chemotherapy.

Specifically, 40 patients received hormone therapy (one patient aromatase inhibitor plus palbociclib), 38 patients received chemotherapy and 9 patients received both. Chemotherapy prescribed included monotherapy agents such as taxane-based chemotherapy (paclitaxel, docetaxel), anthracycline-based and oral fluoropyrimidine. Moreover, chemotherapy regimen was not specified in many reports.

Median overall survival of patients included in this review was available for 46/96 pts (< 50%); median survival estimated from the available data was around 12 mo.

DISCUSSION

Intestinal metastases from BC are rare. Jain *et al*[92] in a large study examined 1238 patients with operable BC and identified metastatic sites. They found that infiltrating ductal carcinoma recurred more often in the lung, pleura and bone, while infiltrating lobular carcinoma metastasized more often to bone marrow and peritoneum. Bone involvement as the initial presentation of distant metastatic disease occurred in over 50% of the women with infiltrating lobular carcinoma, significantly more often than in

Table 1 Demographic and clinical data of patients with intestinal metastasis from breast cancer

	n (%)	Median (range)
Year of publication		
< 2000	5 (5.8)	
2001-2005	12 (13.9)	
2006–2010	18 (20.9)	
2011-2015	36 (41.8)	
> 2016	15 (17.4)	
Age		61 (31-88)
Gender		
Female	94 (97.9)	
Male	2 (2.1)	
Breast cancer histology		
Lobular carcinoma	56(58.3)	
Ductal carcinoma	17 (17.7)	
Phyllodes tumor	3 (0.3)	
Tubular carcinoma	1 (0.1)	
Mixed histology	6 (0.6)	
Unknown	13 (13.5)	
Time of the diagnosis		
After the diagnosis of breast cancer	59 (61.4)	
Concomitant	20 (20.8)	
Before	1 (0.2)	
Unknown	16 (16.6)[1]	
Time after the diagnosis of breast cancer (yr)		7.2 (3 mo-25 yr)
Clinical presentation or main symptom at diagnosis		
Bowel obstruction	39 (40.6)	
Rectal bleeding	10 (10.4)	
Perforation	2 (2)	
Abdominal pain	20 (20.8)	
Change in bowel habit	9 (9.3%)	
Incidental	12 (12.5)	
Unknown	4 (4.1)	
Site of metastatization		
Colon	45 (46.8)	
Small bowel	43 (44.8)	
Colon and small bowel	4 (4)	
Anus	4 (4)	
Diagnostic tool for the detection of the metastasis		
Endoscopy (EGDS or colonscopy or VCE)	54 (56.2)	
Radiography (CT scan, MRI, barium enema, PET)	82 (85.4)	
Unknown	12 (12.5)	
Treatment		

Surgery and medical therapy	69/96 (71.8)	
Hormone therapy and/or chemotherapy	18/96 (18.7)	
Survival after the diagnosis of metastasis (yr)[2]		12 (1 mo-7 yr)

[1]In one case the primary tumor remained occult.
[2]Available for 46 patients. EGDS: Esophago-gastro-duodenoscopy; VCE: Video capsule endoscopy; CT: Computed tomography; MRI: Magnetic resonance imaging; PET: Positron emission tomography.

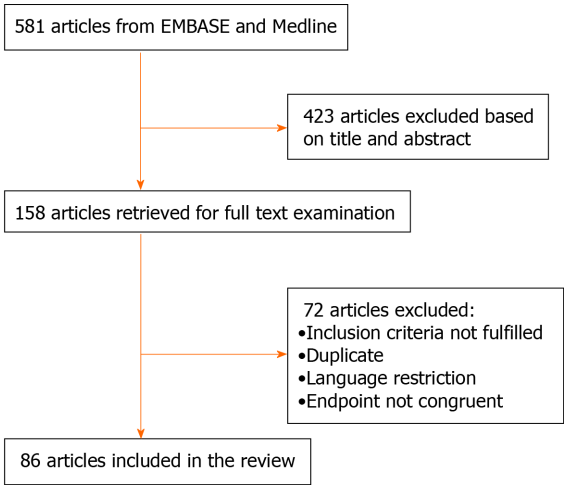


Figure 1 Study identification and selection process.

those with ductal infiltrating carcinoma (34%; $P < 0.01$). Survival was similar for the 2 groups.

Interestingly, metastatic disease to the extrahepatic GI tract from breast carcinoma usually originates from the lobular carcinoma subtype, which accounts for only 8% to 14% of all breast adenocarcinomas, rather than the more common invasive ductal carcinoma[93]. This could be related to a particular tropism of lobular cells. In a large autopsy series[94] of 337 patients who died of BC, GI metastasis presented an incidence of 16.4 % and only 20% of these patients complained of having symptoms. However, in this autopsy series, gastric and intestinal metastases were grouped, preventing an accurate estimation of the latter.

It has been well documented that recurrence in lobular BC can occur several years after the initial diagnosis of BC, even in early stage tumors. Recurrences of lobular BC have been reported up to 30 years from the initial time of diagnosis[95]. Accurate incidence of GI metastasis is hard to establish.

Symptoms depending on metastatic sites are generally not specific and may include abdominal pain, dyspepsia and nausea, acute symptoms such as bowel occlusion and GI bleeding may occur in most cases. Time interval between primary BC cases is wide, mostly years after the first diagnosis but rarely even before.

Endoscopy, radiological exams and histological evaluation are necessary to differentiate primary GI carcinoma from metastatic GI lesion of BC[32].

Endoscopic findings vary significantly and may range from ulcers, mucosal thickening or friability, linitisplastica-like inflammation, stenosis and polyps, to obstructing mass[24]. Barium studies reveal stricture or linitisplastica[41].

CT is indicated for characterizing the extension of the mass and for the re-staging [96]. Recently, Laoutliev *et al*[9] suggested that 18F-fluorodeoxyglucose PET should be considered.

Markers are generally not useful nor specific for the diagnosis of intestinal metastasis, but in an interesting case report by Santini *et al*[8] an increase in CA19.9 was used to diagnose ileocaecal valve metastasis from BC.

Differentiation of breast metastasis from other GI primaries can be difficult and immunohistochemistry is crucial to establishing the accurate diagnosis. Commonly used markers include estrogen receptors and progesterone receptors, CK7, CK20. A CK7-/CK20+ profile favors a large bowel primary, while CK7+/CK20- favors a metastasis[74].

GCDFP-15 and mammaglobin positivity was found to be sensitive and specific markers were used to differentiate a malignant lesion as a metastatic breast carcinoma, with an excellent correlation between GCDFP-15 and mammaglobin positivity and the origin of a metastatic BC[97,98]. It is very important to clarify the diagnosis since the treatment strategy for GI metastasis of BC and for primary GI carcinoma is totally different[99].

However, data on treatment are fragmentary. Surgical treatment, often palliative, should be reserved in case of emergencies (perforation, obstruction and hemorrhage), patients with isolated lesions, and selected cases in which tumor debunking could improve clinical outcome. Medical treatment such as chemotherapy, hormone therapy and anti HER2 therapy may be indicated depending on the biologic features of the primary disease and on prior therapy[100] and it is recommended as first-line treatment in case of multiple metastatic involvement. These therapies can be extremely effective and may help to avoid unnecessary surgery[10]. Radiotherapy is an option in case of anal localization[26,57] and in case of brain metastases[33] or bone metastases.

Long term outcome remains uncertain due to data scarcity and for the rareness of the condition. Nevertheless, some cases of longer survival were also reported[6,30]. Considering the available data, intestinal involvement seems prognostically unfavorable; in fact, progression free survival and overall survival of patients affected with metastatic BC have improved over the years thanks to the new treatment options.

We are aware that this review includes a selection bias as it does not rely on consecutive series of patients, but on many selected single case reports, published in English over a wide lag time (30 years). Therefore, many cases may not have been reported, several clinical data are missing and the outcome is especially not available for many cases even though we tried to contact the authors.

We pointed out that our review includes cases published from 1975 till date and clinical presentation, diagnosis and therapy might have changed over the years with awareness and technological advancement, nuclear medicine and new therapies. In this regard, a tentative analysis to assess the influence of time on several diagnostic and prognostic variables has been performed (data not shown), but no significant difference was found.

Nevertheless, the strength of our paper is its originality and the systematic methodological approach to the literature regarding intestinal localization of metastatic BC. Several reviews have already been published regarding gastric metastasis of BC; but to the best of our knowledge this is the first regarding intestinal involvement. Our paper comprehends all the cases reported so far in English language and summarizes epidemiology, symptoms, diagnostic work-up, therapy and survival of this condition. On this note, an estimate of the problem is given to the best of its knowledge and it forms the basis for the creation of a multicentre prospective study or a registry, which is the best option to investigate this uncommon but relevant issue of BC.

CONCLUSION

Clinicians should consider the possibility of intestinal involvement in case of abdominal symptoms, especially in patients with a histology of lobular carcinoma. Adequate imaging and endoscopic procedure should be performed promptly in order to obtain histological diagnosis. Treatment strategies include surgery, chemotherapy, hormone therapy and radiotherapy. Long term prognosis remains uncertain.

A multicenter study or registry study is required.

ARTICLE HIGHLIGHTS

Research background

Intestinal metastasis from breast cancer (BC) is considered rare.

Research motivation

We conducted a review of the literature regarding intestinal metastasis from BC.

Research objectives

We conducted a review of the literature regarding presentation, diagnosis, treatment and survival of patients with intestinal metastasis from BC.

Research methods

We identified all articles that described patients with intestinal metastasis from BC using MEDLINE and EMBASE electronic databases until 2020.

Research results

We found 96 cases of intestinal metastasis of BC. Metastasization involved large bowel in 51% of the cases, small bowel in 49% of the cases, and anum in less than 1%. Median age of patients was 61-year-old. The most frequent histology was infiltrating lobular carcinoma followed by infiltrating ductal carcinoma. In more than half of patients the diagnosis was made after the diagnosis of BC and in many cases in emergency setting, for bowel obstruction, bleeding or perforation. Diagnosis was achieved through endoscopy, radiological examination or both. In most of the cases patients underwent surgery with or without systemic therapies.

Research conclusions

Although intestinal metastases of BC are considered a rare condition, several cases are reported from the available literature.

Research perspectives

Our paper comprehends summarizes epidemiology, symptoms, diagnostic work-up, therapy and survival of this condition. On this account it gives an estimate of the problem and could lead to and represent the basis for the creation of a multicentre prospective study or a registry.

REFERENCES

1 **DeSantis CE**, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A, Jemal A, Siegel RL. Breast cancer statistics, 2019. *CA Cancer J Clin* 2019; **69**: 438-451 [PMID: 31577379 DOI: 10.3322/caac.21583]

2 **Dafni U**, Tsourti Z, Alatsathianos I. Breast Cancer Statistics in the European Union: Incidence and Survival across European Countries. *Breast Care (Basel)* 2019; **14**: 344-353 [PMID: 31933579 DOI: 10.1159/000503219]

3 **Redig AJ**, McAllister SS. Breast cancer as a systemic disease: a view of metastasis. *J Intern Med* 2013; **274**: 113-126 [PMID: 23844915 DOI: 10.1111/joim.12084]

4 **Jin L**, Han B, Siegel E, Cui Y, Giuliano A, Cui X. Breast cancer lung metastasis: Molecular biology and therapeutic implications. *Cancer Biol Ther* 2018; **19**: 858-868 [PMID: 29580128 DOI: 10.1080/15384047.2018.1456599]

5 **Schwarz RE**, Klimstra DS, Turnbull AD. Metastatic breast cancer masquerading as gastrointestinal primary. *Am J Gastroenterol* 1998; **93**: 111-114 [PMID: 9448188 DOI: 10.1111/j.1572-0241.1998.111_c.x]

6 **Ambroggi M**, Stroppa EM, Mordenti P, Biasini C, Zangrandi A, Michieletti E, Belloni E, Cavanna L. Metastatic breast cancer to the gastrointestinal tract: report of five cases and review of the literature. *Int J Breast Cancer* 2012; **2012**: 439023 [PMID: 23091732 DOI: 10.1155/2012/439023]

7 **Lau LC**, Wee B, Wang S, Thian YL. Metastatic breast cancer to the rectum: A case report with emphasis on MRI features. *Medicine (Baltimore)* 2017; **96**: e6739 [PMID: 28445295 DOI: 10.1097/MD.0000000000006739]

8 **Santini D**, Altomare A, Vincenzi B, Perrone G, Bianchi A, Rabitti C, Montesarchio V, Esposito V, Baldi A, Tonini G. An increase of CA 19.9 as the first clinical sign of ileocecal valve metastasis from breast cancer. *In Vivo* 2006; **20**: 165-168 [PMID: 16433047]

9 **Laoutliev B**, Harling H, Neergaard K, Simonsen L. Rectal metastasis from infiltrating lobular breast carcinoma: imaging with 18F-FDG PET. *Eur Radiol* 2005; **15**: 186-188 [PMID: 15449014 DOI: 10.1007/s00330-004-2394-1]

10 **Yanagisawa K**, Yamamoto M, Ueno E, Ohkouchi N. Synchronous rectal metastasis from invasive lobular carcinoma of the breast. *J Gastroenterol Hepatol* 2007; **22**: 601-602 [PMID: 17376062 DOI: 10.1111/j.1440-1746.2006.04671.x]

11 **Abdalla S**, Macneal P, Borg CM. Metastases of lobular breast carcinoma in the terminal ileum and ileocaecal valve. *J Surg Case Rep* 2015; **2015** [PMID: 25802254 DOI: 10.1093/jscr/rjv028]

12 **Bamias A**, Baltayiannis G, Kamina S, Fatouros M, Lymperopoulos E, Agnanti N, Tsianos E, Pavlidis N. Rectal metastases from lobular carcinoma of the breast: report of a case and literature review. *Ann Oncol* 2001; **12**: 715-718 [PMID: 11432633 DOI: 10.1023/a:1011192827710]

13 **Balakrishnan B**, Shaik S, Burman-Solovyeva I. An Unusual Clinical Presentation of Gastrointestinal Metastasis From Invasive Lobular Carcinoma of Breast. *J Investig Med High Impact Case Rep* 2016; **4**: 2324709616639723 [PMID: 27088099 DOI: 10.1177/2324709616639723]

14 **Yim K**, Ro SM, Lee J. Breast cancer metastasizing to the stomach mimicking primary gastric cancer: A case report. *World J Gastroenterol* 2017; **23**: 2251-2257 [PMID: 28405154 DOI: 10.3748/wjg.v23.i12.2251]

15 **Black M**, Hakam A, Harris C. Metastatic breast carcinoma uncovered in an otherwise unremarkable "random colon biopsy". *Human Pathol Case Rep* 2016; **4**: 23-31 [DOI: 10.1016/j.ehpc.2015.07.004]

16 **Ng CE**, Wright L, Pieri A, Belhasan A, Fasih T. Rectal metastasis from Breast cancer: A rare entity. *Int J Surg Case Rep* 2015; **13**: 103-105 [PMID: 26188979 DOI: 10.1016/j.ijscr.2015.06.023]

17 **Jones C**, Tong AW, Mir M, Coyle Y. Lobular carcinoma of the breast with gastrointestinal metastasis. *Proc (Bayl Univ Med Cent)* 2015; **28**: 50-53 [PMID: 25552798 DOI: 10.1080/08998280.2015.11929185]

18 **Andriola V**, Piscitelli D, De Fazio M, Altomare DF. Massive colonic metastasis from breast cancer 23 years after mastectomy. *Int J Colorectal Dis* 2015; **30**: 427-428 [PMID: 25129525 DOI: 10.1007/s00384-014-1992-x]

19 **Osaku T**, Ogata H, Magoshi S, Kubota Y, Saito F, Kanazawa S, Kaneko H. Metastatic nonpalpable invasive lobular breast carcinoma presenting as rectal stenosis: a case report. *J Med Case Rep* 2015; **9**: 88 [PMID: 25902937 DOI: 10.1186/s13256-015-0568-x]

20 **Gizzi G**, Santini D, Guido A, Fuccio L. Single colonic metastasis from breast cancer 11 years after mastectomy. *BMJ Case Rep* 2015; **2015**: bcr2015211193 [PMID: 26150650 DOI: 10.1136/bcr-2015-211193]

21 **Khairy S**, Azzam A, Mohammed S, Suleman K, Khawaji A, Amin T. Duodenal Obstruction as First Presentation of Metastatic Breast Cancer. *Case Rep Surg* 2015; **2015**: 605719 [PMID: 26266075 DOI: 10.1155/2015/605719]

22 **Wang G**, Wang T, Jiang J, Zhou L, Zhao H. Gastrointestinal tract metastasis from tubulolobular carcinoma of the breast: a case report and review of the literature. *Onco Targets Ther* 2014; **7**: 435-440 [PMID: 24672246 DOI: 10.2147/OTT.S57831]

23 **Shakoor MT**, Ayub S, Mohindra R, Ayub Z, Ahad A. Unique presentations of invasive lobular breast cancer: a case series. *Int J Biomed Sci* 2014; **10**: 287-293 [PMID: 25598762]

24 **Zhang B**, Copur-Dahi N, Kalmaz D, Boland BS. Gastrointestinal manifestations of breast cancer metastasis. *Dig Dis Sci* 2014; **59**: 2344-2346 [PMID: 24748230 DOI: 10.1007/s10620-014-3155-x]

25 **Venturini F**, Gambi V, Di Lernia S, Vanzulli A, Bramerio M, Bencardino K, Amatu A, Cipani T, Forti E, Tarenzi E, Sartore-Bianchi A, Pugliese R, Siena S. Linitis Plastica of the Rectum As a Clinical Presentation of Metastatic Lobular Carcinoma of the Breast. *J Clin Oncol* 2016; **34**: e54-e56 [PMID: 24982453 DOI: 10.1200/JCO.2013.50.6733]

26 **Nair MS**, Phillips BL, Navaratnam R, Fafemi O. Anorectal metastasis from breast carcinoma. *J Gastrointest Cancer* 2013; **44**: 106-107 [PMID: 23397574 DOI: 10.1007/s12029-012-9435-9]

27 **Lv L**, Zhao Y, Liu H, Peng Z. Case report of small bowel obstruction caused by small intestinal metastasis of bilateral breast cancer. *Exp Ther Med* 2013; **6**: 675-678 [PMID: 24137245 DOI: 10.3892/etm.2013.1220]

28 **Maekawa H**, Fujikawa T, Tanaka A. Successful laparoscopic investigation and resection of solitary colonic metastasis from breast cancer (with video). *BMJ Case Rep* 2012; **2012** [PMID: 23152180 DOI: 10.1136/bcr-2012-007187]

29 **Carcoforo P**, Raiji MT, Langan RC, Lanzara S, Portinari M, Maestroni U, Palini GM, Zanzi MV, Bonazza S, Pedriali M, Feo CV, Stojadinovic A, Avital I. Infiltrating lobular carcinoma of the breast presenting as gastrointestinal obstruction: a mini review. *J Cancer* 2012; **3**: 328-332 [PMID: 22866167 DOI: 10.7150/jca.4735]

30 **Rajan S**, Saeed M, Mestrah M. Ductal carcinoma of the breast metastasizing to the rectum. *J Surg Case Rep* 2012; **2012**: 12 [PMID: 24960140 DOI: 10.1093/jscr/2012.5.12]

31 **Gerova VA**, Tankova LT, Mihova AA, Drandarska IL, Kadian HO. Gastrointestinal metastases from breast cancer: report of two cases. *Hepatogastroenterology* 2012; **59**: 178-181 [PMID: 22251535 DOI: 10.5754/hge10681]

32 **Bochicchio A**, Tartarone A, Ignomirelli O, Latorre G, Cangiano R, Gallucci G, Coccaro M, Feudale E, Aieta M. Anal metastasis from breast cancer: a case report and review of the literature. *Future Oncol* 2012; **8**: 333-336 [PMID: 22409468 DOI: 10.2217/fon.12.9]

33 **Zhao R**, Li Y, Yu X, Yang W, Guo X. Duodenal metastasis from recurrent invasive lobular carcinoma of breast: a case report and literature review. *Int J Clin Oncol* 2012; **17**: 160-164 [PMID: 21638025 DOI: 10.1007/s10147-011-0258-7]

34 **Bilen MA**, Laucirica R, Rimawi MF, Nangia JR, Cyprus GS. Jejunal intussusception due to malignant phyllodes tumor of the breast. *Clin Breast Cancer* 2012; **12**: 219-221 [PMID: 22381472 DOI: 10.1016/j.clbc.2012.01.007]

35 **Calò PG**, Fanni D, Ionta MT, Medas F, Faa G, Atzori F. Jejunal obstruction caused by metastasis from an undiagnosed breast cancer: a case report. *Tumori* 2012; **98**: 89e-91e [PMID: 22825527 DOI: 10.1700/1125.12419]

36 **Campanile F**, Maurea S, Mainenti P, Corvino A, Imbriaco M. Duodenal involvement by breast cancer. *Breast J* 2012; **18**: 615-616 [PMID: 23110410 DOI: 10.1111/tbj.12034]

37 **Mistrangelo M**, Cassoni P, Mistrangelo M, Castellano I, Codognotto E, Sapino A, Lamanna G, Cravero F, Bianco L, Fora G, Sandrucci S. Obstructive colon metastases from lobular breast cancer: report of a case and review of the literature. *Tumori* 2011; **97**: 800-804 [PMID: 22322849 DOI: 10.1700/1018.11099]

38 **Kawasaki A**, Mimatsu K, Oida T, Kanou H, Kuboi Y, Fukino N, Amano S. Small intestinal perforation due to metastasis of breast carcinoma: report of a case. *Surg Today* 2011; **41**: 698-700 [PMID: 21533944 DOI: 10.1007/s00595-010-4345-6]

39 **Eljabu W**, Finch G, Nottingham J, Vaingankar N. Metastatic deposits of breast lobular carcinoma to small bowel and rectum. *Int J Breast Cancer* 2011; **2011**: 413949 [PMID: 22295221 DOI: 10.4061/2011/413949]

40 **Mouawad NJ**, Cleary RK. Small bowel obstruction as the primary presentation of undiagnosed metastatic lobular breast carcinoma. *Breast Dis* 2011; **33**: 35-40 [PMID: 21965304 DOI: 10.3233/BD-2010-0317]

41 **Saied A**, Bhati C, Sharma R, Garrean S, Salti G. Small bowel obstruction from breast cancer metastasis: a case report and review of the literature. *Breast Dis* 2011-2012; **33**: 183-188 [PMID: 21778579 DOI: 10.3233/BD-2010-0311]

42 **Cho DH**, Jeon YS, Choi MY, Lee SK, Kim SM, Hur SM, Koo MY, Bae SY, Choe JH, Kim JH, Kim JS, Nam SJ, Yang JH, Lee JE. Ileal metastasis of breast cancer in a patient with a BRCA2 gene mutation: report of a case. *Surg Today* 2011; **41**: 1665-1669 [PMID: 21969203 DOI: 10.1007/s00595-011-4503-5]

43 **Choi JE**, Park SY, Jeon MH, Kang SH, Lee SJ, Bae YK, Kim MK. Solitary small bowel metastasis from breast cancer. *J Breast Cancer* 2011; **14**: 69-71 [PMID: 21847398 DOI: 10.4048/jbc.2011.14.1.69]

44 **Okido M**, Seo M, Hamada Y, Kurihara S, Matsumoto K, Konomi H, Kato M, Ichimiya H. Metastatic breast carcinoma simulating linitis plastica of the colon: report of a case. *Surg Today* 2011; **41**: 542-545 [PMID: 21431489 DOI: 10.1007/s00595-009-4305-1]

45 **Critchley AC**, Harvey J, Carr M, Iwuchukwu O. Synchronous gastric and colonic metastases of invasive lobular breast carcinoma: case report and review of the literature. *Ann R Coll Surg Engl* 2011; **93**: e49-e50 [PMID: 21943448 DOI: 10.1308/147870811X582800]

46 **Amin AA**, Reddy A, Jha M, Prasad K. Rectal metastasis from breast cancer: an interval of 17 years. *BMJ Case Rep* 2011; **2011** [PMID: 22696719 DOI: 10.1136/bcr.01.2011.3683]

47 **Saranovic D**, Kovac JD, Knezevic S, Susnjar S, Stefanovic AD, Saranovic DS, Artiko V, Obradovic V, Masulovic D, Micev M, Pesko P. Invasive lobular breast cancer presenting an unusual metastatic pattern in the form of peritoneal and rectal metastases: a case report. *J Breast Cancer* 2011; **14**: 247-250 [PMID: 22031809 DOI: 10.4048/jbc.2011.14.3.247]

48 **Arrangoiz R**, Papavasiliou P, Dushkin H, Farma JM. Case report and literature review: Metastatic lobular carcinoma of the breast an unusual presentation. *Int J Surg Case Rep* 2011; **2**: 301-305 [PMID: 22096760 DOI: 10.1016/j.ijscr.2011.06.010]

49 **Kumano H**, Hozumi Y, Shiozawa M, Takehara M, Koizumi M, Sata N, Lefor AT, Yasuda Y. Recurrent invasive ductal carcinoma of the breast presenting as a metastasis to the duodenum with long-term survival. *Am Surg* 2011; **77**: e107-e108 [PMID: 21679619]

50 **Razzetta F**, Tassara E, Saro F, Sironi M, D'Ambrosio G. Rare abdominal metastases from occult lobular breast cancer: report of two cases. *Updates Surg* 2011; **63**: 129-133 [PMID: 21286894 DOI: 10.1007/s13304-011-0047-x]

51 **Koleilat I**, Syal A, Hena M. Metastatic male ductal breast cancer mimicking obstructing primary colon cancer. *Int J Biomed Sci* 2010; **6**: 66-70 [PMID: 23675178]

52 **Titi MA**, Anabtawi A, Newland AD. Isolated gastrointestinal metastasis of breast carcinoma: a case report. *Case Rep Med* 2010; **2010**: 615923 [PMID: 20592983 DOI: 10.1155/2010/615923]

53 **López Deogracias M**, Flores Jaime L, Arias-Camisón I, Zamacola I, Murillo Guibert J, Suescun García R, Querejeta Usabiaga J, Martínez García F. Rectal metastasis from lobular breast carcinoma 15 years after primary diagnosis. *Clin Transl Oncol* 2010; **12**: 150-153 [PMID: 20156785 DOI: 10.1007/S12094-010-0481-0]

54 **Ferrari AB**, Pulcini G, Gheza F, Vinco A, Manenti S, Cervi E, Villanacci V, Cervi G. Duodenal metastasis from male breast cancer: a case report and review of the literature. *J Med Case Rep* 2009; **3**: 8331 [PMID: 19830225 DOI: 10.4076/1752-1947-3-8331]

55 **Théraux J**, Bretagnol F, Guedj N, Cazals-Hatem D, Panis Y. Colorectal breast carcinoma metastasis diagnosed as an obstructive colonic primary tumor. A case report and review of the literature. *Gastroenterol Clin Biol* 2009; **33**: 1114-1117 [PMID: 19896312 DOI: 10.1016/j.gcb.2009.05.015]

56 **Kelly RJ**, Barrett C, Swan N, McDermott R. Metastatic phyllodes tumor causing small-bowel obstruction. *Clin Breast Cancer* 2009; **9**: 193-195 [PMID: 19661046 DOI: 10.3816/CBC.2009.n.033]

57 **Puglisi M**, Varaldo E, Assalino M, Ansaldo G, Torre G, Borgonovo G. Anal metastasis from recurrent breast lobular carcinoma: a case report. *World J Gastroenterol* 2009; **15**: 1388-1390 [PMID: 19294770 DOI: 10.3748/wjg.15.1388]

58 **Tang JY**, Rampaul RS, Cheung KL. The use of fulvestrant, a parenteral endocrine agent, in intestinal obstruction due to metastatic lobular breast carcinoma. *World J Surg Oncol* 2008; **6**: 128 [PMID: 19046426 DOI: 10.1186/1477-7819-6-128]

59 **Birla R**, Mahawar KK, Orizu M, Siddiqui MS, Batra A. Caecal metastasis from breast cancer presenting as intestinal obstruction. *World J Surg Oncol* 2008; **6**: 47 [PMID: 18471290 DOI: 10.1186/1477-7819-6-47]

60 **Nihon-Yanagi Y**, Park Y, Ooshiro M, Aoki H, Suzuki Y, Hiruta N, Kameda N, Katoh R. A case of recurrent invasive lobular carcinoma of the breast found as metastasis to the duodenum. *Breast Cancer* 2009; **16**: 83-87 [PMID: 18386118 DOI: 10.1007/s12282-008-0045-0]

61 **Tseng CH**, Wang WL, Lee TC, Lee TH, Wang HP. Ileal obstruction in a 73-year-old woman. *Gut* 2009; **58**: 741, 804 [PMID: 19433590 DOI: 10.1136/gut.2008.164913]

62 **Al-Qahtani MS**. Gut metastasis from breast carcinoma. *Saudi Med J* 2007; **28**: 1590-1592 [PMID:

17914525]
63 **Sato T**, Muto I, Hasegawa M, Aono T, Okada T, Hasegawa J, Makino S, Kameyama H, Tanaka R, Sekiya M. Breast signet-ring cell lobular carcinoma presenting with duodenal obstruction and acute pancreatitis. *Asian J Surg* 2007; **30**: 220-223 [PMID: 17638643 DOI: 10.1016/s1015-9584(08)60026-3]
64 **Uygun K**, Kocak Z, Altaner S, Cicin I, Tokatli F, Uzal C. Colonic metastasis from carcinoma of the breast that mimics a primary intestinal cancer. *Yonsei Med J* 2006; **47**: 578-582 [PMID: 16941751 DOI: 10.3349/ymj.2006.47.4.578]
65 **Asoglu O**, Karanlik H, Barbaros U, Yanar H, Kapran Y, Kecer M, Parlak M. Malignant phyllode tumor metastatic to the duodenum. *World J Gastroenterol* 2006; **12**: 1649-1651 [PMID: 16570365 DOI: 10.3748/wjg.v12.i10.1649]
66 **Nazareno J**, Taves D, Preiksaitis HG. Metastatic breast cancer to the gastrointestinal tract: a case series and review of the literature. *World J Gastroenterol* 2006; **12**: 6219-6224 [PMID: 17036400 DOI: 10.3748/wjg.v12.i38.6219]
67 **Signorelli C**, Pomponi-Formiconi D, Nelli F, Pollera CF. Single colon metastasis from breast cancer: a clinical case report. *Tumori* 2005; **91**: 424-427 [PMID: 16459641]
68 **Yoo JS**, Smith TJ, Ning SM, Lee MJ, Thomas PE, Yang CS. Modulation of the levels of cytochromes P450 in rat liver and lung by dietary lipid. *Biochem Pharmacol* 1992; **43**: 2535-2542 [PMID: 1632812 DOI: 10.1007/s10151-005-0235-0]
69 **Hsieh PS**, Yeh CY, Chen JR, Changchien CR. Ileocecal breast carcinoma metastasis. *Int J Colorectal Dis* 2004; **19**: 607-608 [PMID: 15480710 DOI: 10.1007/s00384-004-0612-6]
70 **Kobayashi T**, Shibata K, Matsuda Y, Tominaga S, Komoike Y, Adachi S. A case of invasive lobular carcinoma of the breast first manifesting with duodenal obstruction. *Breast Cancer* 2004; **11**: 306-308 [PMID: 15550851 DOI: 10.1007/BF02984554]
71 **Lottini M**, Neri A, Vuolo G, Testa M, Pergola L, Cintorino M, Guarnieri A. Duodenal obstruction from isolated breast cancer metastasis: a case report. *Tumori* 2002; **88**: 427-429 [PMID: 12487566]
72 **Daniels IR**, Layer GT, Chisholm EM. Bowel obstruction due to extrinsic compression by metastatic lobular carcinoma of the breast. *J R Soc Promot Health* 2002; **122**: 61-62 [PMID: 11989148 DOI: 10.1177/146642400212200118]
73 **Hata K**, Kitayama J, Shinozaki M, Komuro Y, Watanabe T, Takano T, Iwase S, Nagawa H. Intestinal perforation due to metastasis of breast carcinoma, with special reference to chemotherapy: a case report. *Jpn J Clin Oncol* 2001; **31**: 162-164 [PMID: 11386463 DOI: 10.1093/jjco/hye030]
74 **Cervi G**, Vettoretto N, Vinco A, Cervi E, Villanacci V, Grigolato P, Giulini SM. Rectal localization of metastatic lobular breast cancer: report of a case. *Dis Colon Rectum* 2001; **44**: 453-455 [PMID: 11289296 DOI: 10.1007/BF02234749]
75 **Yokota T**, Kunii Y, Kagami M, Yamada Y, Takahashi M, Kikuchi S, Nakamura M, Yamauchi H. Metastatic breast carcinoma masquerading as primary colon cancer. *Am J Gastroenterol* 2000; **95**: 3014-3016 [PMID: 11051411 DOI: 10.1111/j.1572-0241.2000.03238.x]
76 **Bar-Zohar D**, Kluger Y, Michowitz M. Breast cancer metastasizing to the rectum. *Isr Med Assoc J* 2001; **3**: 624-625 [PMID: 11519394]
77 **Shimonov M**, Rubin M. Metastatic breast tumors imitating primary colonic malignancies. *Isr Med Assoc J* 2000; **2**: 863-864 [PMID: 11344761]
78 **van Halteren HK**, Peters H, Gerlag PG. Large bowel mucosal metastases from breast cancer. *J Clin Oncol* 1998; **16**: 3711-3713 [PMID: 9817292 DOI: 10.1200/JCO.1998.16.11.3711]
79 **Clavien PA**, Laffer U, Torhost J, Harder F. Gastro-intestinal metastases as first clinical manifestation of the dissemination of a breast cancer. *Eur J Surg Oncol* 1990; **16**: 121-126 [PMID: 2157608]
80 **Hansen RM**, Lewis JD, Janjan NA, Komorowski RA. Occult carcinoma of the breast masquerading as primary adenocarcinoma of the small intestine. A case report. *J Clin Gastroenterol* 1988; **10**: 213-217 [PMID: 2843590 DOI: 10.1097/00004836-198804000-00023]
81 **Wang X**, Jin M, Ye Q, Wang M, Hu Y, Yang Y, Yang J, Cai J. Solitary duodenum metastasis from breast cancer with 8 years' latency: A case report. *Medicine (Baltimore)* 2018; **97**: e9550 [PMID: 29480844 DOI: 10.1097/MD.0000000000009550]
82 **Invento A**, Mirandola S, Pellini F, Pollini GP, Grigolato D. Breast cancer and gastrointestinal metastasis. A case report and review of the literature. *Ann Ital Chir* 2018; **89**: 153-156 [PMID: 29848817]
83 **Oh SJ**, Park SY, Kim JY, Yim H, Jung Y, Han SH. Small bowel obstruction from distant metastasis of primary breast cancer: a case report. *Ann Surg Treat Res* 2018; **94**: 102-105 [PMID: 29441340 DOI: 10.4174/astr.2018.94.2.102]
84 **Falco G**, Mele S, Zizzo M, Di Grezia G, Cecinato P, Besutti G, Coiro S, Gatta G, Vacondio R, Ferrari G. Colonic metastasis from breast carcinoma detection by CESM and PET/CT: A case report. *Medicine (Baltimore)* 2018; **97**: e10888 [PMID: 29794798 DOI: 10.1097/MD.0000000000010888]
85 **Numan L**, Asif S, Abughanimeh OK. Acute Appendicitis and Small Bowel Obstruction Secondary to Metastatic Breast Cancer. *Cureus* 2019; **11**: e4706 [PMID: 31355066 DOI: 10.7759/cureus.4706]
86 **Cherian N**, Qureshi NA, Cairncross C, Solkar M. Invasive lobular breast carcinoma metastasising to the rectum. *BMJ Case Rep* 2017; **2017** [PMID: 28775079 DOI: 10.1136/bcr-2016-215656]
87 **Liu M**, Zhang L, Guo L, Lv J, Shi W, Liu B. Intestinal metastasis from breast invasive ductal carcinoma after a long latency: case report and literature review. *Onco Targets Ther* 2018; **11**: 8599-8603 [PMID: 30584319 DOI: 10.2147/OTT.S180949]

88 **Schellenberg AE**, Wood ML, Baniak N, Hayes P. Metastatic ductal carcinoma of the breast to colonic mucosa. *BMJ Case Rep* 2018; **2018** [PMID: 29804074 DOI: 10.1136/bcr-2018-224216]

89 **Meekel JP**, Coblijn UK, Flens MJ, Muller S, Boer den FC. Small bowel obstruction caused by ^{18} FDG-negative ileocecal metastasis of lobular breast carcinoma. *J Surg Case Rep* 2020; **2020**: rjaa167 [PMID: 32760483 DOI: 10.1093/jscr/rjaa167]

90 **Kobayashi M**, Tashima T, Nagata K, Sakuramoto S, Osaki A, Ryozawa S. Colorectal and gastric metastases from lobular breast cancer that resembled superficial neoplastic lesions. *Clin J Gastroenterol* 2021; **14**: 103-108 [PMID: 33159678 DOI: 10.1007/s12328-020-01285-3]

91 **Vande Berg P**, Fonseca S, Al-Awa A, Rezai Monfared M, Delande S, Chamlou R, Etogo-Asse FE, Van Maele P. A rectal metastasis of an unknown lobular breast carcinoma and its management. *Acta Gastroenterol Belg* 2020; **83**: 327-330 [PMID: 32603055]

92 **Jain S**, Fisher C, Smith P, Millis RR, Rubens RD. Patterns of metastatic breast cancer in relation to histological type. *Eur J Cancer* 1993; **29A**: 2155-2157 [PMID: 8297656 DOI: 10.1016/0959-8049(93)90053-i]

93 **Borst MJ**, Ingold JA. Metastatic patterns of invasive lobular versus invasive ductal carcinoma of the breast. *Surgery* 1993; **114**: 637-41; discussion 641 [PMID: 8211676]

94 **Asch MJ**, Wiedel PD, Habif DV. Gastrointestinal metastases from crcinoma of the breast. Autopsy study and 18 cases requiring operative intervention. *Arch Surg* 1968; **96**: 840-843 [PMID: 5647555 DOI: 10.1001/archsurg.1968.01330230148023]

95 **Benfiguig A**, Anciaux ML, Eugène CI, Benkémoun G, Etienne JC. [Gastric metastasis of breast cancer occurring after a cancer-free interval of 30 years]. *Ann Gastroenterol Hepatol (Paris)* 1992; **28**: 175-177 [PMID: 1444182]

96 **Kidney DD**, Cohen AJ, Butler J. Abdominal metastases of infiltrating lobular breast carcinoma: CT and fluoroscopic imaging findings. *Abdom Imaging* 1997; **22**: 156-159 [PMID: 9013524 DOI: 10.1007/s002619900161]

97 **Bhargava R**, Beriwal S, Dabbs DJ. Mammaglobin vs GCDFP-15: an immunohistologic validation survey for sensitivity and specificity. *Am J Clin Pathol* 2007; **127**: 103-113 [PMID: 17145637 DOI: 10.1309/TDP92PQLDE2HLEET]

98 **Wang Z**, Spaulding B, Sienko A, Liang Y, Li H, Nielsen G, Yub Gong G, Ro JY, Jim Zhai Q. Mammaglobin, a valuable diagnostic marker for metastatic breast carcinoma. *Int J Clin Exp Pathol* 2009; **2**: 384-389 [PMID: 19158935]

99 **Taal BG**, Peterse H, Boot H. Clinical presentation, endoscopic features, and treatment of gastric metastases from breast carcinoma. *Cancer* 2000; **89**: 2214-2221 [PMID: 11147591]

100 **Matsui M**, Kojima O, Kawakami S, Uehara Y, Takahashi T. The prognosis of patients with gastric cancer possessing sex hormone receptors. *Surg Today* 1992; **22**: 421-425 [PMID: 1421863 DOI: 10.1007/BF00308791]

Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com