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EDITORIAL

PEDIATRIC CANCER AND THE ENVIRONMENT: A FIFTY-YEAR PERSPECTIVE

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In 1970, when I was the final months of my pediatric residency at Boston Children's Hospital, I spent four weeks on the children's cancer ward. This was a service staffed by some of the most dedicated physicians and nurses in that storied institution, and the care they provided the children was superb. However, the ward was a sad place, because in 1970 a diagnosis of childhood cancer was a death sentence. Chemotherapy was in its infancy. The chemicals were harsh and painful. The best outcome for which we could hope was a remission of a few months' duration. In that era, virtually every child with cancer died.

Since that time, progress in the treatment of childhood cancer has been spectacular. This progress has been the fruit of remarkable advances in medicine, surgery and basic biology. The first five-year survival of a child with pediatric leukemia was reported in the 1970s (1). Today, more than 85% of children with leukemia are cured, and the mortality rate for all forms of pediatric malignancy in the United States has fallen by 70% (2, **figure 1**). This is one of the great triumphs of modern medicine. Unfortunately, this success in the treatment of pediatric cancer is not the entire story. In the same years as childhood cancer deaths were falling because of better treatments, the incidence of childhood cancer – the number of new cases per 1,000 children – was increasing. Leukemia incidence in the United States has increased by 21% since 1976 (3), brain cancer incidence by 45% (3), and testicular cancer incidence by 51% (2). Cancer is now the leading cause of death by disease among American children under the age of 15 years.

The causes of these increases in cancer incidence are only partially understood. They are far too rapid to be of genetic origin. It has been suggested that they may reflect improved access to medical care or the increasingly widespread availability of newer diagnostic technologies such as MRI and CAT scan. That explanation might have accounted for a one-time "bump" in cancer incidence when Medicaid was introduced

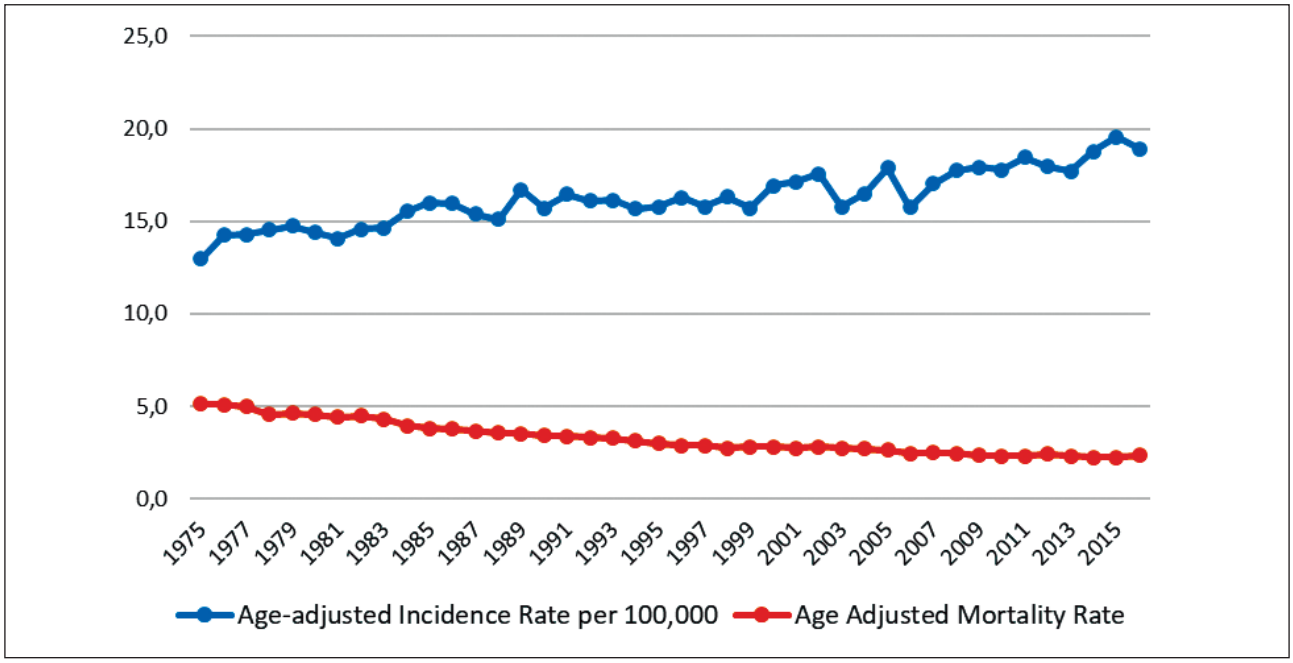

Figure 1. *Childhood cancer, United States - Age-adjusted incidence and mortality, 1974-2016. This figure comes from the National Cancer Institute SEER Program (2).*

or newer imaging techniques first became available. However, it fails to explain the continuing increase in incidence of three different types of childhood cancer over a span of five decades (4). The conclusion becomes inescapable that external, environmental factors must be responsible for at some of the increase.

MANUFACTURED CHEMICALS AND PEDIATRIC CANCER

Evidence is great and growing that environmental exposures, and especially exposures to manufactured chemicals, are in fact important contributors to childhood cancer. Children today are surrounded by an estimated 350,000 manufactured chemicals and chemical mixtures (5). These are novel materials, nearly all of them invented since 1950. They are produced in enormous quantities. Global production volume is on track to double by 2030. Manufactured chemicals now pollute every corner of the planet from the deepest ocean trenches to the high Himalayas. Several hundred are found in measurable quantities in the bodies of almost all persons on earth, including nursing mothers, infants and children (7). Some will persist for centuries. Chemical pollution has become so widespread and complex that an expert body recently concluded that it exceeds societies' abilities to monitor and contain it and thus threatens the safe operating space for humanity (8).

Manufactured chemicals cause disease, disability and death in children. Exposures in the first 1,000 days of life are especially dangerous. Polychlorinated biphenyls (PCBs), organophosphate insecticides, brominated flame retardants and phthalates are all linked to cognitive impairment, reduced intelligence, and behavioral problems (9). Prenatal exposures to phthalates are linked to abnormalities of the reproductive organs in baby boys (10). Early-life exposures to toxic chemicals appear linked to increased risk in later life of cardiovascular and renal disease (11, 12). Manufactured chemicals also cause cancer. The International Agency for Research on Cancer (IARC) has determined through meticulous independent review of the published epidemiological and toxicological data on over 1,000 manufactured chemicals and other environmental hazards that 120 agents are proven causes of cancer in humans (13). The majority of these proven human carcinogens remain in commerce today. Chemicals known to cause cancer in children include benzene, 1, 3-butadiene, and prenatal pesticide exposures (14). Prenatal exposure to DDT is linked to increased risk of female breast cancer in adult life (15).

FAILURE OF CHEMICAL POLICY

The root causes of this chemical crisis are the failure of the chemical industry to take responsibility

for the materials they produce, regulatory failure within countries, and shortcomings in global chemical governance. In most countries, manufactured chemicals are presumed to be harmless until they are proven to cause disease or environmental damage (16). They are brought to market with great enthusiasm but with little or no assessment of their potential dangers. Fewer than 50% of the most widely used manufactured chemicals have been tested for toxicity, and fewer than 20% have been examined for potential developmental toxicity (16). In consequence of this regulatory failure, new chemicals are incorporated into consumer products with no consideration of the hazards they may pose to human health or the environment. Early warnings of danger are ignored or even suppressed (17). The result is that time and again manufactured chemicals have been found – sometimes after years or even decades of use – to have caused great harm to children's health and the environment. Historical examples include tetraethyl lead added to gasoline, DDT, thalidomide, polychlorinated biphenyls (PCBs), diethylstilbestrol (DES) and the chlorofluorocarbons that almost destroyed the earth's stratospheric ozone layer. Newer chemicals that threaten to repeat this sorry history include the phthalates, bisphenols, neonicotinoid insecticides, brominated flame retardants, and perfluorinated substances (PFAS).

A further impediment to the control of hazardous chemicals has been the "risk assessment/risk management" paradigm, introduced in the 1970s (18). With its basis in the presumption that chemicals are harmless until proven to cause harm and its insistence on subjecting each chemical one at a time to exhaustive, multi-year analysis prior to any regulatory action, the "risk assessment/risk management" paradigm has paralyzed chemical control and impeded the protection of public health.

Of great concern to those who care for children is the likelihood that the chemical carcinogens that have been identified to date may account for only a small fraction of the cancers that are caused in children by manufactured chemicals. Almost certainly, there are additional carcinogenic chemicals in the modern environment. They are hidden among the many thousands of manufactured chemicals to which children are exposed every day. However, because most of these chemicals have never been tested for safety or toxicity, we do not know which of them may cause cancer, or which may be driving increases in cancer incidence. We are flying without radar.

The time has come for the oncology and the public health communities to come together to jointly confront the rising incidence of childhood cancer. We can no longer focus almost exclusively on cancer treatments. We can no longer dismiss rising trends in cancer incidence as diagnostic artifacts or the consequence of better reporting. We must instead deploy prevention-oriented research programs designed to discover the environmental causes of pediatric malignancy and implement science-based policies that focus on cancer prevention.

NEED FOR INCREASED RESEARCH INTO PEDIATRIC CANCER

The greatest impediment to discovery of the environmental causes of childhood cancer is lack of funding. In the United States, the National Institutes of Health awards only 3% to 7% of its total funding for childhood leukemia to studies evaluating environmental etiologies, including dietary factors, infections and chemicals (14). The majority of this funding comes from the National Institute for Environmental Health Sciences (NIEHS). The National Cancer Institute directs approximately 1% of its funding for childhood cancer toward research into environmental causes (14).

Increased funding into the environmental causes of childhood cancer has potential to yield a high return on investment. Large, prospective, multi-year birth cohort studies that incorporate assessments of prenatal environmental exposures are especially powerful engines of scientific discovery because they permit unbiased assessment of exposures as they occur before the onset of disease. To bring together data on the preventable, environmental causes of childhood cancer from multiple prospective birth cohort studies in countries around the world, the World Health Organization has organized the International Childhood Cancer Cohort Consortium (I4C) (19). The launching of additional prospective studies would increase this database and further enhance global capacity for discovery of the preventable causes of childhood cancer.

NEED FOR FUNDAMENTAL REVISION OF CHEMICAL POLICY

Chemical policies in all countries need to pivot away from the failed risk assessment/risk management paradigm (18) and its presumption that chemicals

are harmless until proven to cause disease or environmental damage. Chemical management policies must instead be based on the Precautionary Principle, (17) which assumes that all manufactured chemicals are hazardous until they are proven to be safe, and on the Essential Use Doctrine, which states that new chemicals cannot be brought to market unless their use is deemed essential (20).

In short, a new health-protective approach to chemical management must embody the "No Data, No Market' Principle, already articulated in the European Union in its REACH legislation (21) which requires that all new manufactured chemicals be tested for safety and toxicity before they are allowed to enter markets, and that all existing chemicals must be tested – beginning with the worst first – if they are to remain on markets. National chemical policies must require that all manufactured chemicals be subjected to the same degree of scrutiny before they enter markets as chemicals that are intended to be used as pharmaceuticals.

Additional key components of health-protective chemical management policies will be the adoption of strict procedures for full disclosure and elimination of all conflicts of interest and an insistence that testing of chemicals for safety and toxicity be conducted in independent laboratories rather than in laboratories controlled by the chemical industry (22). The current system in which chemical manufacturers generate virtually all data on the potential hazards of new chemicals is broken and must be replaced. New procedures for assessment of the risks of chemicals need also to embody a clearly articulated emphasis on human rights, equity and protection of vulnerable populations**,** including infants and children against the hazards of manufactured chemicals (23). Lastly, they need to incorporate an explicitly articulated intent to reduce unnecessary use of manufactured chemicals and to transition to a more circular economy that emphasizes planetary stewardship (24) and care for the earth, our Common Home (25).

CONCLUSIONS

Need for a new paradigm

The rising incidence of childhood cancer poses a major challenge to our society and to the oncology and public health communities. The time has come for our communities to come together to jointly confront this growing problem. Going forward, we need to insist that all new chemicals and all widely used existing chemicals be tested for safety and toxicity. We can no longer allow our children to be exposed to thousands of manufactured chemicals of unknown hazard. We need to support strong research programs that include epidemiological and toxicological studies. We need to strengthen legislation in all countries to better protect our children and we need to enforce these laws. We need to work with chemical researchers and the business community to develop new green chemicals that will sustain our society without harming future generations. We must act together as wise guardians of our children and of our planet.

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ORIGINAL ARTICLE

PATIENT-REPORTED FINANCIAL TOXICITY WITHIN THE ITALIAN PUBLIC HEALTHCARE SYSTEM: A SINGLE CENTER CROSS-SECTIONAL ANALYSIS IN PATIENTS WITH CANCER

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ABSTRACT

PROFFIT (Patient Reported Outcome for Fighting FInancial Toxicity of cancer) questionnaire has been developed in Italy, within a universal healthcare system, for measuring financial toxicity (FT) in patients with cancer and understanding its determinants. Our aim was to describe the amount of FT in patients with cancer, by using the PROFFIT questionnaire, in subjects treated in a public Italian institution. Between May and July 2021 we administered, on one-off basis with a cross-sectional approach, the PROFFIT questionnaire to 167 outpatients receiving active anticancer treatment at the Oncology Day Hospital of Ordine Mauriziano Hospital, Turin, Italy. Answers were matched with relevant clinical and demographic characteristics.

Median FT score in the overall population was 23.81 (IQR 14.29-47.62). FT score was significantly higher in younger patients, in those with worse educational level, in private employees and unemployed, and in subjects with economically dependent familiars.

No significant differences were found according to gender, time from diagnosis, type of tumor, type of treatment and disease setting. There was a significant association between FT score and the presence of economic damage due to COVID-19 for the patient or the family: median FT score was 14.29, 33.33 and 47.61 for those declaring no damage, a little damage and much damage ($p < 0.001$).
Our analysis, conducted during the COVID-19 pandemic, shows that financial toxicity is not negligible in patients with cancer, also in Italy, a country with universal healthcare system.

KEY WORDS

Financial toxicity; patient-reported outcomes; cancer; Italian health system; PROFFIT.

IMPACT STATEMENT

We administered the PROFFIT questionnaire to 167 outpatients receiving active anticancer treatment at Mauriziano Hospital, Turin, Italy, showing that financial toxicity is not negligible in patients with cancer, also in a country with universal healthcare system.

INTRODUCTION

Financial toxicity (FT) experienced by patients after a diagnosis of cancer has been increasingly discussed and reported worldwide, within countries with different healthcare systems (1-7). Initially, FT has been described in the US, as a factor negatively affecting cancer patients (2). In detail, both QoL and survival have been reported to be worse among patients facing with financial hardships and bankruptcy (8, 9). This is not surprising, considering that the US health system requires out of pocket co-payment of medical expenses and that the cost of cancer treatments has significantly increased in recent years.
The need of a specific instrument to measure FT has been previously addressed in the US, with the development and validation of the Comprehensive Score for Financial Toxicity (COST) instrument (10, 11). However, differently from US, Italy has a public health system, where patients should not directly sustain the expenses related to diagnosis and treatment of cancer. Since 1978, the Italian health care system is designed with a National Health Service model, where the State is the most important financer, via general tax levies. Some years ago, financial difficulties among Italian cancer patients enrolled in 16 clinical trials have been reported in a not negligible proportion of subjects, showing a relevant association with worse quality of life and overall survival (1). Namely, using the answer to item 28 of EORTC QLQ C30 ("Has your physical condition or medical treatment caused you financial difficulties?"), that analysis showed that patients reporting some degree of financial burden at baseline had a higher chance of worsening global quality of life (QoL) during the treatment, and that patients, who developed financial toxicity during treatment, had a statistically significant shorter survival (1).
Therefore, in 2018, in order to develop an instrument for measuring and understanding the determinants of FT in patients with cancer, sensitive to dimensions of a universal healthcare system, the multicentre PROFFIT (Patient Reported Outcome for Fighting FInancial Toxicity of cancer) project was started (12-14). That project led to the production of the PROFFIT questionnaire which is, to the best of our knowledge, the first instrument for FT fully published from a European country.
With the aim of describing the amount of FT in patients with cancer treated in a public Italian institution, we administered the PROFFIT questionnaire to outpatients receiving active treatment at the Oncology Day Hospital of Ordine Mauriziano Hospital, in Turin, Italy.

METHODS

Patients

PROFFIT questionnaire was administered, in paper format, to adult outpatients who were receiving any type of active systemic treatment (chemotherapy, targeted agents, immune checkpoint inhibitors, hormonal treatment) for a solid tumor. Both patients who were starting a treatment and those who were already on treatment were eligi-

ble. Patients were eligible independently of tumor stage, and both patients receiving a (neo)adjuvant treatment and those with advanced disease were included in the analysis.

PROFFIT questionnaire

The PROFFIT questionnaire includes the FT-score (consisting of 7 items) and 9 single items assessing possible determinants of FT. Among the latter ones, 4 items are related to medical expenses (coverage by National Health service; private visits and examinations; medicines and/or supplements; additional expenses), 2 items are related to transportation (distance from hospital and costs of transportation), and 3 items are related to support from health system (doctors and nurses; administrative staff; communication among parties). Responses to PROFFIT items are coded in 4 categories of agreement with the statement of each item, scoring from 1 to 4: 1 (I do not agree at all), 2 (I agree partially), 3 (I agree substantially), 4 (I very much agree).

In addition to the 16 PROFFIT items, information about economically relevant factors (education level, marital status, living alone, presence of dependents among family members, working status, economic damage from COVID-19 pandemic) were collected too, with dedicated questions added to the paper questionnaire. After the collection, each questionnaire was transcripted by an author (G.G.) into an electronic Excel database, and main clinical characteristics (gender, age, time from cancer diagnosis, type of primary tumor, type of anticancer treatment and disease setting) were collected by the same author from patient's electronic medical records.

Statistical issues

PROFFIT results are reported as a FT-score (including items #1 to #7) and 9 separate items for FT determinants. According to the methodology previously described, all the scores are normalised to 0-100%, where 100 indicates the highest toxicity (14). For calculation of the FT-score, including items #1 to #7, the following steps should be followed: (1) reverse the score for Item #1 according to the following formula:

$$X_{1-reverse} = 5 - X_1$$

where X_1 is the response given to item #1; (2) calculate the FT-score according to the following formula:

$$\frac{X_{1-reverse} + X_2 + X_3 + X_4 + X_5 + X_6 + X_7 - Y}{3 \times Y} \times 100$$

where X is the response given for each item and Y is the number of items with valid response; if Y is 3 or less the score should be considered missing. At least 4 valid responses are needed to calculate the FT-score. For calculation of the score for items #8, #14, #15 and #16 use the following formula

$$\frac{4 - X_j}{3} \times 100$$

where X is the response given and j is the item (8, 14, 15, or 16). For calculation of the score for items #9, #10, #11, #12, #13 use the following formula

$$\frac{X_j - 1}{3} \times 100$$

where X is the response given and j is the item (9, 10, 11, 12 or 13).

There was no formal sample size planning for this study. Statistical analyses were essentially descriptive. Categorical variables are described with frequencies and percentages. PROFFIT scores were reported both as mean (and standard deviation) and median (an interquartile range, IQR). FT scores were compared between groups by Mann-Whitney test (for variables with 2 groups) and Kruskal Wallis test (for variables with more than 2 groups). All statistical tests were two-tailed and p-values less than 0.05 were considered statistically significant. Because of the exploratory nature of this analysis, adjustment for multiple item comparisons was not performed. Analyses were performed with SPSS for Windows, version 27.0.

Ethical issues

Our institution was involved in the development and validation of the PROFFIT questionnaire: the study protocol was initially approved by the Ethics Committee of the National Cancer Institute of Naples, which acted as coordinating Ethics Committee, and was subsequently approved by our Ethics Committee.

Following the development of PROFFIT questionnaire within the clinical trial, we administered the same questionnaire to patients routinely treated at our center. Before filling questionnaires, all patients signed a written consent for the treatment of personal data, in anonymous format.

RESULTS

Between May and July 2021, we administered the PROFFIT questionnaire to 170 patients treated at Oncology Day Hospital, Mauriziano Hospital, Turin, Ita-

ly. Three patients were excluded because they were not receiving active anticancer treatment, so the remaining 167 patients were eligible for the analysis. Of them, 24 patients (14.4%) compiled the questionnaire on the day of treatment line start, further 28 (16.8%) had started that line of treatment less than 1 month before, and the remaining 115 (68.9%) had started their line of treatment more than 1 month before.

Main demographic and clinical characteristics of patients included in the analysis are shown in **table I**. Participants were mostly females (110, 65.9%), and median age was 66 years (range 34-87), with 79 patients (47.3%) under 70 years and the remaining 88 (52.7%) older than 70. About half of the patients (82, 49.1%) were resident in the city of Turin, while the remaining 85 (50.9%) were resident outside the city. More than half (59.9%) of the patients had a high level of education (high school or degree), and 108 (65.1%) were married. Forty-one patients (24.6%) lived alone, and 41 patients (24.6%) had 1 or more dependents among family members. In terms of employment status, more than half of the patients (98, 59.4%) were retired, 20 (12.1%) public employees, 19 (11.5%) private employees; 17 (10.3%) were unemployed. Time from tumor diagnosis was lower than 1 year in 90 (53.9%) of patients. The most common tumors were gastrointestinal cancers (71, 42.5%, namely 25 colorectal cancers and 46 upper tract cancers), breast cancer (38, 22.8%), gynecologic cancers (20, 12.0%) and lung cancer (18, 10.8%). Most common treatments received at the time of PROFFIT administration were chemotherapy (123, 73.7%), targeted drugs (20, 12.0%) and immune checkpoint inhibitors (20, 12.0%). Most patients were receiving treatment for advanced disease, as first-line (78, 46.7%) or second-line and beyond (39, 23.4%).

Detailed answers and scores for each of the items included in the PROFFIT questionnaire are reported in **table II**. When asked about their "ability of affording monthly expenses (*e.g.*, rent, electricity, phone) without difficulty", 51 patients (30.5%) declared not agreeing at all or only partially. Proportion of patients declaring substantial or very much agreement was 27.5% for the statement "My illness has reduced my financial resources"; 35.9% for the statement "I am concerned by the economic problems I may have in the future due to my illness" and 15.6% for the statement "My economic situation affects the possibility of receiving medical care". In addition, proportion of patients declaring substantial or very much agreement was 26.3% for the statement "I have reduced my spending on leisure activities such as holidays, restaurants or entertainment in order to cope with expenses related to my illness", 12.0% for the statement "I have reduced spending on essential goods (*e.g.*, food) in order to cope with expenses related to my illness" and 30.5% for the statement "I am worried that I will not be able to work due to my illness". Excluding retired patients from this latter item, proportion of patients declaring substantial or very much worry of not being able to work due to the illness rised to 47.8%.

Based on the above described outcome items, mean FT score in the 167 patients was 29.28 (SD 21.78), and median score was 23.81 (IQR 14.29-47.62), as reported in **table II**. Distribution of FT scores in the whole series of patients is reported in **figure 1**. The association of FT score with patients' characteristics is reported in **table III**. FT score was

	N	%
All patients	**167**	
GENDER		
Male	57	34.1%
Female	110	65.9%
AGE		
Younger than 70 years	79	47.3%
Older than 70 years	88	52.7%
RESIDENCE		
City of Turin	82	49.1%
Outside Turin	85	50.9%
EDUCATION LEVEL		
Primary (elementary)	22	13.2%
Middle school	45	26.9%
High school	72	43.1%
Degree	28	16.8%
MARITAL STATUS (1 MISSING)		
Married	108	65.1%
Divorced	16	9.6%
Cohabiting	7	4.2%
Unmarried	13	7.8%
Widow(er)	22	13.3%
LIVING ALONE		
No	126	75.4%
Yes	41	24.6%
WITH DEPENDENT FAMILY MEMBERS		
No	126	75.4%
Yes	41	24.6%

Table I. *Patients' characteristics. (Continued).*

	N	%
WORKING STATUS (2 MISSING)		
Public employee	20	12.1%
Private employee	19	11.5%
Free lance	11	6.7%
Retired	98	59.4%
Unemployed	17	10.3%
ECONOMIC DAMAGE FROM COVID-19		
Not at all	89	53.3%
Quite a bit	63	37.7%
Very much	15	9.0%
TIME FROM CANCER DIAGNOSIS		
Less than 12 months	90	53.9%
More than 12 months	77	46.1%
TYPE OF TUMOR		
Thoracic	18	10.8%
Breast	38	22.8%
Gastrointestinal, colorectal	25	15.0%
Gastrointestinal, non colorectal	46	27.5%
Genito-urinary	15	9.0%
Gynecologic	20	12.0%
Other	5	3.0%
TYPE OF TREATMENT		
Chemotherapy +/- other	123	73.7%
Targeted agents	20	12.0%
Immunotherapy	20	12.0%
Hormonal treatment	4	2.4%
DISEASE SETTING		
(Neo)adjuvant	50	29.9%
Advanced, first-line	78	46.7%
Advanced, second- / further lines	39	23.4%

Table I. *Patients' characteristics.*

significantly associated with age (worse in younger patients), educational level (better in graduated subjects), occupational status (worse in private employees and in unemployed subjects), presence of economically dependent familiars. On the other hand, there was no significant association of FT score with sex, marital status, time from tumor diagnosis, type of tumor, type of treatment and setting of disease. As expected, there was a significant association between FT score and the presence of economic damage due to COVID for the patient or the family (**figure 2**). Namely, median FT score was 14.29, 33.33 and 47.61 for those declaring no damage, a little damage and much damage.

As for determinants of FT, when asked if "the National Health Service covers all health costs related to their illness", 66 patients (39.5%) declared not agreeing at all or only partially. Proportion of patients declaring substantial or very much agreement was 34.7% for the statement "I have paid for one or more private medical examinations for my illness", 46.7% for the statement "I have paid for additional medicines or supplements related to my illness" and 26.9% for the statement "I have to pay for additional treatment (*e.g.*, physiotherapy, psychotherapy, dental care) myself". As for expenses related to distance from the treatment centre and related costs, proportion of patients declaring substantial or very much agreement was 33.5% for the statement "The treatment centre is a long way from where I live" and 25.1% for "I have spent a considerable amount of money on travel for treatment". As expected, answers to these 2 questions were significantly related to the residence of patients: proportion declaring substantial or very much agreement to the former statement was 11.0% among patients resident within Turin *vs.* 55.3% among those resident outside Turin, while for the latter statement proportion was 13.4% *vs.* 36.5%, respectively. As for support from the health staff, proportion of patients who declared not agreeing at all or only partially was 4.2% for the statement "Medical staff (*i.e.*, doctors, nurses, *etc.*) have been helpful throughout my medical care", 10.8% for "Staff in hospital administration (*i.e.*, for booking appointments, secretaries) have been helpful throughout my medical care" and 12.0% for "Medical staff and medical facilities I attended communicated with each other".

DISCUSSION

This analysis shows that FT in patients with cancer treated at a public institution in Italy, during the COVID-19 pandemic, was not negligible. When testing the association between patients' characteristics and impact of financial toxicity, FT score was significantly higher in younger patients (*i.e.* subjects of working age), in those with worse educational level, in private employees and unemployed patients, and in subjects with economically dependents among their family members. On the other hand, no significant differences were found

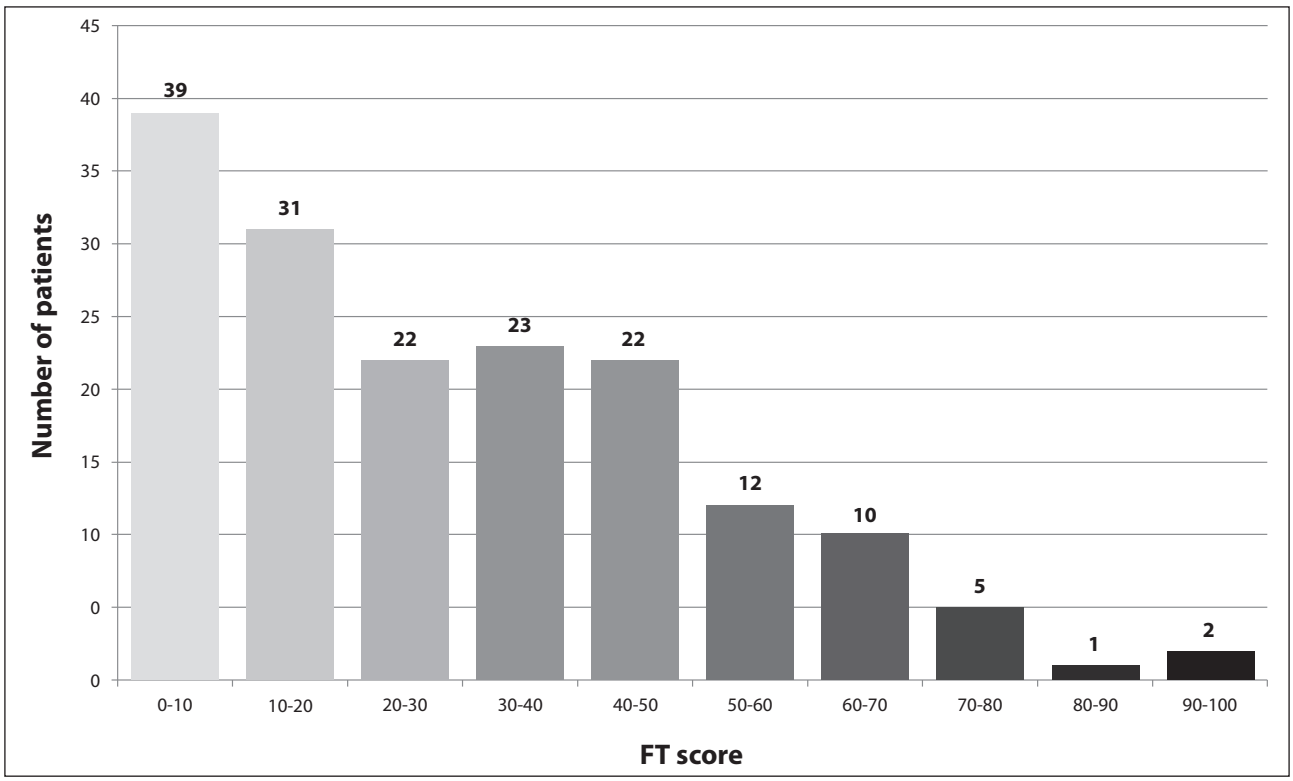

Figure 1. *Distribution of Financial Toxicity score, based on the first 7 items of PROFFIT questionnaire, in the 167 patients included in the analysis. The score is normalised from 0 to 100, where 100 indicates the highest financial toxicity.*

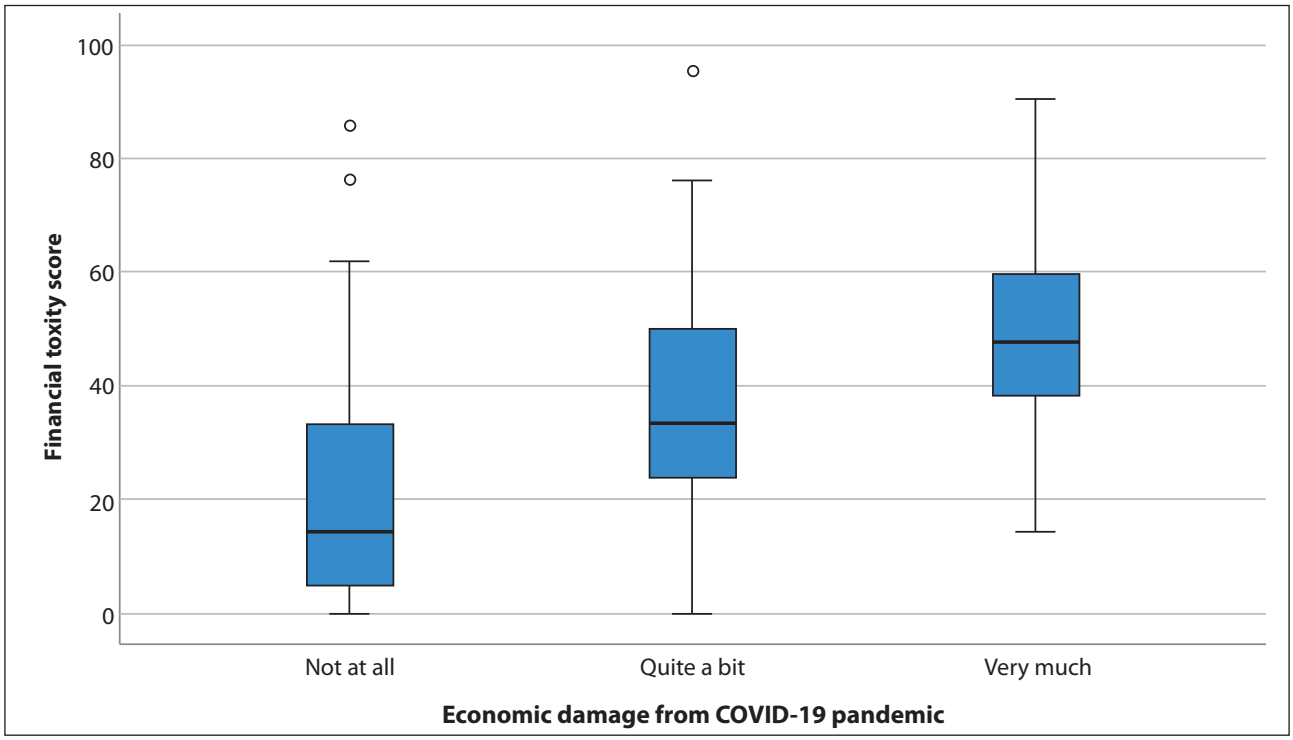

Figure 2. *Box plot of Financial Toxicity score in the 167 patients included in the analysis, according to economic damage from COVID-19 pandemic. The thick line in the middle is the median. The top and bottom box lines show the first and third quartiles. The whiskers show the maximum and minimum values, with the exceptions of outliers (circles).*

according to gender, time from diagnosis, type of tumor, type of treatment and disease setting. As expected, there was a significant association between FT score and the presence of economic

		1 - I DO NOT AGREE AT ALL	2 - I AGREE PAR-TIALLY	3 - I AGREE SUB-STAN-TIALLY	4 - I VERY MUCH AGREE	SCORE	
						MEAN (SD)	MEDIAN (IQR)
	OUTCOME ITEMS (FT SCORE)						
1	I can afford my monthly expenses without difficulty (*e.g.*, rent, electricity, phone...)	16 (9.6%)	35 (21.0%)	69 (41.3%)	47 (28.1%)	29.28 (21.78)	23.81 (14.29-47.62)
2	My illness has reduced my financial resources	64 (38.3%)	57 (34.1%)	36 (21.6%)	10 (6.0%)		
3	I am concerned by the economic problems I may have in the future due to my illness	49 (29.3%)	58 (34.7%)	40 (24.0%)	20 (12.0%)		
4	My economic situation affects the possibility of receiving medical care	94 (56.3%)	47 (28.1%)	18 (10.8%)	8 (4.8%)		
5	I have reduced my spending on leisure activities such as holidays, restaurants or entertainment in order to cope with expenses related to my illness	81 (48.5%)	42 (25.1%)	21 (12.6%)	23 (13.8%)		
6	I have reduced spending on essential goods (*e.g.*, food) in order to cope with expenses related to my illness	123 (73.7%)	24 (14.4%)	18 (10.8%)	2 (1.2%)		
7	I am worried that I will not be able to work due to my illness	87 (52.1%)	29 (17.4%)	25 (15.0%)	26 (15.6%)		
	DETERMINANT ITEMS (SINGLE ITEMS)						
8	The National Health Service covers all health costs related to my illness	26 (15.6%)	40 (24.0%)	65 (38.9%)	36 (21.6%)	44.51 (32.85)	33.33 (33.3-66.67)
9	I have paid for one or more private medical examinations for my illness	65 (38.9%)	44 (26.3%)	32 (19.2%)	26 (15.6%)	37.13 (36.48)	33.33 (0-66.67)
10	I have paid for additional medicines or supplements related to my illness	39 (23.4%)	50 (29.9%)	51 (30.5%)	27 (16.2%)	46.51 (33.92)	33.33 (33.33-66.67)
11	I have to pay for additional treatment myself (*e.g.*, physiothrapy, psychotherapy, dental care)	78 (46.7%)	44 (26.3%)	26 (15.6%)	19 (11.4%)	30.54 (34.60)	33.33 (33.33-66.67)
12	The treatment centre is a long way from where I live	59 (35.3%)	52 (31.1%)	35 (21.0%)	21 (12.6%)	36.93 (34.33)	33.33 (0-66.67)
13	I have spent a considerable amount of money on travel for treatment	78 (46.7%)	47 (28.1%)	25 (15.0%)	17 (10.2%)	29.54 (33.62)	33.33 (0-66.67)
14	Medical staff (*i.e.*, doctors, nurses, *etc.*) have been helpful throughout my medical care	1 (0.6%)	6 (3.6%)	36 (21.6%)	124 (74.3%)	10.18 (18.91)	0 (0-33.33)
15	Staff in hospital administration (*i.e.*, for booking appointments, secretaries, *etc.*) have been helpful throughout my medical care	5 (3.0%)	13 (7.8%)	43 (25.7%)	106 (63.5%)	16.78 (25.58)	0 (0-33.33)
16	Medical staff and medical facilities I attended communicated with each other	9 (5.4%)	11 (6.6%)	41 (24.6%)	106 (63.5%)	17.96 (28.04)	0 (0-33.33)

Table II. *Answers to the 16 items of the PROFFIT questionnaire (n = 167 patients).*

damage due to COVID for the patient or the family. The PROFFIT questionnaire has been developed in Italy, so in this analysis it was used within the specific context which led to its development and validation (12-14). This is particularly important for an instrument which aims to measure determinants of financial burden, which can be largely different among countries with different health systems (16). Moreover, the inclusion in PROFFIT of several items related to determinants of FT may be helpful to identify potential targets

for action, both at a local and a national level. Despite Italian public health system should cover all the needs of cancer patients, we showed that many patients declare some trouble with several potential determinants of FT. For instance, items related to transportation show that a minority of patients declared a long distance between home and the hospital, and relevant costs for transportation, with higher proportion, as

		FINANCIAL TOXICITY SCORE		
	N	MEAN (SD)	MEDIAN (IQR)	
ALL PATIENTS	**167**	**29.28 (21.78)**	**23.81 (14.29-47.62)**	
Sex				0.52
Male	57	27.90 (21.92)	23.81 (9.52-42.86)	
Female	110	30.00 (21.77)	23.81 (14.29-47.62)	
Age				**0.002**
Younger than 70 years	79	34.54 (22.33)	14.29-47.62)	
Older than 70 years	88	24.57 (20.27)	19.05 (9.52-36.90)	
Residence				0.99
City of Turin	82	29.97 (23.84)	23.81 (9.52-47.62)	
Outside Turin	85	28.63 (19.71)	23.81 (14.29-42.86)	
Education level				**0.024**
Primary (elementary)	22	29.65 (19.82)	33.33 (14.29-48.81)	
Middle school	45	31.32 (21.06)	33.33 (14.29-47.62)	
High school	72	32.28 (23.76)	30.95 (14.29-42.86)	
Degree	28	18.03 (15.68)	14.29 (9.52-19.05)	
Marital status				0.58
Married	108	28.53 (21.63)	23.81 (14.29-42.86)	
Divorced	16	35.42 (19.67)	40.48 (16.67-52.38)	
Cohabiting	7	27.21 (27.45)	9.52 (4.76-57.14)	
Unmarried	13	34.07 (26.36)	23.81 (14.29-64.29)	
Widow(er)	22	26.84 (20.50)	21.43 (9.52-44.05)	
Living alone				0.38
No	126	28.38 (21.47)	23.81 (14.29-42.86)	
Yes	41	32.06 (22.76)	33.33 (11.90-50.00)	
With dependent family members				**0.006**
No	126	26.76 (21.30)	23.81 (9.52-42.86)	
Yes	41	37.05 (21.65)	33.33 (21.49-52.38)	
Working status				**0.011**
Public employee	20	26.90 (21.92)	16.67 (14.29-38.10)	
Private employee	19	40.85 (21.26)	42.86 (23.81-52.38)	
Free lance	11	33.77 (17.75)	23.81 (19.05-47.62)	
Retired	98	24.83 (20.07)	23.81 (8.33-39.29)	
Unemployed	17	36.69 (24.44)	33.33 (16.67-57.14)	
Economic damage from COVID-19				**< 0.001**
Not at all	89	20.81 (19.47)	14.29 (4.76-33.33)	
Quite a bit	63	36.73 (19.68)	33.33 (23.81-52.38)	
Very much	15	48.25 (20.90)	47.61 (33.33-66.67)	
Time from cancer diagnosis				0.44
Less than 12 months	90	30.58 (22.37)	26.19 (14.29-47.62)	
More than 12 months	77	27.78 (21.12)	23.81 (9.52-42.86)	

Table III. *Financial toxicity score in the whole population and according to patients' characteristics. (Continued).*

		FINANCIAL TOXICITY SCORE		
	N	MEAN (SD)	MEDIAN (IQR)	
ALL PATIENTS	**167**	**29.28 (21.78)**	**23.81 (14.29-47.62)**	
Type of tumor				0.38
Thoracic	18	28.31 (28.92)	23.81 (4.76-48.81)	
Breast	38	34.84 (23.49)	35.71 (14.29-52.38)	
Gastrointestinal	71	29.18 (21.07)	28.57 (9.52-42.86)	
Colorectal	*25*	*22.29 (20.05)*	*14.29 (4.76-38.10)*	
Non colorectal	*46*	*32.92 (20.86)*	*33.33 (14.29-47.62)*	
Genito-urinary	15	21.27 (16.58)	19.05 (4.76-33.33)	
Gynecologic	20	25.24 (17.69)	23.81 (14.29-33.33)	
Other	5	32.38 (13.64)	33.33 (19.05-45.24)	
Type of treatment				0.63
Chemotherapy +/- other	123	29.11 (21.39)	23.81 (14.29-47.62)	
Targeted agents	20	30.24 (23.60)	33.33 (5.95-46.43)	
Immunotherapy	20	31.90 (23.44)	23.81 (14.29-46.43)	
Hormonal treatment	4	16.67 (19.25)	16.67 (0-33.33)	
Disease setting				0.96
(Neo)adjuvant	50	29.05 (21.39)	23.81 (14.29-47.62)	
Advanced, first-line	78	29.73 (21.95)	26.19 (14.29-44.05)	
Advanced, second- /further lines	39	28.69 (22.48)	23.81 (9.52-42.86)	

Table III. *Financial toxicity score in the whole population and according to patients' characteristics.*

expected, among those living outside the city of Turin. The majority of patients treated at Mauriziano Hospital come from Turin city and neighbouring municipalities, but this issue can be even higher at institutions which treat a higher number of patients coming from other provinces or regions (17). As a general rule, with the exception of those patients who are included in a clinical trial which is only available at our center, we usually propose all patients to be treated in the hospital closest to home, to avoid a negative impact on quality of life and to reduce financial and logistical burden related to transportation issues. As for additional medical expenses not covered by the public health system, these have been declared by a not negligible proportion of patients included in our analysis. On the other hand, we were particularly satisfied by the overall answers to the last 3 items of PROFFIT, pertaining to the quality of assistance by medical and administrative staff and to the efficiency of communication among the different operators. Our Hospital is in Turin, in a Region where the oncologic network ("Rete Oncologica") is considered well established since many years, and this should, at least in principle, assure the efficiency of the diagnostic, therapeutic and assistance path for patients which come to our hospital with a suspect of cancer diagnosis. Of course, this does not necessarily reflect this issue in all other Italian Regions, considering that the degree of implementation of oncologic networks is not the same in the whole country. From this point of view, the larger study currently ongoing within the PROFFIT project (NCT03473379), involving our hospital among many other Italian institutions, distributed among the Italian macro-regions (North, Centre, South, Islands) could be helpful to describe differences, if any, among different parts of the country.

Beyond the single-center dimension discussed above, our analysis has some important limitations. Firstly, it was based on a single questionnaire, administered on a one-off basis, and patients reported about their FT in different moments of their disease trajectory. All respondents were on active treatment (mostly chemotherapy, but not exclusively), but time from cancer diagnosis, time from treatment start and disease setting (adjuvant *vs.* advanced) were quite heterogeneous. Of course, the cross-sectional approach adopted in this analysis allows a rough comparison between different categories (*e.g.* adjuvant *vs.* advanced, shorter *vs.* longer time from cancer diagnosis), but not the description of changes over time in the same patient. Within

the PROFFIT project, an ongoing study with the repeated administration of questionnaires during the course of patients' treatment will allow a better description of the changes over time of FT.

Second, our results are unavoidably conditioned by the impact of COVID-19 emergency. The COVID-19 pandemic in Italy, like in all other countries, has produced a dramatic impact on the management of patients with cancer (15). Beyond the impact on patients' management in terms of treatment decisions, rules for access in the hospital *etc*., COVID-19 pandemic, with social restrictions and limitations of economic activities, had major consequences on economic income of many people, potentially including patients with cancer and their families. All the questionnaires described here were administered between May and July 2021, just after the second and third waves of the pandemic emergency. This could represent a major limitation for the generalization of our results. Results could have been sensibly different 2 years before, and could be (hopefully) different in the near future, with the evolution / resolution of the pandemic emergency. However, we documented a strong association between FT score and the economic damage from COVID-19 declared by patients.

In conclusion, our analysis confirms that FT is not negligible in patients with cancer, also in a country with universal healthcare system like Italy, and in a Region like Piedmont, where the oncologic network is considered well established since many years. The PROFFIT questionnaire was successfully administered, with optimal compliance. This approach will hopefully provide insights on how to fight against FT, in order to improve the outcomes of cancer patients.

ETHICS

Fundings

There were no institutional or private fundings for this article.

Conflict of interests

Massimo Di Maio reports personal fees from AstraZeneca, Pfizer, Novartis, Roche, Takeda, Janssen, Eisai, Astellas, Merck Sharp & Dohme, Boehringer Ingelheim, grants from Tesaro - GlaxoSmithKline, outside the submitted work. Francesco Perrone reports grants and personal fees from Bayer, Astra Zeneca, Pierre Fabre, Roche, Incyte, MSD, Janssen Cilag, personal fees from Daichii Sankyo, Clovis, Bristol Myers Squibb, Astellas, Ipsen, Seagen, Eli Lilly, GSK, grants from Tesaro, Pfizer, Exelixis, Aileron, outside the submitted work. All remaining authors declared no conflicts of interest.

Availability of data and materials

The raw dataset used for the analysis reported in this article is available online as **Supplementary materials**.

Code availability

N/A.

Authors' contributions

FDV, GG, MDM contributed to the study conception and design. Material preparation and data collection were performed by FDV, GG, AB, LF, LP, VA and ST. The first draft of the manuscript was written by FDV, GG and MDM, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical approval

Our institution was involved in the development and validation of the PROFFIT questionnaire: the study protocol was initially approved by the Ethics Committee of the National Cancer Institute of Naples, which acted as coordinating Ethics Committee, and was subsequently approved by our Ethics Committee. Following the development of PROFFIT questionnaire within the clinical trial, we administered the same questionnaire to patients routinely treated at our center.

Consent to participate

Before filling questionnaires, all patients signed a written consent for the treatment of personal data, in anonymous format.

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ORIGINAL ARTICLE

THE INCIDENCE OF CANCER AT THE TIME OF COVID-19 IN NORTHERN ITALY

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ABSTRACT

Recent studies have assessed the impact of the COVID-19 pandemic and related control measures on the number of new cancer diagnoses. The aim of this work was to evaluate the real impact of the lockdown on new cancer diagnoses in 2020. To compare the incidence of tumors in 2020 with that in 2019, we used data collected by the Reggio Emilia Cancer Registry. We reported the variations (number of cases and % values) of all tumors and of the main sites by sex and period of lockdown. We calculated the standardized incidence and mortality rate of the last twenty years (2001-2020) for all tumor sites and the main sites (breast, colorectal, lung and prostate) by sex. In 2020, 4,031 cases of cancer were recorded, 669 fewer than in 2019 (-14.2%). The sites that recorded the largest decline compared to 2019 were: skin (non-melanoma) (-281 cases), prostate (-110 cases), melanoma and bladder (-53 cases) and colorectal (-38 cases). The incidence trend in males decreased from 491.74 cases per 100,000 p/y in 2001 to 471.58 in 2019 and dropped to 386.59 in 2020. Mortality also decreased over the years from 250.8 cases per 100,000 p/y in 2001 to 164.4 cases in 2019 and 161.9 in 2020. In women, the incidence remained almost constant over the years, whereas there was a decline in mortality. The decrease in cancers recorded, especially during the lockdown, has been widely reported in the literature, but the data usually only cover the months leading up to September 2020. The COVID-19 pandemic has caused delays in the diagnosis of new cancers. However, it is necessary to document with data the real impact the pandemic has had on new diagnoses, taking into account the tumor site, gender, the presence of cancer screening, and in general the organization of healthcare of the territory in question.

KEY WORDS

Cancer; Covid-19; impact; incidence.

IMPACT STATEMENT

The pandemic has caused a decline in new cancer diagnoses but there is a strong variability linked to sex, tumor site and health organization.

INTRODUCTION

The incidence of tumors in Italy is monitored by the constant activity of the Cancer Registries (1). Every year in Italy there are about 376,000 new diagnoses of malignant cancer: breast, colorectal and lung are the most frequent cancers in women; prostate, lung and colorectal are the most frequent sites in men. For the majority of cancers, the rates are progressive. Melanoma (due to greater exposure to ultraviolet rays) and pancreatic tumors are on the increase in both sexes. Among women, the incidence of lung cancer continues to increase (largely linked to smoking), and breast cancer diagnoses have increased, due to more widespread screening throughout the national territory and to an extension of the target population age range (from 50-69 to 45-74).

But the situation changed quickly. The outbreak of the global pandemic dramatically changed our lives, and the impact of this phenomenon on new cancer diagnoses was not long in coming. The first analysis, published by Liang *et al.* (2), highlighted the impact of the SARS-CoV-2 infection on cancer patients in China; in particular, Intensive Care Unit (ICU) admissions and deaths were higher in cancer patients, especially if the cancer had been diagnosed in recent years. Subsequently, several papers were published on the subject and on the impact of infection on new cancer diagnoses. An Italian study (3) showed that during the lockdown (March-May 2020) in Italy there was a 45% reduction in new cancer diagnoses compared with the same months of 2018-19. In particular, the decrease concerned skin cancers and melanomas (-57%), and colorectal (-47%), prostate (-45%) and bladder (-44%) cancer. A subsequent study evaluated the impact that the lockdown (and the suspension of screening) had on new cancer diagnoses (4), highlighting a 35% decrease in new diagnoses compared to the previous year. In particular, there was a 35% reduction in diagnoses of breast cancer, 32% in prostate cancer and 53% in colorectal cancer.

The same attention was also given in Italy to studying the association between COVID-19 infection and cancer diagnosis. A study in the Veneto region (5) confirmed that cancer patients had a greater chance of being hospitalized and dying from COVID-19 than the general population, in particular for lung, breast and hematological cancers. Similar results were observed in a population study in Reggio Emilia (6), which confirmed the higher risk in patients with cancer compared to the general population (OR 1.45, 95% CI 1.12-1.89). The risk increased in the presence of distant metastases and if the patient had been diagnosed with cancer less than 2 years prior, and was higher for hematological cancers (excluding lymphoma), melanomas and cancers of the female genital organs.

The aim of this work is to describe the impact of Covid-19 on the incidence of tumors in a province of northern Italy, over a long period of time and using population data.

MATERIALS AND METHODS

This is a population-based cohort study using data from the Reggio Emilia Cancer Registry (CR) approved by the provincial Ethics Committee of Reggio Emilia (Protocol no. 2014/0019740 of 04/08/2014). The main information sources of the RE-CR are anatomic pathology reports, hospital discharge records, and mortality data, integrated with laboratory tests, diagnostic reports, and information from general practitioners. The RE-CR covers a population of 532,000 inhabitants and is considered a high-quality CR thanks to the fact that its data are up to date (the incidence data extend to the end of 2020), and it has a high percentage of microscopic confirmation (for example, 98.8% for breast cancer and 93.4% for colon cancer, and the percentage of Death Certificate Only is below 0.1%) (7). The study included cancer data for 2001-2020 obtained from the RE-CR and specifically compares the 2019-2020 data by site, gender and

for breast, colorectal and cervical cancer, also the in-situ forms were reported. The difference between the cases registered in 2020 and 2019 was calculated, also reporting the percentage of variation. Data are presented in both aggregate form and stratified form for males and females.

The standardized incidence and mortality rate of the last twenty years (2001-2020) was calculated divided by males and females for all sites (excluding the skin) and for the main tumor types: breast, prostate, lung and colorectal. We performed the annual percent change (APC) analysis in age-standardized rates with 95% confidence intervals using Joinpoint regression.

Population estimates, which were used to derive rates, are represented by the general population of the Province of Reggio Emilia recorded on January 1st of each year. Incidence rates and incidence-based mortality rates were adjusted to the 2013 European standard population and calculated per 100,000 person-years.

RESULTS

82,564 diagnosed patients in the period 2001-2020 were considered. The distribution of cases by gender, age at diagnosis, tumor site and period of incidence is shown in **table I**.

From the comparison of the cases registered in 2020 compared to 2019, it is clear that in 2020, 4,031 cases of cancer were recorded, 669 fewer than in 2019 (-14.2%). The sites that showed the greatest decline compared to 2019 were: skin (non-melanoma) (-281 cases), prostate (-110 cases), melanoma and bladder (-53 cases) and colorectal (-38 cases) (**table II**).

VARIABLES	N	%
ALL	**82,564**	
Sex		
Male	44,182	53.5
Female	38,382	46.5
Age at diagnosis		
< 50	9,785	11.8
50-69	29,388	35.6
70 +	43,391	52.6
Sites		
Breast female	9,271	11.2
Prostate	6,346	7.7
Lung	7,214	8.7
Colorectal	3,214	3.9
Other sites	56,519	68.5
Years of diagnosis		
2001-2005	18,612	22.5
2006-2010	19,959	24.2
2011-2015	21,886	26.5
2016-2020	22,107	26.8

Table I. *Number and percentage of cases, period 2001-2020.*

In males, the largest decline involved non-melanoma skin cancers (-159 cases; -24.3%), prostate (-110 cases; -28.4%), lung (-46 cases; -17.6%), colorectal (-34 cases; -18.7%), and melanoma -24 cases; -22.4%) and bladder (-24 cases; -13.4%) **table III**). In females, the decline primarily involved non-melanoma skin cancers (-122 cases; -25.4%), followed by *corpus uteri* (-31 cases; -31%), bladder (-29 cases; -26.6%), melanoma (-29 cases; -27.65), and stomach tumors (-25 cases; -44.6%) (**table III**). There was no decline for breast cancer in situ (+2 cases); on the other hand, for the cervix and colon in situ, fewer cancers were diagnosed than in 2020 (-68 and -22 cases, respectively) (**table IV**).

Considering 20 years of incidence and mortality, it is observed that the incidence trend in males (**figure 1 *A***) decreased from 491.74 cases per 100,000 persons/year in 2001 to 471.58 in 2019 and dropped to 386.59 in 2020, with a decline especially in the last year (APC -1.1; 95% CI -1.5 to -0.7). Mortality also decreased over the years, from 250.8 cases per 100,000 p/y in 2001 to 164.4 cases in 2019 and 161.9 in 2020 (APC -2.3; 95% CI -2.8 to -1.8). In females, the incidence remained almost constant over the years (APC 0.1; 95% CI -0.1 to 0.3), while there was a significant decline in mortality rate (APC -1.5; 95% CI -1.9 to -1.0) (**figure 1 *B***).

The incidence of breast cancer slightly increased in the last year (from 126.35 cases per 100,000 p/y recorded in 2019 to 133.23 cases in 2020) (APC 0.0; 95% CI -0.4 to 0.3), while mortality slightly decreased in the last period (**figure 2 *A***) (APC -2.4; 95% CI -3.5 to -1.4). For prostate cancer, there was a sharp increase in incidence until the mid-2000s (APC 2001-2003, 18.4; 95 % CI -16.9 to -68.8), due to the excessive use of PSA testing; in the last two years, instead, the incidence showed a significant decline (from 94.98 cases per 100,000 p/y in 2019 to 67.79 cases in 2020) (APC 2003-2020, -1.9; 95% CI -3.0 to -0.8) (**figure 2 *B***).

There has been a constant and significant decline in the incidence (APC -2.7; 95% CI -3.3 to -2.1) and mortality (APC 2001-2012, -5.6; 95% CI -7.5 to -3.7) of lung cancer over the years in males: the incidence dropped from 83.67 cases per 100,000 p/y in 2001 to 61.87 in 2019 and 49.16 cases in 2020; mortality declined from 73.9 cases in 2001 to 38.4

SITE	YEAR		DIFFERENCE 2020 *VS.* 2019	
	2019	2020		
	N.	N.	N.	%
Head-neck*	53	50	- 3	- 5.7
Esophagus	17	16	- 1	- 5.9
Stomach	124	102	- 22	- 17.7
Small intestine	14	16	2	14.3
Colorectal	325	287	- 38	- 11.7
Liver	77	77	0	0.0
Gallbladder and bile ducts	25	30	5	20.0
Pancreas	152	143	- 9	- 5.9
Larynx and nasal cavity	35	40	5	14.3
Lung and other thoracic organs	397	370	- 27	- 6.8
Bone	9	7	- 2	- 22.2
Skin, melanoma	212	159	- 53	- 25.0
Skin, non-melanoma	1133	852	- 281	- 24.8
Mesothelioma	18	27	9	50.0
Soft tissue and Kaposi sarcoma	18	16	- 2	- 11.1
Breast	509	524	15	2.9
Cervix uteri	12	18	6	50.0
Corpus uteri	100	69	- 31	- 31.0
Ovary	49	53	4	8.2
Other female genitals	10	12	2	20.0
Penis	5	4	- 1	- 20.0
Prostate	387	277	- 110	- 28.4
Testicle and other genitals	22	19	- 3	- 13.6
Bladder (including not malignant)	248	195	- 53	- 21.4
Kidney and urinary duct	117	107	- 10	- 8.5
Eye	6	0	- 6	- 100.0
Brain (including not malignant)	132	111	- 21	- 15.9
Thyroid	110	97	- 13	- 11.8
Other endocrine glands	10	5	- 5	- 50.0
Hodgkin Lymphoma	15	22	7	46.7
Non-Hodgkin Lymphoma	146	120	- 26	- 17.8
Myeloma	50	48	- 2	- 4.0
Leukemia	57	67	10	17.5
Other MPD and MDS**	75	46	- 29	- 38.7
Other sites	31	45	14	45.2
TOTAL	**4700**	**4031**	**- 669**	**- 14.2**

Table II. *Number of cases by cancer site and year of diagnosis 2019-2020.*
**(C00-C14, C30, C31, C32); **myeloproliferative disorders, myelodysplastic syndromes.*

and 38.5 cases in 2019 and 2020, respectively (**figure 2 *C***). In females, however, the situation is the opposite, where both incidence (APC 1.8; 95 % CI 0.9 to 2.7) and mortality (APC 0.9; 95% CI -0.4 to 2.2) are slightly but steadily increasing (**figure 2 *D***). For colorectal cancer, there was a peak of incidence in both sexes around 2006 due to the more extensive use of screening, and then decreasing in both sexes over the years (APC Males, -3.2; 95 % CI -4.1 to -2.2; APC Females, -2.4; 95% CI -3.5 to -1.3).

SITE	MALES				FEMALES			
	2019	2020	DIFFERENCE 2020 *VS.* 2019		2019	2020	DIFFERENCE 2020 *VS.* 2019	
	N.	N.	N.	%	N.	N.	N.	%
Head-neck*	38	38	0	0.0	15	12	- 3	- 20.0
Esophagus	11	9	- 2	- 18.2	6	7	1	16.7
Stomach	68	71	3	4.4	56	31	- 25	- 44.6
Small intestine	8	9	1	12.5	6	7	1	16.7
Colorectal	182	148	- 34	- 18.7	143	139	- 4	- 2.8
Liver	55	59	4	7.3	22	18	- 4	- 18.2
Gallbladder and bile ducts	13	19	6	46.2	12	11	- 1	- 8.3
Pancreas	81	71	- 10	- 12.3	71	72	1	1.4
Larynx and nasal cavity	30	31	1	3.3	5	9	4	80.0
Lung and other thoracic organs	262	216	- 46	- 17.6	135	154	19	14.1
Bone	6	4	- 2	- 33.3	3	3	0	0.0
Skin, melanoma	107	83	- 24	- 22.4	105	76	- 29	- 27.6
Skin, non-melanoma	653	494	- 159	- 24.3	480	358	- 122	- 25.4
Mesothelioma	12	22	10	83.3	6	5	- 1	- 16.7
Soft tissue and Kaposi sarcoma	14	8	- 6	- 42.9	4	8	4	100.0
Breast	7	5	- 2	- 28.6	502	519	17	3.4
Cervix uteri	-	-	-	-	12	18	6	50.0
Corpus uteri	-	-	-	-	100	69	- 31	- 31.0
Ovary	-	-	-	-	49	53	4	8.2
Other female genitals	-	-	-	-	10	12	2	20.0
Penis	5	4	- 1	- 20.0	-	-	-	-
Prostate	387	277	- 110	- 28.4	-	-	-	-
Testicle and other genitals	22	19	- 3	- 13.6	-	-	-	-
Bladder (including not malignant)	179	155	- 24	- 13.4	69	40	- 29	- 26.6
Kidney and urinary duct	78	66	- 12	- 15.4	39	41	2	5.1
Eye	4	0	- 4	- 100.0	2	0	- 2	- 100
Brain (including not malignant)	61	49	-12	- 19.7	71	62	- 9	- 12.7
Thyroid	33	31	- 2	- 6.1	77	66	- 11	- 14.3
Other endocrine glands	6	2	- 4	- 66.7	4	3	-1	- 25.0
Hodgkin Lymphoma	12	11	- 1	- 8.3	3	11	8	266.7
Non-Hodgkin Lymphoma	78	57	- 21	- 26.9	68	63	- 5	- 7.4
Myeloma	27	23	- 4	- 14.8	23	25	2	8.7
Leukemia	30	35	5	16.7	27	32	5	18.5
Other MPD and MDS**	38	25	- 13	- 34.2	37	21	- 16	- 43.2
Other sites	16	19	3	18.8	15	26	11	73.3
TOTAL	**2523**	**2060**	**- 463**	**- 18.4**	**2177**	**1971**	**- 206**	**- 9.5**

Table III. *Number of cases by cancer site and sex, years 2019-2020.*
**(C00-C14, C30, C31, C32); **myeloproliferative disorders, myelodysplastic syndromes.*

In 2020 the incidence declined more in males, as a result of lack of diagnosis probably due to the COVID-19 pandemic. Mortality also declined over the years in both sexes: it decreased by about 50% from 2001 to 2020 (from 24.5 cases per 100,000 p/y in 2001 for males and 13.6 for females to 12.5

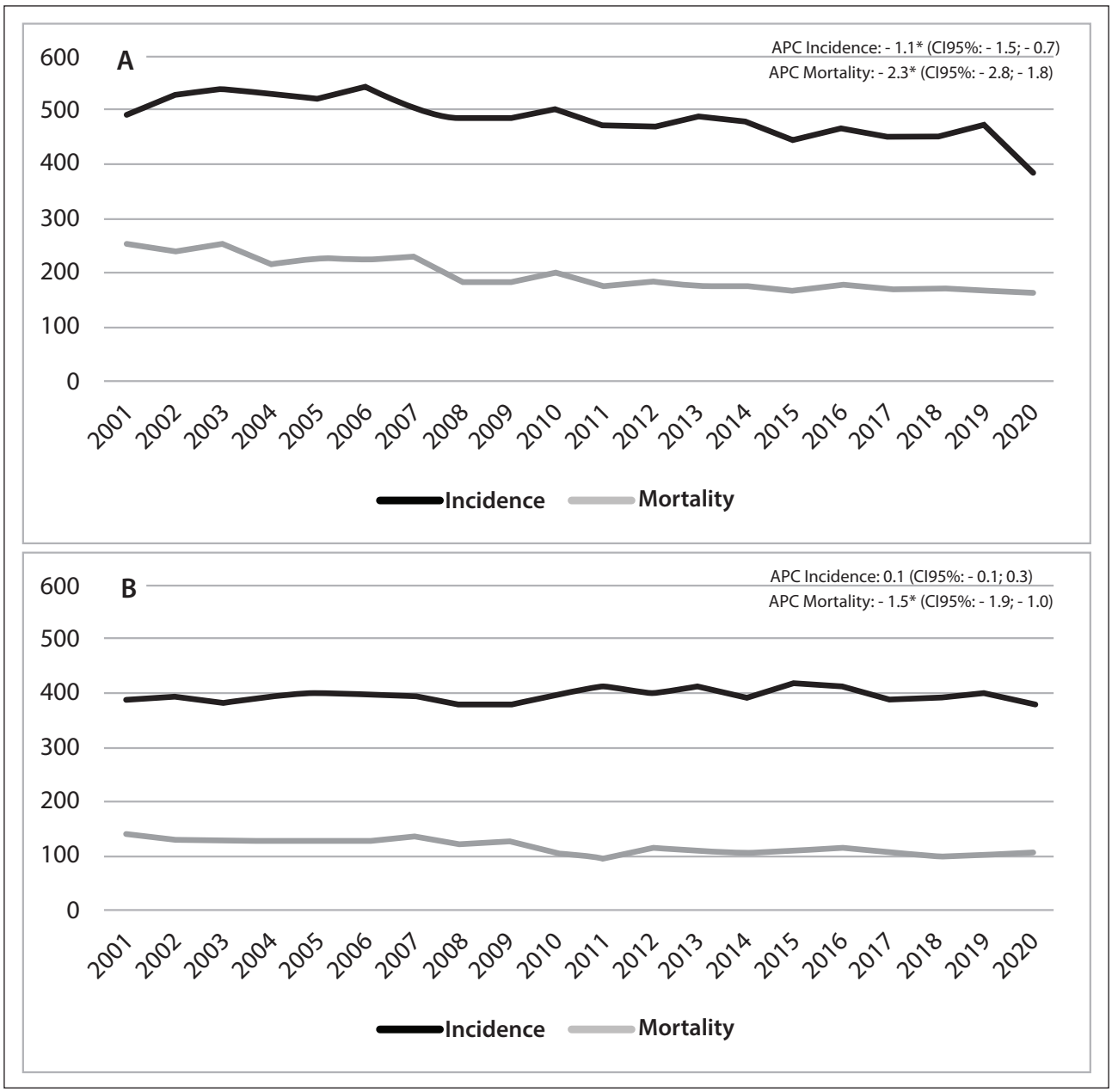


Figure 1. *Incidence and mortality trend of all cancer sites (excluding skin) years 2001-2020;* ***A****. In males;* ***B****. in females.*

	2019	2020	DIFFERENCE 2020 *VS.* 2019
Breast	75	77	2
Cervix	288	220	- 68
Colorectal	28	6	- 22
TOTAL	**391**	**303**	**- 88**

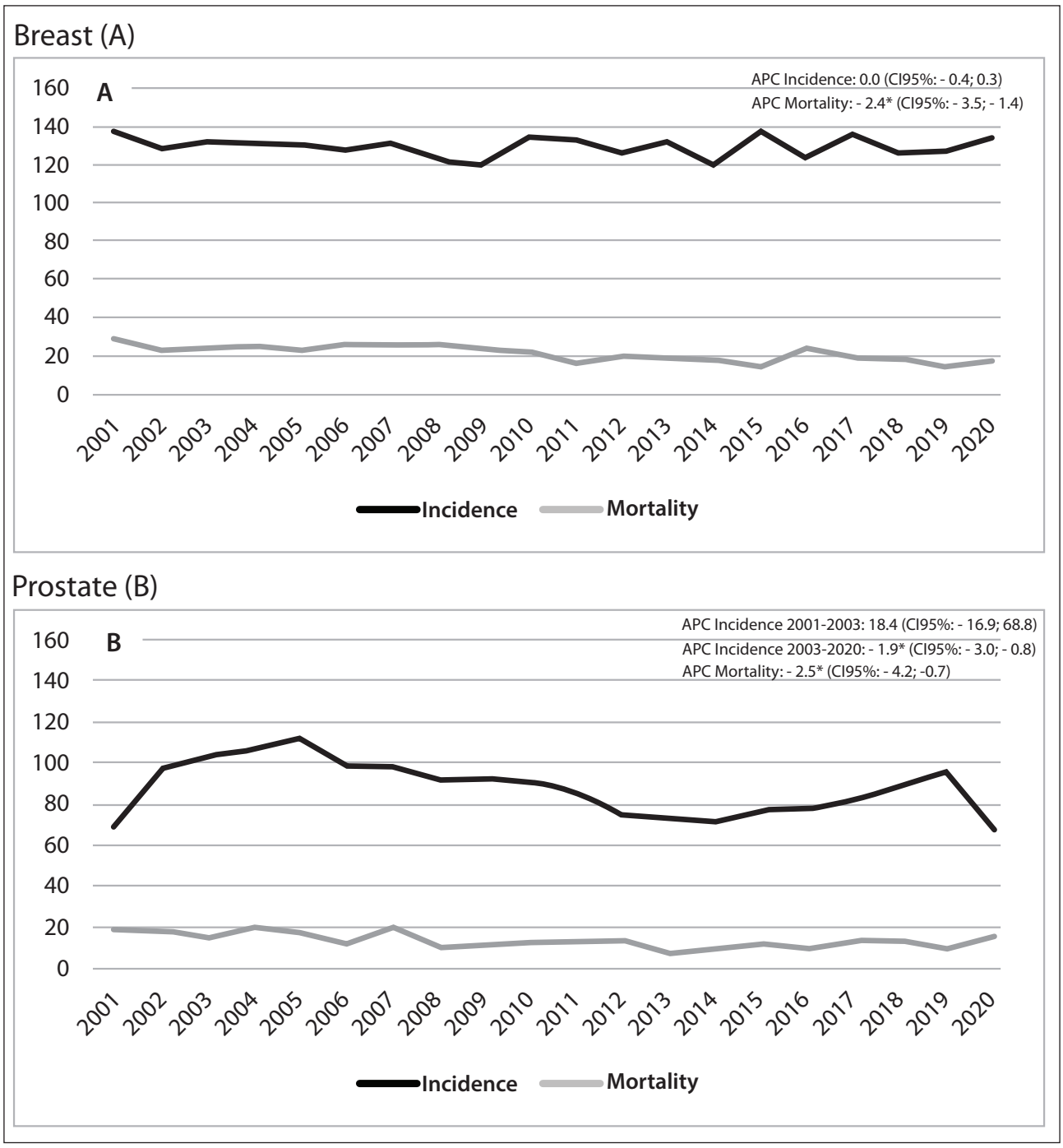
Table IV. *Number of in situ cases by cancer site in the three screened cancers, years 2019-2020.*

cases in males and 7.6 in females, respectively) (APC Males, -3.0; 95 % CI -4.2 to -1.8; APC Females, -2.4; 95% CI -3.5 to -1.3) (**figure 2** ***E***, ***F***).

DISCUSSION

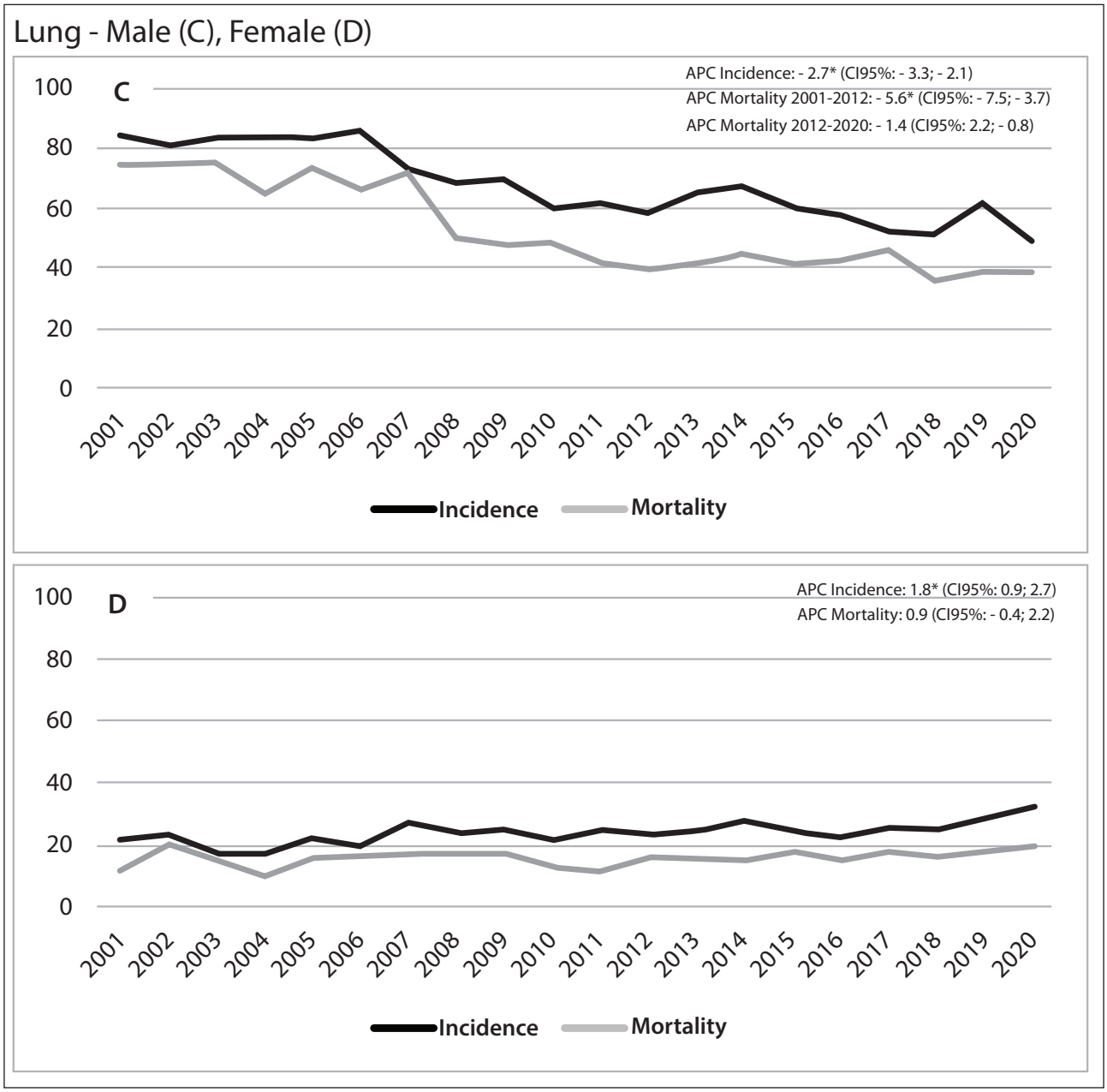
The aim of this work was to compare the tumors incidence in 2020 with those of 2019 and describe the incidence and mortality trends relating to 20 years of registration, to better understand the phenomenon in recent years, for all sites and for the main tumor sites.

The first interesting data is the decrease in cancers recorded in 2020 compared to 2019: -669 cases, equal to 14.2% less. The decline, especially during the lockdown, has been widely reported in the literature but usually covers the months leading up to September 2020, recording first a decrease and



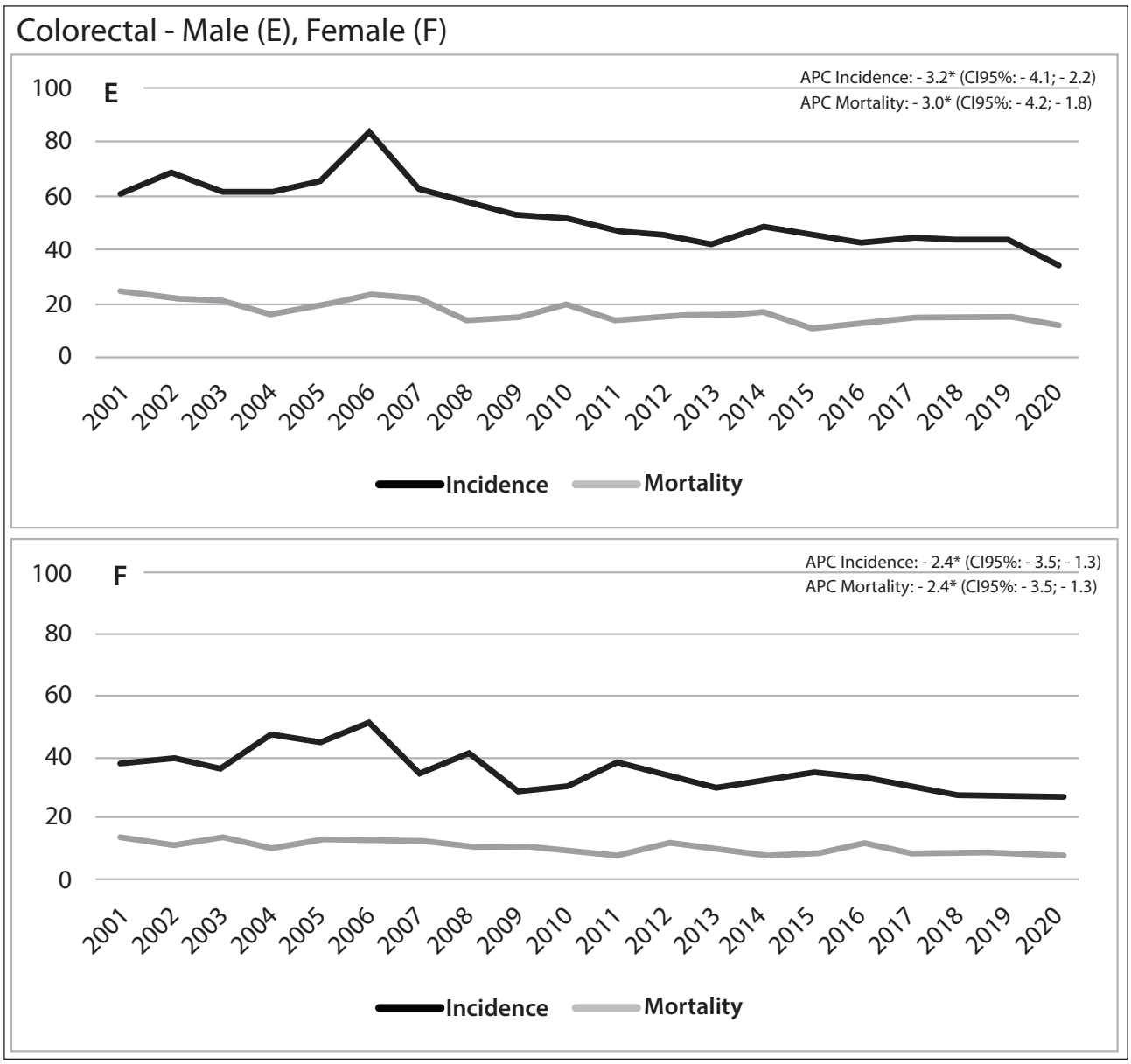
Figures 2 ***A, B****. Incidence and mortality trend for main site of cancer by sex, years 2001-2020.*

then a recovery in the incidence (8). In particular, the decline concerned cancers of the skin (-25%), prostate (-28.4%), melanoma (-25%), bladder (-21.4%), colorectal (-11.7%) and the body of the uterus (-31%). A decrease in skin cancers (-74%) and melanoma (-54%), probably due to diagnostic failures, has already been reported in the English literature in a recent study by Venables *et al*. (9) and by Eskander *et al*. (8) as regards melanoma. The decrease in prostate cancer (-28.4%) has been widely reported in other studies: -54.7% in Eskander (8) and -64% in Venables (9). This decrease was not confirmed, however, in a German study (10). It should be noted that the two studies cited above, Eskander (8) and Venables (9), refer to the incidence up to September 2020, thus not allowing a possible resumption of diagnoses. A decrease in diagnoses was observed in all countries and for almost all tumor sites (3), in particular those subject to screening (11). The recovery of the post-lockdown diagnoses almost never compensates for the decrease observed during the lockdown (12), with a few exceptions (4).



Figures 2 ***C, D****. Incidence and mortality trend for main site of cancer by sex, years 2001-2020.*

For the three cancers screened, our study did not show any decrease in breast cancer diagnoses (+15 cases equal to +2.9%) since after the interruption of screening during the lockdown, a rapid resumption of screenings, and therefore of diagnoses, followed in the target population. The interruption of screening already reported in the literature (13) seems to have had a greater impact on the decrease of early forms (tumors in situ and T1 and stage I tumors) but not on the increase of advanced forms. Our study does not report delays in the diagnosis of cervical cancer, though referring to small numbers, from 12 to 18 cases in the two-year period considered, while the literature shows a decrease in the incidence (8) or a delay in HPV-negative patients (14). Rather, the delay led to problems in the management and treatment of cervical cancers. As regards colorectal cancers, the decline concerned primarily colon cancers and mainly in males (-20 cases), while in females the incidence remained almost stable (-7 cases). A decrease in colorectal cancers had already been reported by the Ferrara study (3). A shift in the diagnosis of these tumors could have a greater impact, given the natural history of this cancer, with an increase in advanced forms from 26% to 29% for a delay of 7-12 months and from 26% to 33% for a delay of 12 months (15).



Figures 2 ***E, F.*** *IIncidence and mortality trend for main site of cancer by sex, years 2001-2020.*

The impact this will have on the next few years can only be predicted with estimates. Ward *et. al* (16) report that after a decline in 2020 there will be a recovery in 2021 and that in the future there will be above all an increase in advanced stages. The decline in lung cancers reported in the literature finds a strong difference between genders in our study: it decreased in men and increased in women, in this case attributing the incidence exclusively to the main risk factor, cigarette smoking (10). Finally, little or no impact was seen on hematological cancers, which continued to be diagnosed unaffected by the pandemic.

The absolute numbers are also confirmed by the standardized incidence rates. In males there was a decrease in tumors from 471.6 in 2019 to 386.6 in 2020, while in females the incidence is almost constant, largely linked to the "resistance" of tumors of the breast. In males, in addition to lung cancer, there was a sharp decline in prostate cancer, from 95.0 in 2019 to 67.8 in 2020. Lung cancer continued to record a downward trend in males: 61.9 in 2019 and 49.2 in 2020, while in females it rose from 28.6 to 32.3, respectively. Mesothelioma continues to increase in males, as reported by a recent published paper (17).

Finally, colorectal cancer incidence shows a sharp increase in 2006 after population screening was started in 2004. The trend then steadily declined over the years and in the last year it dropped from 43.4 to 34.5 in males but not in females (26.6 in 2019 and 26.8 in 2020). The mortality trend in re-

cent years has been stable in males and females. Breast cancer incidence showed a slight increase in 2020 (from 14.7 in 2019 to 17.3 in 2020) as did prostate cancer (from 9.5 in 2019 to 15 in 2020).

CONCLUSIONS

The aim of this work was to describe the impact of Covid-19 on the incidence of tumors in a province of northern Italy, over a long period of time and using population data. Our study confirmed that in 2020 there were nearly 700 fewer cancer diagnoses than the previous year: the decline affected almost all sites, especially skin cancers and prostate cancer. Breast cancer did not show a decline in incidence and, unlike what emerged in the literature, no decline in early stage tumors.

ETHICS

Fundings

There were no institutional or private fundings for this article.

Conflicts of interests

The authors have declared no conflict of interests.

Availability of data and materials

The data underlying this article can be shared just before a reasonable request to the corresponding author.

Authors' contribution

LM: conceptualization, investigation, writing - original draft, visualization, supervision; FM: formal analysis, methodology; IB: writing - review and editing, and visualization; CP: conceptualization, writing - original draft, investigation, and supervision.

Ethical approval

Protocol no. 2014/0019740 of 04/08/2014.

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REVIEW

INCIDENCE OF BREAST CANCER IN ETHNIC MINORITY GROUPS IN NORTH AMERICA AND POPULATIONS IN WESTERN EUROPE

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ABSTRACT

Breast cancer (BC) is one of the most prevalent cancer types among women, and among the top for cancer deaths. Research to address this worldwide issue has been conducted to identify risk factors associated with development and treatment. It was identified that risk factors not only included age, other underlying diseases, environmental factors, but also socioeconomic factors, language barriers and ethnic background. Unfortunately, due to low socioeconomic status groups that are affected the most in the United States are African American and Hispanic women while in Western Europe, such as in Italy, discrimination was based on geographical location rather than racial background. Previous studies indicate that discrimination and racial disparities are relevant factors affecting women battling against breast cancer. By analyzing and highlighting the pitfalls of the current medical approaches to treatment among various ethnic groups in North America and Western Europe, researchers and medical professionals will be better able to tailor treatments and improve prognosis among all BC patients, regardless of race and ethnicity.

KEY WORDS

Breast cancer; triple negative breast cancer; minorities; racial disparities; tailored treatment.

IMPACT STATEMENT

Despite considerable advantages in research for treatments and therapies for breast cancer there is a noticeable lack of resources which emphasizes racial disparities and socioeconomic status.

INTRODUCTION

Breast cancer (BC) is the second-leading cause of cancer death, after lung cancer, and the most common cancer type among women worldwide at 24.5% (1). The greatest incidence, in females, is found in Asia (45.4%), followed by Europe (23.5%) and then by North America (12.5%) (1). **Figure 1** shows the estimated age-standardized incidence rates of BC across all ages. BC exhibits substantial variability among women of differing ancestries. For this reason, it is critical to analyze the incidence of BC in various ethnic groups, observe standards of care and tailor treatment and possible therapeutics in the hopes of improving quality of life and survival. The classification of breast cancers reflects the current state of knowledge; thus, it is an ever-evolving process. BC is a genetically and clinically heterogeneous disease with different biological, clinical and molecular characteristics (2). The molecular classifications divide breast cancer into six groups: luminal A, luminal B, HER-2, basal, normal breast like and claudin-low (3). There are three main subtypes of BC that are based on immunohistochemistry cellular markers (IHC) or a combination of IHC and microarray expression methods (gene signatures): Hormone receptor positive (ER+ or PR+), HER2 positive, and Triple-negative (absence of ER, PR, and HER2 amplification) (4). **Figure 2** shows the molecular classification of breast cancers (5). More recent data for molecular classification of BC indicate prognostic associations which include intrinsic subtypes, integrative cluster subtypes, triple-negative sub-classification and mutation-based profiling (6). Triple-negative breast cancer (TNBC) accounts for 10-20% of all invasive breast cancers (7).

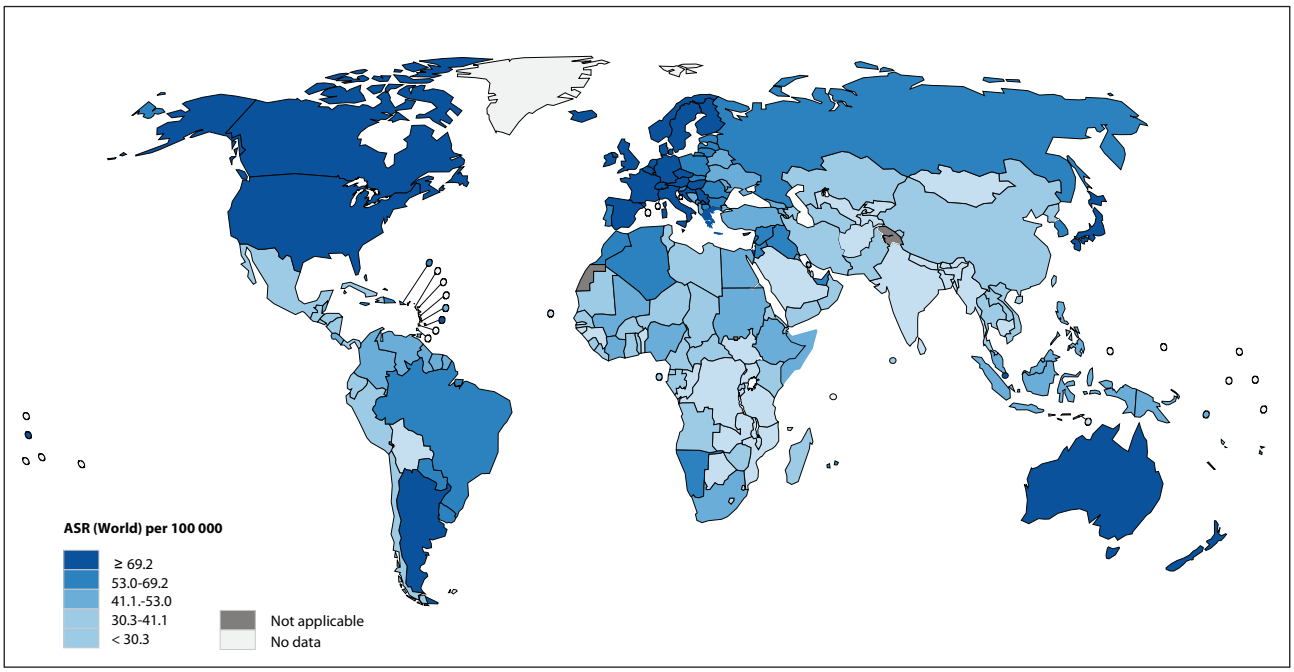


Figure 1. *Breast cancer (BC) estimated age-standardized incidence rates (World) in 2020, all ages female. World map illustrating the age-standardized incidence rates in 2020 of breast cancer in women. The darker blue colored countries have a higher age-standardized rate (ASR), which include Belgium (113.2), France (99.1), Australia (96.0), United States of America (90.3), Italy (87.0), United Kingdom (87.7). While countries with lighter blue color have lower ASR. Graphic taken from International Agency for Research on Cancer, 2020, WHO.*

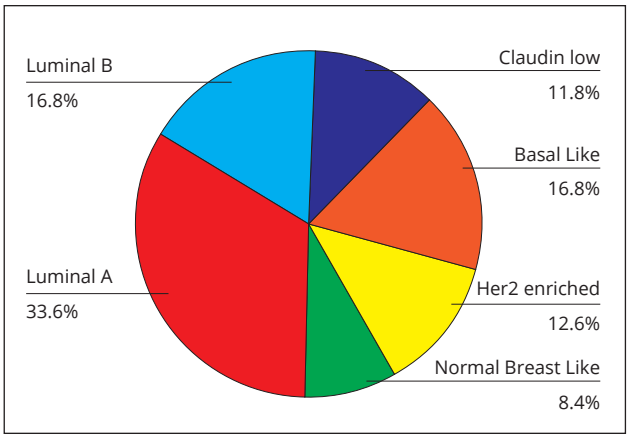


Figure 2. *Molecular Classification of breast cancer. Breast Cancer (BC) is a heterogeneous disease. It can be classified based on different biological, clinical and molecular characteristics. The molecular classifications can be divided into six classes: claudin low, basal like, HER2, Normal breast like, Luminal A and luminal B. Luminal A and B are characterized by a cellular marker of Estrogen Receptor (ER) positive. HER2 enriched BC subtype express HER2 protein and no ER. Basal-like BC is characterized as having no ER, no Progesterone Receptor (PR) and no HER2 present, thus it is called Triple Negative Breast Cancer (TNBC). Because of the absence of these receptors and proteins, TNBC does not respond well to hormone therapies, thus it is difficult to treat and has a poor prognosis*

Among the subtypes, TNBC is associated with high mortality, early and more frequent recurrence and poor treatment response, regardless of ethnic background and social standing.

Despite the commonality of molecular characteristics among BC patients, the available treatment and therapy, overall survivorship and quality of life greatly differ among different ethnic groups especially within the United States. Due to the socioeconomic status and other economic disparities some ethnic groups, *e.g.*, African American and Hispanic women, do not have access to routine screenings, medical care, treatment and therapies. Unfortunately, these shortcomings in treatment are not only prevalent in the US but also within some countries in Western Europe, such as Italy.

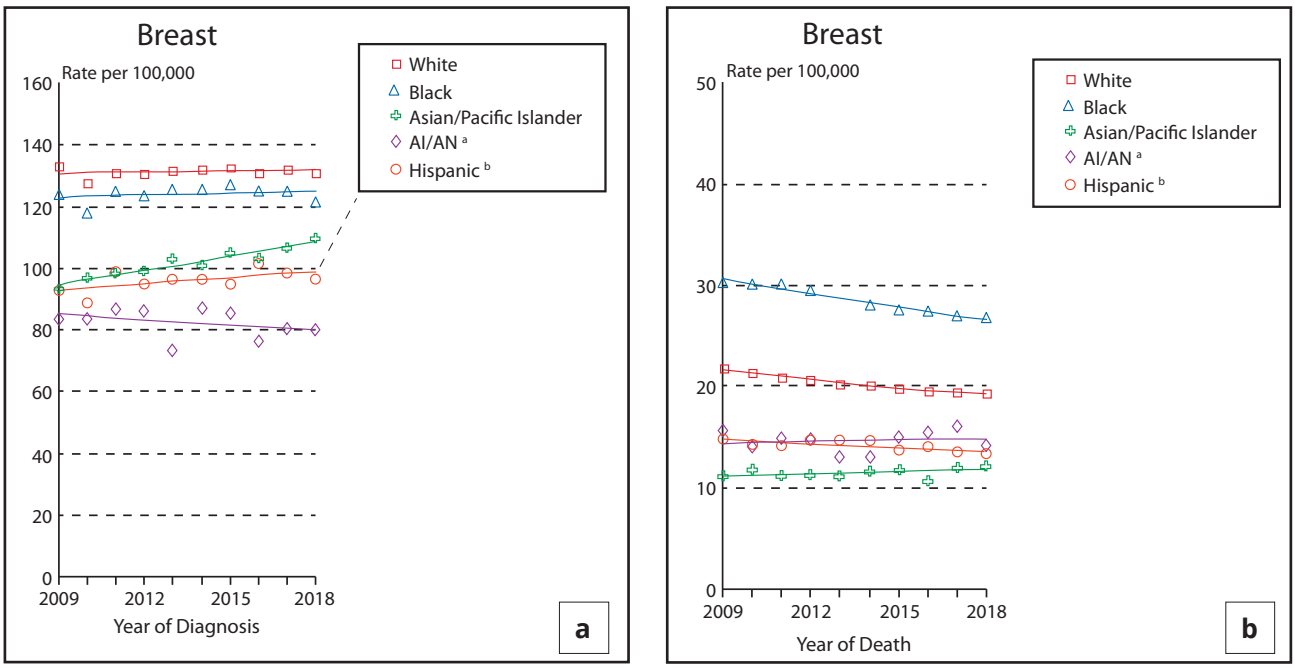
AFRICAN AMERICAN WOMEN

While the incidence of BC in African American (AA) women is lower when compared to European American (EA) women, the mortality rate is higher which may be caused by disparities in the socioeconomic status and in the environment-related conditions, as shown in the **figures 3 *a, b*** (8). The data from the Surveillance, Epidemiology, and End Results (SEER) database report the incidence trends across different races and ethnic groups within the United States. As a direct consequence of unhealthy living conditions in areas with low income, AA women are exposed to breast carcinogens that are present in the environment (9). AA women are consistently diagnosed at a more advanced stage of the disease and usually express a triple negative or ER-negative BC phenotype, which is more aggressive and has a poor prognosis (10). Resources such as screening and early detection procedures which could potentially improve survival rates, are less likely to be available for AA women. Friebel-Klingner (11) observed that TNBC was also less likely to be screen detected in AA women. In cases where a diagnosis is available, treatment, *e.g.*, surgery and chemotherapy, may be economically infeasible (12).

Underlying diseases, *e.g.*, obesity and diabetes, may potentially increase the risk to develop BC. Obesity is associated with advanced BC at diagnosis, high tumor proliferation rates, and more triple-negative phenotypes, indicating that it may adversely contribute to prognosis (13). Friebel-Klingner (11) investigated the associations of known BC risk factors, including breast density, with TNBC among black women and concluded that breast density was more strongly associated with TNBC than other subtypes, and obesity was associated with greater risk of TNBC among this group.

Therapeutics are usually tailored to a specific demographic. The majority of clinical trials groups are represented by Caucasian women. Some clinical trials neglect to take into consideration factors such as genetic background and environment-related conditions in the recruitment process, thus affecting in particular AA women. Additionally, a percentage of AA women perceive research as biased to benefit solely Caucasians (14). Multiple preclinical and clinical studies suggest inherent genetic risk factors and aberrant activation of oncogenic pathways in AA TNBC (15). In an effort to provide more inclusive therapeutics, these genetic risk factors and oncogenic pathways may be further researched with the goal to tailor precision medicine to AA TNBC.

In order to address these socioeconomic disparities and racial differences, it is critical to educate and inform with preventative screenings and to improve treatment adherence and efficacy in AA women with TNBC.



Figures 3. ***a.*** *SEER Incidence 2009-2018 Females by Race/Ethnicity;* ***b.*** *US Mortality 2009-2018 Females by Race/Ethnicity. These graphics present the incidence and mortality rate trends in female breast cancer across different races and ethnic groups within the United States from 2009 to 2018. The incidence of female breast cancer was higher in White women followed by Black and then Asian/Pacific Islanders while the mortality rate was highest in Black women followed by White and then Hispanics. Graphics taken from National Cancer Institute: Surveillance, Epidemiology, and End Results (SEER) Program, 2020.*

Source: SEER 21 areas (San Francisco, Connecticut, Detroit, Hawaii, Iowa, New Mexico, Seattle, Utah, Atlanta, San Jose-Monterey, Los Angeles, Alaska Native Registry, Rural Georgia, California excluding SF/SJM/LA, Kentucky, Louisiana, New Jersey, Georgia excluding ATL/RG), Idaho, New York and Massachusetts. Rates are age-adjusted to the 2000 US Std Population (19 age groups – Census P25-1103). Regression lines are calculated using the Joinpoint Regression Program Version 4.9, March 2021, National Cancer Institute.

[a] Incidence rates for American Indian/Alaska Native (AI/AN) are based on the CHSDA (Contract Health (Service Delivery Area) counties.

[b] Hispanic is not mutually exclusive from whites, blacks, Asian/Pacific Islanders, and American Indians/Alaska Natives. Incidence data for Hispanics are based on NHIA and exclude cases from the Alaska Native Registry.

Source: US Mortality Files, National Center for Health Statistics, Centers for Disease Control and Prevention. Rates are age-adjusted to the 2000 US Std Population (19 age groups – Census P26 – 1103). Regression lines are calculated using the Joinpoint Regression Program, Version 4.9, March 2021, National Cancer Institute.

[a] Mortality rates for American Indian/Alaska Native (AI/AN) are based on the CHSDA (Contract Health Service Delivery Area) counties.

[b] Hispanic is not mutually exclusive from whites, blacks, Asian/Pacific Islanders, and American Indians/Alaska Natives.

HISPANIC WOMEN

Another minority group facing discrimination and lack of financial stability in BC treatment and therapeutics is Hispanic American women. Urban Hispanic women who survive BC are exposed to more risk factors due to low SES, unsafe neighborhood conditions, and limited access to treatment resources (9). Unfortunately, among ethnic minorities (*e.g.*, African American and Hispanic) BC survivors, the association of neighborhood context has a significantly negative impact on health outcomes (16). Although rarely taken into account, the importance of neighborhood context may aid in examining determinants of health, survivorship and quality of life outcomes among cancer patients (16). Howell (17) analyzed Philadelphia's urban poor and concluded that although the financial impact and neighborhood context were not unique to urban Hispanic women, this minority group was at greater risk for poorer survivorship because of lower incomes as compared with other racial and ethnic groups. Unlike AA women, the overall rate of BC has declined for Hispanic women. However, similar to AA women, they are diagnosed with more advanced breast cancers (18). This later diagnosis creates a severe setback for these women even prior to treatment. Furthermore, there are additional factors which impede the delivery of proper treatment and care to Hispanic women: health literacy and language barrier. Health literacy is multifaceted. Ineffective communication and lack of health literacy may affect a

patient's ability to access healthcare, follow advice and receive proper treatment (9). This factor further subjects this group of individuals to discrimination. There is an increased risk for health disparities if a patient's primary language is not English and/or if they migrated to the United States (19). If a patient's primary language is Spanish, they have more difficulties with the continuity of their cancer care (20). Various measures have been put into practice in order to address this issue facing Hispanic women. For instance, the U.S Department of Health and Human Services, Office of Minority Health, has developed and established fluency standards for healthcare professionals to implement culturally and linguistically appropriate services (CLAS) (21).
The main goal in discussing these issues is to establish standards and guidelines with the ultimate aim of advancing health equity, improve quality of life for all cancer patients and eliminate disparities among specific ethnic groups.

WESTERN EUROPE - ITALY

With regard to Western European countries, Germany, France and Italy experienced the greatest incidence of BC cases (1). **Figure 4** shows the estimated number of new cases and number of deaths caused by breast cancer in European women in 2020. As stated earlier, a risk factor associated with cancer incidence may be the presence of other underlying diseases such as obesity and diabetes. A cross-sectional study conducted in 2010 provided information on the prevalence of overweight and obesity in Europe (22). Gallus (22) observed that out of the 16 European countries analyzed in the study, two Mediterranean countries, Italy and France, showed the lowest prevalence of obesity. The prevalence of obesity significantly increased with age and decreased with level of education (22). Despite the lower prevalence of obesity in Mediterranean countries, Italy and France were among the top countries with the greatest number of BC incidences, indicating that other factors contribute to the development of cancer and survivorship.
In Italy, breast cancer is the most frequent neoplasm, with almost 55,000 new cases per year (23). As in the US, in Italy certain risk factors are associated with SES and location which determine access to screening and treatment (24). Rossi's (2020) time-trend study, conducted from 1990 to 2016, showed a decrease of age-standardized mortality rate in Italy. Additionally, in this study, various factors were taken into account, *e.g.*, fertility rates, routine and mammographic screenings, breastfeeding and mean age at birth. These factors were compared and contrasted among the various regions in Italy and trends indicated either a decrease or increase in BC incidence (24). For instance, southern regions saw a decrease in participation in mammographic screenings while in northern regions an increase was observed. Other factors analyzed that contribute to BC development were breastfeeding and mean age at birth, which both saw an increase throughout Italy (24). Certain risk factors that influence BC incidence outcome include the stage at diagnosis and access to effective and timely treatments, which are directly correlated to individual socioeconomic and geographic differences.
In Italy, there is drastic geographic inequality between the Northern and Southern regions. In recent years this gap has been reduced. Differences in mortality rate and prevalence of risk factors are diminishing between the north and the south (24). However, between 1990 and 2017, an increase in cancer death was observed, with BC being one of the major causes of cancer death among women. This increase was likely due to the progressive aging of the Italian population (25).

FUTURE DIRECTIONS

Developments in personalized medicine should be encouraged and pursued. Specific areas such as accessibility to modern diagnostic technologies, improvements in surgery and introduction of innovative treatment approaches are critical to address BC and especially TNBC in order to give patients hope and thus improve their quality of life.
Previous shortcomings, *e.g.*, discrimination and inequality in treatment and therapeutics, may even further underline the need for the scientific community to collaborate globally in an effort to advance treatments that could benefit the individuals who need care, regardless of gender, race and ethnicity. The new era of personalized medicine in cancer therapy should be accessible to all.

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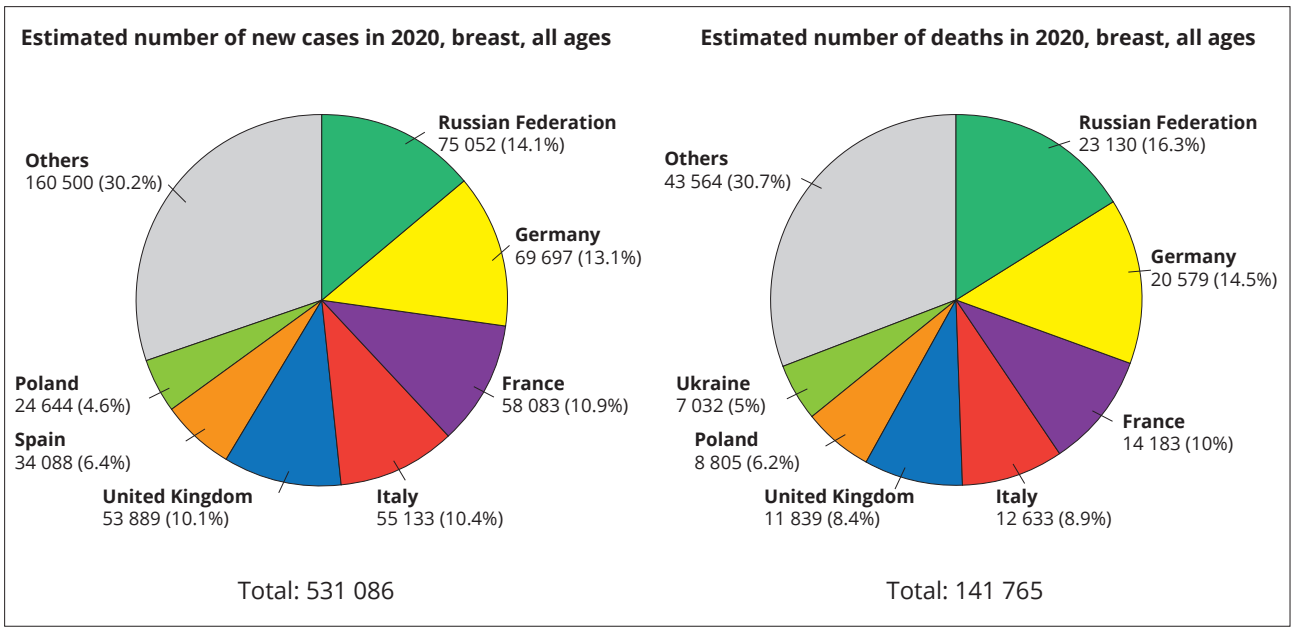

Figure 4. *Estimated number of new cases and number of deaths caused by breast cancer in European women in 2020. Pie charts illustrating the incidence and mortality rates of breast cancer in women in different European countries. As observed, the estimated number of new cases in 2020 was most in the Russian Federation (14.1%), Germany (13.1%), France (10.9%) followed by Italy (10.4%) while the estimated number of deaths in 2020 showed a similar pattern with the Russian Federation (16.3%) showing the greatest percentage of mortality, followed by Germany (14.5%), Frances (10%) and Italy (8.9%). Pie chart taken from International Agency for Research on Cancer, 2020, WHO.*

ETHICS

Fundings

This work was supported by the Sbarro Health Research Organization (SHRO).

Conflict of interests

The authors have declared no conflict of interests.

Availability of data and materials

The data underlying this article are available in the public domain, using various datasets primarily from Pubmed, GCO, SEER, *etc*.

Authors' contribution

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

Ethical approval

Ethical approval was not necessary for this study because it does not involve patients.

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REVIEW

FEIJOA SELLOWIANA FRUIT, AN AMAZING SOURCE OF ANTICANCER MOLECULES

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ABSTRACT

Feijoa sellowiana O. Berg is a tropical plant with edible fruits and characterised by a high content of flavonoids. Several studies have shown that *Feijoa* contains many bioactive components such as flavonoids, vitamin C, and essential minerals that contribute to multiple health benefits, such as antimicrobial, anti-inflammatory, antioxidant, and anticancer activities. Regarding anticancer activity, several authors have shown that the *Feijoa* fruit acetonic extract and the molecules isolated by it have a selective cytotoxic effect, induce apoptosis, modulate cell cycle against solid and hematologic tumours and are effective against sensitive and resistant cancer cells. This review summarizes *Feijoa* fruit biological activities that have so far been identified with a focus on anticancer activity.

KEY WORDS

Flavone; antitumoural; antioxidant; anti-inflammatory; MDR cells.

IMPACT STATEMENT

Feijoa sellowiana, due to its peculiar chemical characteristics, can give an important contribute in cancer prevention and therapy.

INTRODUCTION

Cancer is a pathological condition characterised by cells that resist apoptosis, respond abnormally to cell cycle regulation mechanisms, are self-sufficient about growth factors, can present impaired differentiation, and contact inhibition is suppressed (1). It is estimated that around three out of ten cancers are caused by poor eating habits (American Institute for Cancer Research) (2). Several epidemiological studies have highlighted that natural products such as fruits, vegetables, spices, and cereals are foods containing active ingredients capable of having beneficial effects on health, in particular having anti-tumour activity; in fact, the lack of consumption of these foods is linked to a series of neoplasms (3). In light of this, research is showing more and more interest in fruits rich in polyphenols, in particular the flavonoids that humans cannot synthesize, and must be taken with diet. Flavonoids are particularly known for their innumerable properties such as antioxidant, anti-inflammatory, as well as antiproliferative and pro-apoptotic for cancer cells, thanks to their ability to modulate different biological processes that characterize these cells (i.e. blocking apoptosis, migration, resistance to chemotherapeutic agents) (4, 5).

Feijoa sellowiana O. Berg, also known as *Acca sellowiana* or *Pineapple guava*, is a tropical evergreen shrub belonging to the *Myrtaceae* family, whose fruits are rich in interesting secondary metabolites such as flavonoids (**figure 1**). *F. sellowiana* is native to the area of South America between northern Argentina, southern Brazil, Uruguay, and Paraguay, where it grows spontaneously and luxuriantly, but it is cultivated in many other countries such as New Zealand, France, Israel, Italy, California, and Florida (6).

The *Feijoa* fruit is edible, oval and has a size of 4-8 cm, with a robust green exocarp, the pulp is white-yellowish translucent, gelatinous and very hard small seeds (**figure 1**). In Italy, the fruit harvest begins in October and ends in November. Its flavour is sweet-sour, a mixture of pineapple, strawberries, and guava and can be eaten fresh, in the form of yoghurt, juice, jam, *etc*. (7). The flower is white-pink and has numerous, very showy red-violet stamens. The petals are crunchy and sweet and can be used for salads. The leaves are 5 cm long; they are dark, thick, elliptical, and opposite. The dried leaves can be used to make infusions.

In traditional medicine, the infusion of *Feijoa* leaves was mainly given to children to treat bacterial and fungal diseases in general and in particular cholera (8). In vitro studies have shown that the acetonic extract of *Feijoa* leaves has antibacterial and antifungal activity; confirming these applications (9). The fruit is rich in pectin, vitamin C (28 mg 100 g^{-1} fresh weight), and essential minerals such as potassium, phosphorus, magnesium, calcium, and iodine (3 mg 100 g^{-1} of fresh fruit) (10,11). In addition, the fruit contains dietary fibre, quinones, terpenes, tannins, and steroid saponins; the aroma that characterizes *Feijoa* is largely due to the volatile esters of ethyl benzoate, ethyl butanoate, and the high amount of methyl benzoate (12). Furthermore, the *Feijoa* fruit contains bioactive phytochemicals, such as large amounts of polyphenols (flavones, catechins, procyanidins B1 and B2, quercetin-glycoside, flavonols, naphthoquinones, leucoanthocyanins, and proanthocyanidins) (13).

Different studies have reported potential therapeutic properties of Feijoa such as acetylcholine and butyrylcholine esterase inhibition (14), antifungal (15), and antibacterial (16-18).

Epidemiological data report that the populations of tropical and subtropical countries that habitually consume the fruits of *Feijoa* have a lower incidence of cancer in the gastrointestinal tract (19).

Numerous studies have been carried out on the anticancer properties and chemical characterisation of *F. sellowiana* (**table I**), as reported below:

- antibacterial activity on *Helicobacter pylori*, the presence of which is one of the causes of gastric cancer;
- immunomodulatory effect;
- antioxidant activity, in order to protect the populations who regularly consumed this fruit from

oxidative stress;
- anti-inflammatory activity;
- cytotoxic activity;
- anti-tumour action on different cell lines of solid and haematological tumours;
- chemical characterisation and activity-guided fractionation;
- selectivity of anti-tumour activity against tumour cells;
- antiproliferative and apoptotic effects on cancer gastric cells;
- anticancer activity against cancer cells that had developed multidrug resistance.

Figure 1. *Details of the Feijoa sellowiana fruits, flowers and leaves*

BIOACTIVITIES

Anti-Helicobacter pylori activity

Despite a sharp decline in incidence and mortality, stomach cancer is still the fourth most common cancer in the world. The best-known risk factor for stomach cancer is *H. pylori* infections (20) which are considered the leading cause of distal gastric adenocarcinoma, and gastric lymphoma (MALToma). Furthermore, *H. pylori* is associated with several diseases, including chronic gastritis and peptic ulcers (21, 22). The pathogenesis depends on the virulence of the strain, the genetic susceptibility of the host, and the environmental cofactors (23). Already in 1994, the International Agency for Research on Cancer classified *H. pylori* as a carcinogen, or cancer-causing agent, in humans (NIH, National centre institute). *H. pylori* is a spiral-shaped Gram-negative bacterium that grows in the mucus layer that coats the inside of the human stomach. Some studies on the inhibitory activity of the active components of plant extracts against *H. pylori* are reported (21, 22, 24-27).

Regarding *F. sellowiana*, Motohashi's group (28) subjected the fruit peel to extraction with hexane, acetone, MeOH and 70% MeOH at room temperature, obtaining 26 fractions (**table I**). All fractions were tested against Gram-positive and Gram-negative bacteria, and *Candida albicans*. The data obtained showed that the acetone extract and the MeOH extract have inhibitory activity against the microorganisms tested.

Basile *et al.* (9) and Vuotto *et al.* (29) also evaluated the antibacterial activity of *Feijoa* fruit extracts against Gram-positive and Gram-negative but using the various parts of the fruit (whole fruit, pulp, and peel). Subsequently, the *Feijoa* fruit was subjected to extraction with acetone, obtaining 11 fractions (A-M). All fractions were tested against *H. pylori*, and by activity-guided fractionation, it was possible to identify the compound responsible for anti-*H. pylori* activity. The substance most responsible for this activity is the Flavone (6). Therefore, these works confirm that flavonoids of natural origin, and in particular Flavone, could be considered a natural therapy in the treatment of infections, having an interesting therapeutic potential for the treatment of gastrointestinal diseases associated with *H. pylori* infection, as well as their generic antibacterial activity against various Gram-positive and Gram-negative bacterial strains (30).

Immunomodulant activity

Flavonoids are a heterogeneous group of plant phenolic compounds widely used in the medical field because they have numerous biological activities, including antioxidant and immunomodulating activity. Ielpo *et al.,* (31) tested natural catechin, and two of its derivatives (+)-3-O propionylcatechin and (-)-3-O-valerylcatechin, extracted from the *Feijoa* fruit on the oxidative metabolism of phagocytes through the luminol-dependent chemiluminescence emitted by resting human phagocytes and activated by PMA (phorbol myristate acetate) (**table I**). Chemiluminescence is a simple method to study the oxidative metabolism of phagocytes and indirectly phagocytosis; in fact, light is emitted following a chemical reaction of cells activated as granulocytes, when phagocytosis is activated as the first immune response to protect the body from invaders. The results demonstrated that the low concentrations of

BIOACTIVE COMPONENT	CELL LINES	EVALUATION METHOD/ TREATMENT	EFFECTS	REFERENCE
Natural catechin; (+)-3-*O*- propionylcatechin; (-)-3-*O*- valerylcatechin extracted from *Feijoa*	Human leukocytes induced by PMA	(50 µM) Luminol-dependent CL	Inhibition of ROS release	(25)
Aqueous extract of *Feijoa* fruit	Human whole blood phagocytes (1.0 microliters); PMN (1 × 10^5 cells ml^{-1})	(1 m/ mL–12 ng/ mL). Basal CL 0.5 mg OZ-stimulated. 150 nmol PMA-stimulated	Inhibition of emission of CL	(23)
Feijoa acetonic extract	J774 (macrophage cell line)	(50, 250, 750 µg/mL) LPS stimulation (10 µg/mL) for 24 h MTT assay Griess assay	Decrease of nitrite production in a concentration-dependent manner (attenuating the activation of NF-KB and/or MAPK)	(6)
[A3] fraction benzene-AcOEt (1:1) from *Feijoa* peel	HSC-2; HSG	MTT assay (IC50 > 100 µg/mL)	Cytotoxic activity	(22)
Feijoa acetonic extract	Caco-2	BrdU assay (50, 500 µg/mL for 24 h) MTT assay (5, 50, 500 µg/mL for 24 h) H2O2 1mM and 5, 50, 500 µg/mL for 24 h Dahlqvist test and glucose oxidase assay (5-500 µg/mL for 24 h)	Decrease in cell proliferation rate No significant cytotoxic effect Significant reduction of MDA Improved lactase and sucrase-isomaltase activity	(30)
Feijoa acetonic extract	HT-29	BrdU assay (50, 500 µg/mL for 24 h) MTT assay 5mg/mL (5, 50, 500 µg/mL for 24 h) H2O2 1mM and 5, 50, 500 µg/mL for 24 h Dahlqvist test and glucose oxidase assay (5-500 µg/mL for 24 h)	Decrease in cell proliferation rate No significant cytotoxic effect Significant reduction of MDA No improvement in lactase and sucrase-isomaltase activity	(30)
PAOF-1 derived from *Feijoa* fruits	OSCC cell lines: HSC2; HSC-3; HSC-4; CAS9-22 HGF; HPC; HPLF	MTT assay CC_{50} of PAOF-1 against OSCC cell lines:151 µM CC_{50} of PAOF-1 against HGF, HPC, HPLF: 477 µM	Selective cytotoxicity against OSCC cell lines	(12)
Feijoa acetonic extract	HeLa; MCF-7; SKBR3; MDA-MB231; NB4	Feijoa acetonic extract (5-3 mg/mL) Crystal violet assay Trypanblue assay	Anti-proliferative activity dose-dependent	(13)

Table I. *List of the bioactivities of F. sellowiana acetonic extracts, flavone and catechins.*

BIOACTIVE COMPONENT	CELL LINES	EVALUATION METHOD/ TREATMENT	EFFECTS	REFERENCE
Feijoa acetonic extract	HeLa; MCF7; U937; NB4 LnCap	(0,5-1-3-5 mg/mL) for 3 days Western blot FACS RT-PCR	Apoptosis in a dose dependent manner HeLa blocked in G1 phase MCF7, U937, NB4 blocked in S or G2/M phases Less sensitive to treatment.	(13)have been often claimed, although the corresponding molecular mechanism(s
Pure flavone (FP) *Feijoa* acetonic extract	NB4	Pure flavone (0,037 mg/mL 170M). Western blot. FACS RT-PCR	Apoptosis: NB4 blocked in G1 phase Induction of p16, p21, and TRAIL Inhibition of HDAC	(13)have been often claimed, although the corresponding molecular mechanism(s
Feijoa acetonic extract FS or FP	AML primary blasts; CD34+	Feijoa acetonic extract (1-3 mg/mL) FS, FP (0,37 mg/mL). FACS	Apoptosis in AML primary blasts increasing histone H3 acetylation levels	(13)have been often claimed, although the corresponding molecular mechanism(s
Feijoa acetonic extract	BALB/c 3T3 (nonmalignant murine cell line); SVT2 (malignant counterpart) HRCE (human primary renal cortical epithelial cells); HEK-293 (transformed human embryonic kidney)	MTT assays (0-5 mg/mL for 24, 48, 72 h)	Cytotoxic activity: IC_{50} 48h (2,5 mg/mL for SVT2; 1 mg/mL for HEK-293) Cytotoxic activity: IC_{50} 48h (4,5 mg/mL for BALB/c 3T3; 2,5 mg/mL for HRCE	(36)
Feijoa acetonic extract	hBMSC (human bone marrow mesenchymal stem cell)	5 ng/mL for 4 days MTT assay 5 ng/mL for 7 days MTT assay	Improved proliferation and reduction of PDT Reduction of proliferation	(11)
Feijoa acetonic extract Synthetic flavone (FS)	SNU-1	MTS and Annexin V FITC assays (5, 50, 500 µg/mL for 24/48 h) MTS and Annexin V FITC assays (5, 50, 100 µg/mL for 24/48 h)	Antiproliferative and apoptotic effect in a time- and dose-dependent manner	(31)
Feijoa acetonic extract Synthetic flavone (FS)	AGS; KATOIII	MTS and Annexin V FITC assays (5, 50, 500 µg/mL for 24/48 h) MTS and Annexin V FITC assays (5, 50, 100 µg/mL for 24/48 h)	No growth inhibitory effects Antiproliferative and apoptotic effect in a time and dose-dependent manner	(31)

BIOACTIVE COMPONENT	CELL LINES	EVALUATION METHOD/ TREATMENT	EFFECTS	REFERENCE
Feijoa acetonic extract Synthetic flavone (FS)	PMN (polymorphonuclear leukocytes)	Feijoa acetonic extract (567,7 µg/mL) Synthetic flavone (21,6 µg/mL) SOD (superoxide dismutase) CAT (catalase); GPx (glutathione peroxidase)	Improved antioxidant enzymes activity The activity of SOD, CAT GPx enzymes was greater in PMN cells treated with flavone	(31)
1) Whole flower ethanolic extract 2) Petals ethanolic extract 3) Petal juice	*In vitro* antioxidant activity	FRAP CUPRAC DPPH ABST Total polyphenols	Antioxidant activity: whole flower > petals > petals juice. Total polyphenols: whole flower > petals juice > petals. See reference for more details	(46)
Fruit ethanolic extract (80:20 v/v)	*In vitro* antioxidant activity	1) ORAC 2) ABST 3) Deoxyribose assay	1) 148.8-272.7 µM Trolox equivalent 2) IC_{50} = 10.8-52.5 µg/ml 3) IC_{50} = 67.5-174.5 µg/ml	(49)
Leaves methylene choloride: methanolic extract (80:20 v/v)	*In vitro* antioxidant activity	1) DPPH 2) ABTS 3) FRAP 4) CUPRAC	1) 90.58 ± 0.89 2) 113.80 ± 0.02 3) 102.58 ± 0.41 4) 180.23 ± 0.44 mg Trolox equivalent/g.	(14)

Table I. *List of the bioactivities of F. sellowiana acetonic extracts, flavone and catechins.*

the *Feijoa* acetonic extracts were able to inhibit the release of ROS in human leukocytes induced by PMA. The catechins [50 µM] inhibited the chemiluminescence emission of resting phagocytes in a dose-dependent manner. In particular, the inhibitory effect is more evident when valerylcatechin is used. The authors hypothesized that this effect might be due to myeloperoxidase, lipoxygenase, or inhibition of NADPH-oxidase. Flavonoids can inhibit the release of β-glycuronidase acting on A2 phospholipase, and they also inhibit the phosphorylation of proteins that mediate the activation of PMNs induced by PMA.

Antioxidant activity

Oxidative stress is the result of the imbalance between ROS production and levels of antioxidant systems. Normally, cells can maintain a balance between ROS production and removal. When the equilibrium shifts toward the production of ROS or the levels of antioxidant systems, a condition of oxidative stress is established (32), which damages crucial biomolecules such as nucleic acids, proteins, lipids, and carbohydrates (33).

Several studies have shown that oxidative stress can play a crucial role in human pathophysiological diseases (34) and, in particular, ROS influence cancer evolution by initiating tumorigenesis, causing cell death, or inducing cell proliferation (33). Plants are rich in antioxidants compound and, currently, an increasing focus is on flavonoids (35).

Schmidt *et al.* reported that the ethanolic fruit extracts (80:20 v/v) had antioxidant effects against

OH radical, ROO– radical and ABTS radicals. The authors tested the *in vitro* antioxidant properties of *Feijoa* hydroethanolic extracts from three different locations. All the measured antioxidant activities were correlated to the fruit phenolic contents (**table I**). Both the antioxidant activities and the total phenolic content varied among the three harvested *Feijoa* fruits, suggesting that edaphoclimatic conditions, cultivation techniques and plant management can affect the phenols contents and consequently the antioxidant activities of the extracts. Vuotto *et al.,* (29) tested the aqueous extract of *Feijoa* fruit at various concentrations (1 mg mL^{-1} –12 ng mL^{-1}) on the oxidative burst in human whole blood phagocytes (1.0 microliters) and on isolated polymorphonuclear leukocytes (PMN) (1 × 10^5 cells mL^{-1}) by measuring chemiluminescence without (basal CL) or with 0.5 mg of opsonised zymosan (OZ-stimulated) or 150 nmol of phorbol myristate acetate (PMA-stimulated), in 1.0 mL final volume (**table I**). In addition, to exclude the toxic activity of *Feijoa* in PMNs, the Trypan blue exclusion test was carried out before and after the chemiluminescence evaluation, which showed that leukocytes were viable at all concentrations of the extract. When OZ was used as the stimulant, CL activity was affected only by the highest concentrations of *F. sellowiana* extract. Whereas, when PMA was used, CL inhibition was still statistically significant at low *Feijoa* extract concentration (≈ 15 mg L^{-1}). It was hypothesized that the aqueous extract of *Feijoa* was able to inhibit the emission of CL of native and stimulated human leukocytes by OZ and PMA and that this action can be explained by the scavenger effect on free radicals. Subsequently, the acetonic extract of *Feijoa* was tested on human intestinal epithelial cells (Caco-2 and HT-29) to evaluate their viability, cell proliferation, sucrase-isomaltase activity and lactase, and the membrane lipid peroxidation induced by H_2O_2 (36). The *Feijoa* extract (5, 50 and 500 µg mL^{-1}) after 24 h improved the activity of lactase and sucrase-isomaltase, in Caco-2 cells, but not in HT -29. Furthermore, it was shown that *Feijoa* acetonic extract also exerts antioxidant activity when cells are treated with H_2O_2, used to mimic an oxidative environment. The results obtained highlighted that the *Feijoa* acetone extract did not cause oxidative damage, on the contrary, it was able to have a significant protective and curative effect against the damage induced by H_2O_2. When Caco-2 and HT-29 cells were treated with *Feijoa* extract (5, 50, and 500 µg mL^{-1}) 2 h before and 2 h after exposure to H_2O_2, a decrease in MDA (malondialdehyde, a marker of lipid peroxidation) was observed.

The antioxidant activity of *Feijoa* has also been demonstrated by Russi *et al.,* (37) by testing the enzymatic activity of SOD, CAT and GPx on PMN cells (polymorphonuclear leukocytes). In particular, this study highlighted that the activity of antioxidant enzymes in PMN increases when cells are treated with *Feijoa* extract, and the greatest effect occurs when the flavone is used.

Other studies have investigated the antioxidant activity of non-edible parts of *Feijoa* such as leaves. Saber *et al*., 2021 reported the *in vitro* antioxidant activity of methylene chloride: methanol extracts (80:20 v/v) of *Feijoa* leaves. The leaves extracts showed a good *in vitro* antioxidant activity, as shown in Table 1. Furthermore, the authors isolated several pure compounds from the extracts and tested them for *in vitro* antioxidant activity. The results showed that quercetin, avicularin, flavone, and α-tocopherol were the main contributors to the antioxidant activity of the extracts (for more details see supplemental materials in (14)).

Piscopo *et al.* (17) investigated the *in vitro* gastrointestinal digestion of *Feijoa* fruit proteins. Interestingly, the results showed that the antioxidant activity increases 19-fold after digestion (0.731 ± 0.056 mmol TE mg^{-1}) with respect to the non-digested protein sample (0.039 ± 0.005 mmol TE mg^{-1}), indicating the release of small peptides with strong antioxidant activities during the in vitro gastrointestinal digestion. The ability of Feijoa to protect reproductive tissues from oxidative stress has been studied by Horri *et al.* (38) in mice treated with cadmium. The researchers analysed the sperm parameters, testis morphology, testis histopathology, and serum hormone levels in mice after an intraperitoneal exposure to 0.1 mg kg^{-1} cadmium only, and after cadmium plus 400 mg kg^{-1} *Feijoa* fruit extract. Mice exposed to cadmium showed loss of testis volume, testis weight, sperm viability, sperm number, and histological alterations such as disruption of the epithelium of seminiferous tubules. The treatment with 400 mg kg^{-1} *Feijoa* fruit extracts had a significant effect on the above-mentioned parameters to a level closer to controls.

Anti-inflammatory activity

To explain the action mechanism of the anti-inflammatory activity of *Feijoa sellowiana*, Rossi *et al.* (7) used the cell line of murine macrophages J774 stimulated with an iNOS inducer, lipopolysaccharide (LPS), which determines the overproduction

of NO in inflammatory processes (**table I**). When macrophages were pretreated with 10 µg mL^{-1} LPS for 24h, a very significant increase in NO in the cell medium was observed (63.70 nmol/106 cells *vs*. 2.95 nmol/106 cells). Nitric oxide (NO) is known to play a key role in the physiological and pathological functions of many organs, such as vascular tone regulation, neurotransmission, microorganisms, tumour cell killing, and other homeostatic processes. Several pathophysiological processes such as inflammation and carcinogenesis are correlated with high levels of NO (39). Then the acetonic extracts of *Feijoa* fruit were tested for their anti-inflammatory properties since previous studies exhibited the highest antioxidant and antibacterial activities among different *Feijoa* extracts (9, 29). The addition of the acetonic extract of *Feijoa sellowiana* coincided with dose-dependent inhibition of NO production (35.6, 75.8, and 92.5% inhibition at 50, 250, and 750 µg mL^{-1}). To further investigate the NO modulation, western blot analysis of iNOS, IkBα, and pERK-1/2 was performed. The results showed that the *Feijoa* fruit acetonic extract was able to inhibit the expression of iNOS in a dose-dependent manner (50, 250 and 750 µg mL-1). Simultaneously, IkBα and pERK-1/2 decreased, indicating that the acetonic extract acted as a transcriptional control on the expression of iNOS by blocking the activation of NF-kB via IkBα degradation. In fact, in macrophages, LPS activates the transcription factor nuclear factor-κB (NF-κB), which controls the expression of many early immediate genes, including iNOS. The same experimental procedure was applied to determine which chromatographic fraction of the acetonic extract was the most active. Aliquots of the acetonic extract were dissolved in methanol and separated chromatographically with different proportions of n-hexane/EtOAc or EtOAc/MeOH. Only two of 11 fractions, B (eluted with n-hexane/EtOAc 60:40) and C (eluted with n-hexane/EtOAc 50:50) were the most active, showing modulation of iNOS (extract B from 0.30 µM to 4.5 µM and extract C from 1.30 µM to 3.9 µM. The molecules responsible for the fractions activity were identified as the flavone (B) and stearic acid (C), which were thus the most active compounds in the *Feijoa* fruit. Interestingly, the authors found that *Feijoa* acetonic fruit extract was not cytotoxic to murine macrophages J774 at the concentrations tested (50, 250 and 750 µg mL^{-1}), suggesting low toxicity to normal cells. In summary, the study showed that *Feijoa* acetonic extract, thanks to Flavone and stearic acid, was able to inhibit NO production in J774 cells by attenuating NF-KB and / or MAPK activation. In a study by Mahmoudi *et al.* (40), mice with carrageenan-induced edema were treated with *Feijoa* leaves and fruit extracts. Carrageenan-induced edemas were significantly inhibited by the extract at 50-400 mg kg^{-1}. The researchers tested the antinociceptive effects of both extracts. The leaves extracts showed an activity equivalent to diclofenac at 50 mg kg^{-1}. Fruit extracts showed higher activity than diclofenac at 400 mg kg^{-1} doses. In all tested doses, the extract significantly augmented the pain threshold in the hot plate thermal test. Furthermore, the extracts were demonstrated to be safe up to a dose of 1 g kg^{-1}.

Cytotoxic activity

Motohashi's group (28) subjected the *Feijoa* peel of the fruit to extraction with hexane, acetone, MeOH and 70% MeOH at room temperature (**table I**). All fractions were tested against two tumour cell lines, HSC-2 (human oral squamous carcinoma cells), HSG (human oral salivary gland tumour cells), and against the healthy cell line HGF (human oral gingival fibroblast). Most fractions showed low cytotoxicity against tested cells (IC50 > 100 µg mL^{-1}); only the A3 fraction (benzene-AcOEt 1:1) showed a relatively cytotoxic action for tumour cell lines and the healthy cell line.
By increasing the solubility in water, there was a decrease in cytotoxic activity against the healthy cell line (SI = HGF/HSG-2).
Turco *et al.* (36) evaluated the cytotoxic activity of the *Feijoa* acetonic extract of fruit on Caco-2 and HT-29 (**table I**). The MTT assay showed that with 5, 50 and 500 µg mL^{-1} of extract (for 24 h) no significant cytotoxic effects occurred. Aoyama *et al.* (13) identified and quantified different polyphenols in the various botanical parts of *Feijoa* (fruit, leaves, flowers, and branches) through High-Performance Liquid Chromatography coupled (HPLC-MS) and Nuclear Magnetic Resonance (NMR). They purified from leaves, fruits, and flowers of *Feijoa sellowiana*, in addition to other substances, proanthocyanidin oligomer PAOF-1. PAOF-1 derived from *Feijoa* fruits was tested on OSCC cell lines (HSC2, HSC-3, HSC-4, CAS9-22) and healthy oral cell lines (HGF, HPC, HPLF) and the data obtained showed selective cytotoxicity against OSCC cell lines.

Antiproliferative effects of *Feijoa* acetonic extracts on HeLa, MCF-7, SKBR-3, MDA-MB231, NB4 U937, LnCap

Bontempo *et al.* (19) tested the acetonic extract of *Feijoa sellowiana* fruit in solid and hematolog-

ic tumour cell lines (**table I**). The acetonic extract showed antiproliferative activity, measured with the Trypan blue viability test, on several tumour cell lines: HeLa, SKBR-3, MCF-7, MDA-MB231, while treatment of prostate cancer cell lines (LnCaP) registered the lowest decrease in viability. The highest antiproliferative activity was observed when using 5-3 mg mL^{-1} of raw *Feijoa* acetonic extract. As for the effect of the acetonic extract on the cell cycle and apoptosis of solid cancer cells and haematological cancer cells, HeLa, U937, MCF7 and NB4 cells responded to the extract with dose-dependent apoptotic action but with different sensitivity; while the prostate cells (LnCap) responded less sensitively, indicating that the action of the *Feijoa* acetonic extract has a certain specificity and confirming the results of the viability test. Furthermore, increasing amounts of *Feijoa* acetonic extract resulted in blockade of the cell cycle in the phases S or G2 / M in U937, MCF7, and NB4 cells, while in HeLa cells the blockage occurred in the phase G1. The difference in the blocking of these cells at various stages of the cell cycle may have to do with the cellular context. The activity test of caspases 8 and 3, 7 was also performed on NB4 cells, demonstrating that the cell block was followed by apoptosis. Furthermore, the measurement of CD11c and CD14 constituted a clear signal of the restoration of granulocytic differentiation activity in the NB4 line, indicating that treatment with *Feijoa* caused cell cycle block followed by differentiation and cell death.

The first activity-guided fractionation was performed by Bontempo *et al.* (19). Activity-guided fractionation was performed to understand which substance or group of substances was able to explain the anticancer action of acetonic *Feijoa* fruit extract. Eleven fractions (A, B, C-E, F-H, I-M) were produced and of these only the fraction B, consisting of pure flavone (0.75% by dry weight), was able to induce apoptosis in NB4 cells. Unlike, however, the complete extract of *Feijoa* which induced a cell cycle block in the S or G2 / M phases in NB4 cells, the pure flavone induced the cell cycle block in the G1 phase. This difference was probably due to the presence of other components in the acetonic extract of *Feijoa* that modulated the activity of the cell cycle. Subsequently, the action of the pure flavone (FP) was compared with the commercial flavone (FS) and both flavones had the same effect, that is, blocking the proliferation and inducing apoptosis of cancer cells. Their activity is maximised at concentrations of 100-200 μM. Hence, flavone was the most active compound against the treated cancer cell lines in the *Feijoa* extract. Then, to understand the molecular mechanisms underlying cell cycle block and apoptosis, *Feijoa* extract and FP or FS were tested on NB4 cells, focusing attention on key factors of cell cycle and apoptosis. Both the acetonic extract and flavone (FP-FS) caused overexpression of p21 and p16 (cell cycle inhibitors) and TRAIL (the TNF ligand that induces apoptosis) in NB4 cells, both at the RNA and protein levels; furthermore, they induced hyperacetylation of histone H3 and α-tubulin (which was used as an example of a non-histone target of acetylation) and finally the enzymatic assays showed that both *Feijoa* acetonic extracts and FP-FS were able to inhibit HDAC activity. Further investigations were carried out by Scafuri *et al*., 2020 (41), studying the in-silico docking of flavone and its derivatives apigenin and luteolin to HDAC1 and HDAC 2. The authors observed that flavone, apigenin and luteolin have binding energies similar to a known inhibitor of HDAC 1 and HDAC 2, suggesting that these molecules can target HDAC 1 and HDAC 2. These results indicated that the anti-tumour activities of the Flavone can act through epigenetic modulation (19).

Chemical characterization and activity-guided fractionation

Numerous chemical studies showed that *Fejioa* contains many bioactive components such as flavonoids, phenolic acids, vitamin C, dietary fibre, and potassium (16, 42-44), which contribute to several beneficial health effects such as antimicrobial, anti-inflammatory, antioxidant, and anticancer activities. Furthermore, different organs such as flowers, fruits and leaves have shown different phytochemical profiles. In particular, Monforte *et al.* (45) showed that *Feijoa* pulp is rich in ellagic acid, gallic acid, quercetin, pyrocatechol, rutin, syringic acid, catechin, eriodictyol and eriocitrin.

Aoyama *et al.* (13) identified and quantified different polyphenols in the various botanical parts of *Feijoa* (fruit, leaves, flowers, and branches) through -HPLC-MS and NMR. They purified gossypetin-3-O-α-L-arabinofuranoside, gossypetin-3-O-α-rhamnopyranoside, gossypetin-3-O-β-xylopyranoside, naringenin glycoside from leaves, fruits, and flowers of *Feijoa sellowiana*, aromadendrin glycoside, cyanidin glycoside, quercetin, kaempferol glycoside, ellagic acid and its derivatives, flavone, peduncolagin and proanthocyanidin oligomer PAOF-1 testing the latter compound on oral squamous cell carcinoma

cell lines (HSC-2, HSC-3, HSC-4, CAS9-22). Interestingly, flavone was the main constituent of the leaf extract.

Similar results were obtained by Saber *et al.* (14), where flavone was the most abundant compound in leaves followed by avicularin, and quercetin. Mosbah *et al.* (42) analyzed the phenolic fingerprint of the aqueous extract of *Feijoa* leaves through HPLC-DAD-MS. The results showed that *Feijoa* leaves extracts contained mainly flavan-3-ols, procyanidins and catechins; flavonols such as quercetin glycosides and ellagitannins.

Recently, Montoro *et al.* (46) investigated the phytochemical profile and antioxidant activity of *Feijoa* comparing the whole flower, petals only and petals juice. *Feijoa* is known for its massive flower production, which can be a valuable molecule source for the food, pharmaceutical and nutraceutical industries (46). The researchers found that *Feijoa* flowers showed a different phytochemical profile with respect to fruits and leaves. The whole flower ethanolic macerate and the two analysed fractions contained various amounts of ellagitannins, flavonoids and anthocyanidins. Ellagitannins were higher in the whole flower than the petal macerate (15 *vs*. 0.4 mg L^{-1}), while < LOQ in petal juice. Flavonols were found in comparable concentrations in whole flower and petals macerates (42.9 *vs*. 45.1 mg L^{-1}) and lower in petals juice (4.7 mg L^{-1}). The whole flower ethanolic macerate showed the highest polyphenolic content (395.14 mg GAE L^{-1} *vs*. 98.59 in petals and 114.53 mg GAE L^{-1} in petals juice), with a consequent higher antioxidant activity compared to petals macerate and petal juice, measured with various *in vitro* assays (FRAP, CUPRAC, DPPH, and $ABST^{+}$) (**table I**).

Smeriglio *et al.* (8) showed interest in the phytochemical profile and biological activity of essential oils (EO) extracted from the peel of the *Feijoa* fruit. Through GC-FID and GC-MS analyses, they identified and quantified 40 compounds belonging to sesquiterpenes (76.89%), monoterpene hydrocarbons (3.26%), and oxygenated monoterpenes (0.34%). The main compounds were γ-selinene (17.39%), α-caryophyllene (16.74%), β-caryophyllene (10.37%) and Germacene D (5.32%).

In a study carried out by Tuncel and Ylmaz (47), syringic and trans-cinnamic acids were identified in the pulp of the *Feijoa* fruit. Phan *et al*., 2019 (16) analysed whole fruit, peel and pulp methanolic extracts through UHPLC-PDA searching for phenolic compounds. The researchers found that *Feijoa* fruit peel contained the highest amounts of both free and bound phenolic compounds such as catechin, dihydroxyflavone, ellagic acid, p-coumaric acid, and ferulic acid.

However, it is important to note that the phytochemical profile of *Feijoa* can change according to the variety, depending on the portion of the fruit used, the ripeness, the climate, the origin of the plants, environmental conditions and the extraction method (8, 48). Schmidt *et al.* (49) found that *Feijoa* hydroethanolic extracts (80:20 v/v) of whole fruit collected in different sites showed a variable content in phenolic compounds. Furthermore, the authors reported for the first time the presence of castalagin, catechin and epicatechin.

Another research by Magri *et al.* (50) measured the phenolic content of *Feijoa* flowers at different flowering stages. The results indicated that *Feijoa* flowers in the early flowering stage (*i.e*., during petals opening) are characterized by the highest phenolic content.

Furthermore, the phenolic content can change between different Feijoa cultivars. In a study by Peng *et al.* (51), total phenolic contents of four Feijoa cultivars juice: Apollo, Wiki Tu, Unique, and Opal Star were investigated. The results showed that the Wiki Tu and Unique had the highest TPC (1.89 ± 0.01 mg GAE mL^{-1} juice) among the four cultivars, and the Opal Star cultivar had a significantly lower TPC (1.17 ± 0.01 mg GAE mL^{-1} juice).

Regarding environmental conditions, by comparing the composition of the essential oils of *Feijoa* fruits grown in polluted sites with those collected in nonpolluted sites, it was shown, by GC-MS, that the essential oils of *Feijoa* in polluted sites were characterised by a greater quantity of antioxidant compounds, in particular Flavone (the compound responsible for antitumoural and antioxidant activity), respect to the control site. Sixty compounds, representing 96.6% and 97.8% (unpolluted site and polluted site, respectively) of the oils were identified. The main constituents were β-caryophyllene (12.4% and 16.8%), ledene (9.6% and 11.1%), α-humulene (6.3% and 8.2%), β-elemene (4.9% and 5.3%) and δ-cadinene (4.7% and 5.2%) at the control site and the polluted site, respectively (48).

Selective cytotoxic activity of *Feijoa* extract

The activity of acetonic extract and flavone has been proven to be very specific as it does not manifest itself toward non-tumour cells.

In this regard, Dell'Olmo *et al.* (52) demonstrated

the selective cytotoxicity of *Feijoa* acetonic extract using healthy and cancerous eukaryotic cells, such as the nonmalignant murine cell line BALB/c 3T3 and its malignant counterpart, mouse fibroblasts SVT2, HRCE cells, and the malignant counterpart HEK-293 (Tab. 1).

In all cell lines, both time-dependent and dose-dependent inhibition of cell viability was shown. But the most surprising thing is that the extract was found to be more cytotoxic on cancer cells than on untransformed cells. Indeed, the 48-hour IC50 values were significantly lower for tumour cells (2.5 and 1 mg mL^{-1} for SVT2 and HEK-293 cells, respectively) than for untransformed cells (4.5 and 2.5 mg mL^{-1} for BALB / c 3T3 and HRCE cells, respectively). The goal of chemotherapy is to inhibit cell proliferation and tumour multiplication, thus avoiding invasion and metastasis. But, most conventional chemotherapy agents are toxic to both cancer cells and normal cells (53); in light of this, the selective, albeit partial, toxic action exerted by the *Feijoa* extract could represent an interesting feature for the future design of innovative chemotherapy strategies.

Bontempo *et al*. (19) studied, in addition to the selectivity of the acetonic also extract the activity of both Flavone and acetonic extract on AML primary blasts and CD34+ (**table I**).

The study showed that both *Feijoa* extract and FS or FP tested on AML samples induced apoptosis characterised by the overexpression of some molecular effectors (**table I**), namely p16, p21 and TRAIL; moreover, inhibition of deacetylases and, therefore, an increase in histone acetylation was found. The addition of FS or FP on the CD34+ did not result in significant biological effects, indicating that *Feijoa* and the flavones have a selective cytotoxic activity.

More recently, in a study by Rasekh *et al.* (12) the acetonic extract of *Feijoa sellowiana* was also tested in stem cells derived from human bone marrow (hBMSC) to assess their proliferative and apoptotic activity. The results obtained showed that with 5 ng mL^{-1} of *Feijoa* acetonic extract an increase in the proliferation of hBMSC was obtained up to day 4 thanks to the presence of bioactive components of the fruit (vitamins, polyphenols, essential minerals); after 7 days there was a decrease in proliferation due to the anticancer activity of *Feijoa*. Furthermore, overexpression of the Bax gene (pro-apoptotic protein) and a decrease of Bcl-2 (anti-apoptotic protein) were highlighted, confirming the role of *Feijoa* in the pro-apoptotic process.

Green synthesized silver nanoparticles (SNPs) prepared with *Feijoa* methanolic extract have shown selective antiproliferative activity against MCF-7 and AGS cells (18). The data indicated that the SNPs prepared with *Feijoa* methanolic extract at a concentration of 1.56 and 3.12 μg mL^{-1} were cytotoxic to MCF-7 and AGS cells, while no cytotoxicity was observed in human foreskin fibroblasts.

Antiproliferative and apoptotic effects on cancer gastric cells

Turco *et al.,* (36) measured the proliferation of intestinal epithelial cells by measuring the incorporation of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) into DNA (**table I**). The analysis showed that 50 and 500 μg mL^{-1} of *Feijoa* acetonic extract caused a decrease in the proliferation rate of Caco-2 cells, while a significant decrease in the proliferation rate of HT-29 cells was obtained using 500 μg mL^{-1}.

In another study, Russi *et al.,* (37) evaluate the proliferative and pro-apoptotic activity of *Feijoa* in gastric tumour cell lines (SNU-1, AGS, KATOIII). Cell lines were treated with *Feijoa* acetonic extract (5, 50, and 500 μg mL^{-1}) or flavone (5, 50, and 100 μg mL^{-1}) for 24 and 48 h. By MTS and Annexin V FITC assays, it was found that among the three cell lines tested, SNU-1 showed a significant decrease in cell proliferation and induction of apoptosis; in contrast, AGS and KATOIII were weakly influenced by treatment, confirming that gastrointestinal cancer is a disease characterised by cellular heterogeneity.

Effectiveness of *Feijoa* against multidrug-resistant cancer cells (MDR)

The phenotypic expression of MDR is the frustrating outcome of an initially successful chemotherapy treatment that affects and seriously compromises the effectiveness of conventional drugs, thus determining a consequent poor prognosis (54). Those responsible for drug resistance are the ATP-binding cassette transporters (ABCs), which pump a variety of drugs out of cells at the expense of ATP hydrolysis.

The P-glycoprotein (P-gp) is the most studied among ABC transporters and is responsible for transporting various xenobiotics out of cells by using ATP (55). It is now established that this protein is also expressed in many normal tissues at low levels (56), but the interest in this protein began when it was understood that its overexpression in cancer cells caused the MDR phenotype.

The analysis carried out in the study by Dell'Olmo *et al.* (52) highlighted that the *Feijoa sellowiana* extract can inhibit cell proliferation (measured by the MTT assay) of KB-3-1 (drug-sensitive cancer cell line) KB-C1, and KB - A1 (drug-resistant cells) in a dose-time dependent manner, thus indicating the ability of the *Feijoa* extract to also act on MDR tumour cells (**table I**).

This property of *Feijoa* leads us to consider the possible applicability of this natural extract to treat neoplasms characterised by multi-resistance.

However, specific studies on the modulation of MDR- related proteins (*e.g.,* P-glycoprotein) by *Feijoa* extracts would be advisable. Identification of compounds that are effective in MDR cancer cells could greatly contribute to the future design of alternative therapeutic approaches capable of overcoming this huge obstacle.

CONCLUSIONS

There is increasing evidence that polyphenols may protect cell constituents against oxidative damage and provide significant protection against the development of several chronic diseases (35).

Indeed, the *Feijoa* acetonic extract, (in particular the catechins), is able to inhibit the release of ROS in human PMNs induced by PMA, probably thanks to myeloperoxidase, lipoxygenase or inhibition of NADPH-oxidase. Furthermore, the aqueous extract of *Feijoa* was able to inhibit the emission of CL from native and stimulated human leukocytes by OZ and PMA. This action can be explained by the scavenger effect on free radicals (29). Since immune and inflammatory cells are also affected by diet, foods rich in flavonoids, such as vegetables, should promote good health (31).

Furthermore, it has been shown that the acetonic extract of the *F. sellowiana* fruit, in particular the flavone, exerts a powerful antifungal (*Candida albicans*), antibacterial activity against some Gram-positive and Gram-negative bacterial strains (28) and in particular, the action of the flavone was significantly effective against *H. pylori* (6). Therefore, these works confirm that flavonoids of natural origin can be considered a natural therapy in the treatment of infections.

In summary, *Feijoa* has been shown to have antioxidant activity when Caco-2 and HT-29 cells have been treated with H_2O_2 (36); anti-inflammatory activity, due to NO inhibition by attenuating the activation of NF-κB and/or MAPK in J774 cells (7); anti-tumour action blocking the cell cycle of cancer cells in S, G2/M or G1 phases and inducing apoptosis due to flavone, responsible for the overproduction of p16, p21 and TRAIL and inhibiting HDAC in cancer cells. Flavone has been demonstrated to act on epigenetic processes via HDAC. Currently, for many natural compounds, it is not completely clear whether for some observed beneficial effects, such as antineoplastic activity, a transcriptional action is necessary or whether they are mainly related to epigenetic action. For this reason, further studies should be carried out to evaluate whether some of these biological activities described could be attributable to a possible epigenetic action exerted by the second metabolites present in the bryophytes as demonstrated for other compounds of natural origin (57).

Russi *et al.,* (37) evaluated the antiproliferative and pro-apoptotic activity of *Feijoa* on gastric tumour cell lines. Dell'Olmo *et al.* (52) demonstrated the selective cytotoxic activity of the acetonic *Feijoa* extract using healthy and cancerous eukaryotic cells. Finally, the acetonic extract of *Feijoa sellowiana* is effective on sensitive and MDR tumour cells (**table I**) (52). The efficacy of *Feijoa* acetonic extract against cells with MDR phenotype is very interesting because although various anticancer drugs have been developed, the toxic effect even on healthy cells and the presence of the MDR phenotype are the main obstacles to the success of cancer chemotherapy treatment. Hence, the identification of compounds that are effective in MDR tumour cells could greatly contribute to the future design of combinatorial therapeutic approaches that are effective against disease states that now inevitably lead to death. Taken together, these data provide a new perspective for the use of plant products in alternative anticancer treatments, thanks to their ability to counteract the MDR phenotype and have a selective cytotoxic effect.

In conclusion, the ability of the *Feijoa sellowiana* fruit extract to induce selective proliferative arrest, cell differentiation, and apoptosis, together with the ability to counteract the MDR phenotype, opens interesting prospects for its future applicability in cancer therapy.

Furthermore, *Feijoa* can be considered a safe nutraceutical to improve pathologies characterised by reduced disaccharidase activity (lactose and sucrase-isomaltase) and having antioxidant properties that can have beneficial effects in diseases

caused by oxidative stress such as cancer (36, 37). All the evidence obtained here could contribute to the future identification of new compounds effective in pathologies that require innovative strategies.

ETHICS

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There were no institutional or private fundings for this article.

Conflict of interests

The authors have declared no conflict of interests.

Availability of data and materials

The data underlying this article are available in the article.

Authors' contribution

All authors contributed to write and revise the manuscript.

Ethical approval

N/A.

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REVIEW

BIOMARKERS OF HOMOLOGOUS RECOMBINATION DEFICIENCY IN THE ERA OF PARP INHIBITORS

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ABSTRACT

Homologous Recombination Deficiency (HRD) was initially described in cancers with germline mutations of *BRCA1* and *BRCA2* and thereafter in both sporadic and hereditary cancers carrying muta-tions or epigenetic inactivation of other genes in-volved in HR. Since cancers harbouring HRD are particularly susceptible to PARP inhibitors (PARPi), identifying methods to detect HRD that can accu-rately predict clinical sensitivity to PARPi beyond *BRCA1/2* mutations has been challenging. In this review, we describe the HRD biomarkers identified up to now, pointing out strengths and weakness-es of each associated assay.

Multigene panel test-ing, genomic scar assays and the most recent func-tional assays developed in the last ten years are associated with several drawbacks, mainly due to the possible restoration of HR proficiency and tu-mor heterogeneity. The use of functional assays on samples obtained from liquid biopsy could overcome these issues, providing a dynamic readout of HRD status and helping in clinical decision-making especially in the recurrent setting. Composite HRD scores involving two or more biomarkers would be probably required to define "HRDness" and to predict response to PARPi alone or in combination regimens.

KEY WORDS

Omologous recombination deficiency; PARP inhibitors; genomic scar; BRCA1; BRCA2.

IMPACT STATEMENT

The current available biomarkers to infer the presence of HRD, including multigene panel testing, genomic scar and functional assays, are inadequate predictors of response to PARPi.

INTRODUCTION

Homologous recombination (HR) is a fundamental pathway that allows error-free repair of double-stranded DNA breaks (DSBs). HR operates during S and G2 phase of the cell cycle when a homologous sister chromatid is available as template and relies on many proteins including BRCA1 and BRCA2, MNR complex (MRE11/RAD50/NBS), RAD51, ATM, ATR, PALB2, BRIP1, and BARD1 (1). HR deficiency (HRD) induces activation of the more error-prone template-independent non-homologous end-joining (NEJH) pathway, which results in the accumulation of additional mutations and chromosomal instability (2).

HRD was initially described in cancers with germline mutations of *BRCA1* and *BRCA2* (*BRCA1/2*) (3). However, germline or somatic mutations or epigenetic inactivation of other genes involved in HR can lead to HRD in both sporadic and hereditary cancers, broadly termed BRCAness (4, 5). Cells whit HRD are particularly susceptible to the DNA damage induced by DSBs and crosslinks generating agents like platinum compounds (6, 7). Moreover, cells with mutant *BRCA1/2* are exquisitely sensitive to poly-(ADP-ribose) polymerase (PARP) enzyme PARP inhibitors (PARPi) (8, 9). The PARP1 subunit binds single-stranded DNA breaks (SSBs) and then organizes their repair by synthesising PAR chains on target proteins (the so-called PARylation) (10). Inhibition of PARP1 promotes SSBs, which, if unrepaired, consequently lead to DSBs by collapsing of the stalled replication fork during DNA replication (11). PARPi act mainly in a double way: by inhibition of the catalytic activity of PARP1, which results in synthetic lethality in cells with impaired HR, and by trapping PARP1 at sites of DNA damage (12, 13).

Other mechanisms of HR impairment beyond *BRCA1/2* mutations can similarly confer PARPi sensitivity; however, identifying methods to detect HRD that can accurately predict clinical sensitivity to PARPi has been challenging (14-16). *BRCA1/2* mutations and/or HRD status have been evaluated in clinical trials with PARPi (16-18). Multiple genomic biomarkers have been evaluated to presume the presence of HRD; although promising, these biomarkers are inadequate predictors of response to PARPi, with clinical benefits observed both with and without HRD as measured by current clinical assays (19). In this review, we aim to describe the HRD biomarkers identified up to know, pointing out strengths and weaknesses of each associated assay, and key challenges in the clinical use of HRD testing. An overview of HRD assays and their biological principles are summarized in **figure 1**.

GERMLINE AND SOMATIC MUTATIONS IN HR-RELATED GENES

Testing for germline and somatic mutations in *BRCA1/2* and other HR-related genes may be used to infer the presence of HRD. Germline *BRCA1/2* (*gBRCA*) mutations are present in 13-15% of epithelial non-mucinous ovarian cancer (OC) patients and an additional 5-7% of OC harbour somatic *BRCA1/2* (*sBRCA*) mutation that have arisen during cancer development or progression (20, 21). *BRCA1/2* mutant cells show clear evidence of HRD in vitro (22, 23). The main randomised clinical trials indicate that *gBRCA* mutations remain the best clinical biomarker for response to PARPi while limited data are available on *sBRCA* mutations alone, although the clinical outcomes for patients with *sBRCA* mutations were similar to those with *gBRCA* mutations (24-33). Retrospective analysis from Study 19 identified bi-allelic inactivation in > 80% of cases of *sBRCA* mutation and mutations were predominantly clonal, suggesting that *sBRCA* mutations arise early in tumorigenesis (34).

In vitro studies showed that beyond *BRCA1/2*, mutations in other HR-related genes can also confer an HRD phenotype and increased sensitivity

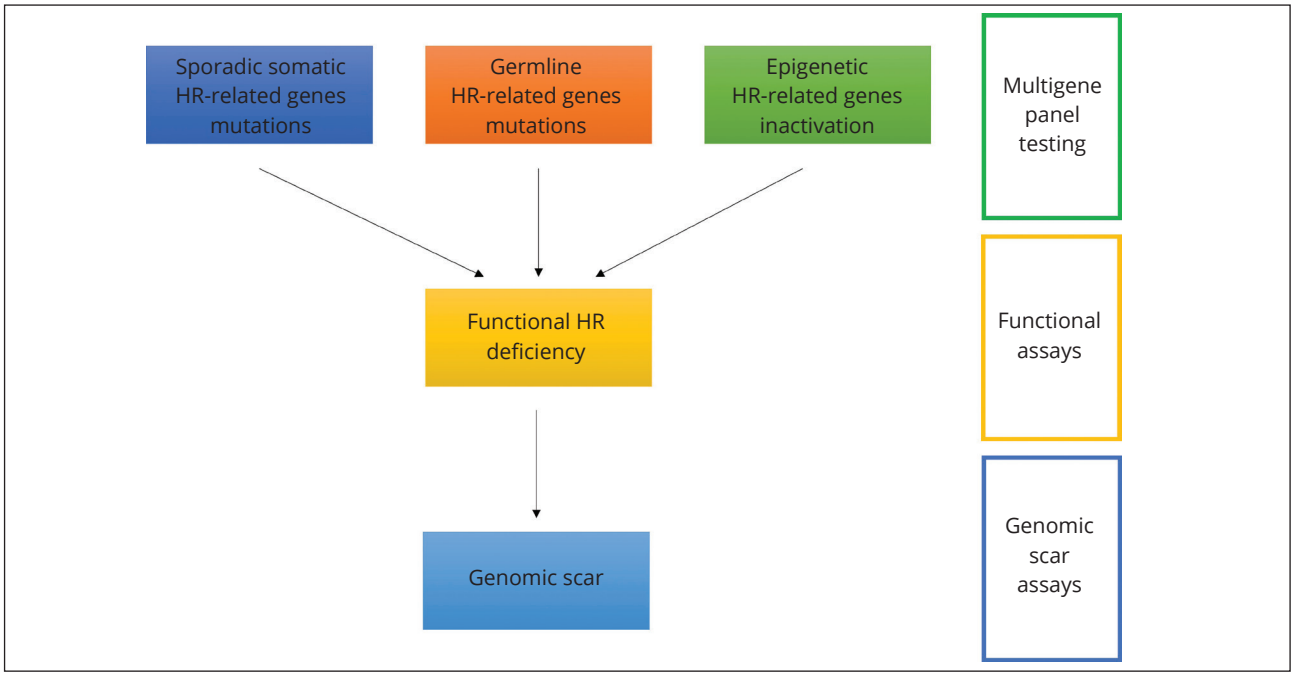

Figure 1. *An overview of HRD assays and their biological principles. Sporadic somatic or germline mutations (that can be detected with multigene panel testing) as well as epigenetic inactivation of HR-related genes induce a functional deficiency of HR recombination that can be revealed by functional assays. HRD and consequent defective DNA repair induces chromosomal aberrations, called genomic scars, detectable by specific assays.*

to platinum and/or PARPi (35). Cancer-associated mutations in *PALB2*, *BARD1*, *BRIP1*, *RAD51B*, *RAD51C*, *RAD51D*, *ATM*, *FAAP20*, *CHEK2*, *FAN1*, *FANCE*, *FANCM*, and *POLQ* (20,36,37), are potential biomarkers of HRD in cancer but how much these genes impact on PARPi response *in vivo* is still being defined due to the relative rarity of non-*BRCA* HR-related genes mutations (19). For example, mutations or methylation of *RAD51C* were identified in OC patients with clinical PARPi responses (38), and patients harbouring *RAD51C/D* mutations had long-term responses with rucaparib (39). *ATM* pathogenic variants are associated with olaparib response in OC and prostate cancer (40, 41).

Germline genetic testing is recommended for all women with OC, ideally with genetic counselling (18, 42). The blood-based assay Myriad Genetics BRACAnalysis CDx platform (Myriad Genetics; Salt Lake City, UT) has been FDA approved to identify OC patients with suspected pathogenic *gBRCA* variants eligible for treatment with olaparib (43). The phase III studies of PARPi in OC (Study 19 (33) and the NOVA trial (24)) and breast cancer (BC) (OlympiAD (25)) used BRACAnalysis to establish *gBRCA* mutation status (**table I**). Multigene germline panels, which extend the analysis to other genes as-

TRIAL	GBRCA TEST	SBRCA TEST	HRD TEST
SOLO1 (30)	Myriad BRCAnalysis	FoundationFocus BRCA	NA
PRIMA (29)	Local testing	Myriad myChoice HRD	Myriad myChoice HRD
PAOLA-1 (32)	NA	Myriad myChoice HRD	Myriad myChoice HRD
VELIA (27)	Myriad BRCAnalysis	Myriad myChoice HRD	Myriad myChoice HRD
Study 19 (33)	Myriad BRCAnalysis or local testing	Foundation medicine NGS	NA
SOLO2 (31)	Myriad BRCAnalysis	NA	NA
NOVA (24)	Myriad BRCAnalysis	Myriad myChoice HRD	Myriad myChoice HRD
ARIEL2 (37)	BRCA-HR Sequencing	FoundationFocus BRCA	FoundationFocus BRCA LOH
ARIEL3 (28)	Myriad BRCAnalysis	Foundation medicine NGS	FoundationFocus BRCA LOH

Table I. *HRD biomarkers and relative assays used in clinical trials of PARPi in ovarian cancer.*

sociated with increased cancer risk such as *BRIP1*, *RAD51C/D*, and *PALB2*, include both commercial and academic laboratory tests (44). There is currently no approved diagnostic assay for HRD based on germline mutations of other HR-related genes. Using germline mutations in HR genes to classify tumors as HRD has several disadvantages. In fact, is not always clear if a mutation truly disrupts gene function or is benign: the American College of Medical Genetics and Genomics provides guidelines for variants interpretation but in case of variants of uncertain significance (VUS) the genotype-phenotype correlation remains unclear (45). Somatic reversion mutations in *BRCA1/2* could restore HR function and confer platinum and PARPi resistance even with germline mutation (46).

The tissue based FoundationFocus CDx BRCA assay (Foundation medicine; Cambridge, MA) detects both *gBRCA* and *sBRCA* mutations in the tumor and is FDA approved as a companion diagnostic to rucaparib based on the ARIEL trials (28, 38) (**table I**). Multigene panels detecting somatic mutations in other genes than *BRCA1/2* may add additional information although mutations in non-*BRCA* HR-related genes are not currently part of an FDA-approved test to assess PARPi eligibility in OC (19). The limits of somatic testing include not only the difficult interpretations of VUS and the possibility of reversion mutations as for germline testing, but also the impossibility to analyse the intratumoral heterogeneity in a single tumor specimen.

PLATINUM SENSITIVITY

Platinum sensitivity *in vitro* is a feature of HRD, and *BRCA1/2* mutant OC and BC have increased platinum sensitivity (7, 47). As platinum is a key component of first line chemotherapy in OC, prior platinum sensitivity has been considered a surrogate clinical marker for prediction of PARPi efficacy (48). For example, in the phase III NOVA trial, Niraparib conferred a benefit in all subsets of platinum-sensitive OC, also in non-*BRCA* mutated patients (24). However, PARPi sensitivity does not completely overlap with platinum sensitivity in all cases (**figure 2**) (26). Considering cancers with defects in nucleotide excision repair, the response to platinum therapy does not confer a concurrent PARPi sensitivity (49). On the other hand, there is also a fraction of platinum-resistant patients who maintain PARPi sensitivity (50).

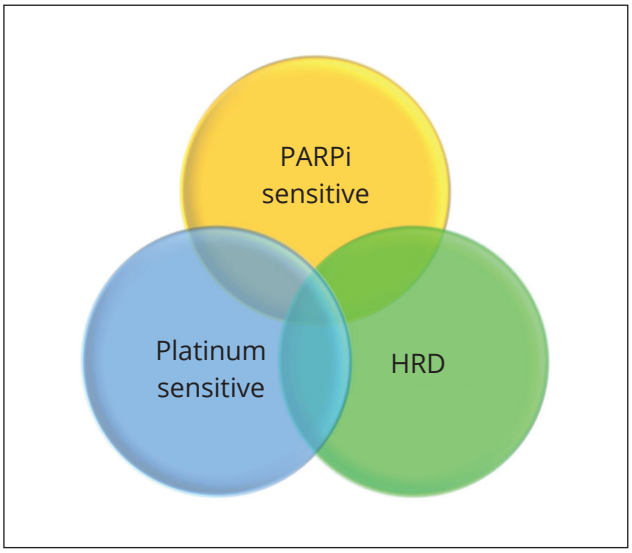

Figure 2. *HRD, platinum and PARPi sensitivity. Tumors with evidence of HRD, determined by the current available tests, are more likely to respond to platinum compounds and PARPi. However, PARPi sensitivity does not completely overlap with platinum sensitivite in all cases.*

GENOMIC SCAR ASSAYS

The loss of HR function and consequent defective DNA repair induces chromosomal aberrations, irrespectively of which component of the pathway was lost. "Genomic scars" of HRD consist of specific patterns of mutations and structural chromosomal aberrations, including rearrangements, insertions, and deletions in the genome (51). Current genomic scar assays are based on a combination of different genomic profiling techniques including array-based comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) genotyping, and next generation sequencing (NGS).

aCGH of structural chromosomal rearrangements

The aCGH assay detects genomic copy number variation (CNV) in tumors (52). An aCGH genomic profiles analysis of primary BC identified four subgroups, two of which were enriched for *BRCA1/2* deficiency (53). However, only two-thirds of *BRCA1*-like tumors harbour either *BRCA1* mutation or promoter methylation. A *BRCA1*-like aCGH signature predicted favourable response to platinum, suggesting that this signature identifies a wider spectrum of HRD tumors (54). The *BRCA1*-like and *BRCA2*-like profiles were later combined to create a *BRCA*-like aCGH score that was evaluated ret-

rospectively in a BC clinical trial, where *BRCA*-like aCGH patients showed a statistically significant benefit from high-dose platinum-based therapy (55). Up to now, aCGH assays have not been evaluated in the context of PARPi.

SNP-based "genomic-scar" assays

In 2012, three studies reported SNP-based CNV assays to assess up to three types of genomic scarring patterns (56-58). Loss of heterozygosity (LOH) is the absence of one of two gene alleles at a heterozygous site or uniparental disomy due to inaccurate repair of sister chromatids during the S/G2 phase of cell cycle. A study in OC detected that a "HRD-LOH" score defined by the number of LOH regions of more than 15 Mb and shorter than the whole chromosome was associated with *BRCA1/2* deficiency (56). Large-scale transitions (LST) are chromosomal breaks between adjacent genomic regions longer than 10 Mb (after exclusion of region shorter than 3 Mb). Break points may be caused by chromosomal inversions, deletions, duplications, translocations, or other rearrangements. *BRCA1/2* and *RAD51C* deficient BC show higher LST than sporadic cancers (57, 59). Telomeric allelic imbalance (TAI) considers subchromosomal regions displaying allelic imbalance extended to one of the telomeres but not crossing the centromere longer than 11 Mb. TAI is consequence of aberrant chromosomal end fusion due to inappropriate end-joining during mitosis. TCGA data show elevated TAI in *gBRCA* mutated OC and higher levels of TAI correlates significantly with response to neoadjuvant platinum-based chemotherapy in triple-negative BC (58). HRD-LOH, LST, and TAI are correlated each other (60) and are associated with *BRCA1/2* deficiency independently and when combined into a single score (56-61).

Several combination HRD score have been described (62), with most data for the three-factor combination scar assay by Myriad Genetics (60). The Myriad myChoice test joins a combined HRD score called "Genomic Instability Score" (GIS) with mutation and rearrangement analysis of *BRCA1/2*. GIS consists of the unweighted sum of LOH, LST, and TAI which produces a continuous score between 0 and 100. A threshold for GIS was decided on a pooled set of BC and OC in which HRD was defined as biallelic *BRCA1/2* loss of function. A score of 42 corresponded to the 5th percentile of the set of known *BRCA*-mutant tumors, therefore a score of ≥ 42 was established to denote HRD and a score of < 42 was considered HR-proficient (63). Several PARPi clinical trials have incorporated the myChoice test (**table I**), with a score of ≥ 42 considered HR deficient in most trials (24, 29, 32). This assay was FDA-approved as a companion diagnostic for niraparib in relapsed OC and for olaparib with bevacizumab in newly diagnosed patients following front-line therapy (19).

The FoundationFocus™ CDx BRCA LOH assay was applied in clinical trials of rucaparib (28, 38) (**table I**) and it has been approved as a complementary diagnostic to determine tumor HRD status. In this assay, a percent genomic LOH is calculated based on the fraction of genome regions with LOH. The optimal cut-off from analysis of OC (56) was 14% genomic LOH, which was prospectively validated in the ARIEL2 study, where progression-free survival was longer in the LOH-high subgroup compared with LOH-low (38). In a subsequent phase III study of rucaparib ARIEL3, the cut-off was adjusted to 16% genomic LOH as the threshold to identify HRD tumors (28).

The combined HRD score and the percent genomic LOH only partial correlated in predicting HRD status (64). Both HRD tests have several drawbacks, since they estimate the likelihood of HRD in the tumor based on evidence of genomic scarring. However, genomic alterations induced by HRD are permanent, even if functional capacity of HR is restored, for example in case of reversions in *BRCA1/2*, hence HRD testing via one of these assays on archival tumor may not represent the current HRD status of the cancer cells. Furthermore, HRD test results may not perfectly predict PARPi response due to PARPi resistance mechanisms which overcome HRD. Finally, HRD tests can have false positives or negatives due to technical factors, empiric threshold to classify HRD patients not accurate for all and heterogeneity in HRD between biopsy site and other disease sites (19).

NGS-based mutational signatures

Cancer types carry distinct mutational signatures which reveal the impact of different mutational processes including aging, UV light, and DNA damage repair and replication defects. A set of mutational signatures were detected from whole-exome sequencing of human tumors using NGS and computational technologies (65, 66). One of these, "signature 3" is enriched in cancers with *BRCA1/2* mutations and other mechanisms of HRD and has

been shown to exist in several cancers, including BC, OC, pancreatic, prostate, and gastric. It has been proposed as a biomarker for HRD (67, 68). A computational tool called Signature Multivariate Analysis (SigMA) can identify the presence of signature 3 on targeted gene panel data and does not require whole-exome sequence data. However, the sensitivity for identification of signature 3 is only 74% (67).

HRDetect (69) was developed using whole genome sequence data from *BRCA* mutant and wild-type (control) BC samples. The algorithm uses information from all four aCGH genomic profiles and incorporates a weighted score of microhomology mediated deletions, base substitutions/rearrangements signatures and the HRD score (as used in Myriad myChoice HRD). Using a probabilistic cut-off of 70%, HRDetect predicted *BRCA* deficiency with a sensitivity of 98.7% in BC and reaching 100% in OC and pancreatic cancer validation cohorts. There is some evidence that the HRDetect score can predict clinical outcome and response to platinum therapy in BC but its ability to predict PARPi benefit has not yet been established (70, 71).

There is strong pre-clinical evidence that mutation-based assays that use information from multiple mutation types could outperform existing scar assays (for example the GIS had a sensitivity of 60% (63)). A major limitation, however, is the need of fresh frozen material while most trial samples are formalin fixed paraffin embedded (FFPE). A second limitation is that mutation-based assays remain genomic scar assays, so they by definition reflect the historical presence of HRD and do not provide information about current HR status that can be restored through different mechanisms as above mentioned.

FUNCTIONAL ASSAYS

Functional assays have the potential to provide a dynamic indicator of the actual HR status, giving the challenge of measuring a single downstream event that would reflect proficiency of multiple upstream components of HR (16). The most used experimental system in this setting has been quantification of RAD51 nuclear foci. RAD51 is a DNA recombinase which act as a downstream HR protein facilitating DNA strand invasion into the sister chromatid and consequent faithful DSBs repair. Reduced DNA damaged-induced nuclear RAD51 foci has been associated with *BRCA1/2* deficiency as well as PARPi responses, both in OC and BC laboratory models and in small cohorts of patient samples (72-74).

One of the most frequently used RAD51-based functional HRD tests that has been validated on different tumor and specimen types is the REcombination CAPacity (originally termed Repair CAPacity) or RECAP test (75-78). However, this test relies on the use of fresh tumor tissue and requires *ex vivo* induction of DNA damage; so, a RAD51 score on FFPE tumor tissue has been developed (79-81). The RAD51 score is dependent on the combination of two parameters: the percentage of geminin-positive GMN+ cells (an S/G2 phase cell proliferation marker (82)) with RAD51 foci and the number of RAD51 foci per nucleus. In BC samples, an RAD51 score threshold of 10% GMN+ cells with RAD51 foci and a cut-off of five foci/nucleus showed the best correlation with PARPi response in *gBRCA1* patient-derived xenografts and *gBRCA1/2* patient samples (79). This outcome was confirmed by a second study that identified all *BRCA1/2*-deficient BC tumors as HRD (80). An RAD51 score threshold of 15% GMN+ cells with RAD51 foci in combination with a RAD51 foci number cut-off of two foci/nucleus yielded the highest sensitivity, identifying 90% of *BRCA*-deficient and 87% of RECAP-HRD cases on endometrial and OC specimens (81). More recently, Pellegrino *et al.* (83) using a panel of patient-derived tumor xenografts models from BC, OC and pancreatic cancer demonstrated that a RAD51 score ≤ 10% predict PARPi response more accurately than HR-related gene mutations and genomic scar analysis. This work furnished a preclinical *in vivo* validation of the RAD51-immunofluorescence test for dynamic identification of tumors with HRD, differentiating PARPi-sensitive tumors from those that become PARPi-resistant after restoration of functional HR.

Drawbacks of RAD51 foci as a surrogate of HRD include the impossibility to identify defects in HR downstream of RAD51 loading on to DNA and technical aspects, such as the possibility of non-informative results (due to insufficient number of proliferating tumour cells) (82). Retrospective analyses of larger clinical cohorts are also needed to clinically validate the RAD51 score thresholds above mentioned and prospective trials selecting patients according to their RAD51 score are also awaited.

FUTURE PERSPECTIVES

Although promising, the current available biomarkers (multigene panel testing, genomic scar and functional assays) are inadequate predictors of response to PARPi, with clinical benefits observed both with and without HRD (84). Moreover, the current HRD assays do not provide a dynamic readout and are only valid for the time point at which the cancer tissue sample is obtained, usually at diagnosis, and do not consider tumor heterogeneity. Considering cancer's capacity to continuously evolve and to develop therapy resistance, functional assays are expected to be able to detect acquired resistance to PARPi due to HR restoration in HRD tumors. The so-called liquid biopsy that sample circulating tumor cells or circulating tumor DNA may overcome these issues. Hence, analysing feasibility of functional assays on multiple and serial samples obtained from liquid biopsy could majorly impact in clinical decision-making in the recurrent setting.

Several mechanisms of resistance to PARPi have been suggested, with only reversions in *BRCA1/2* clinically proved; however, other mechanism different from HR restoration have been described in preclinical models (85). Moreover, several ongoing clinical trials are investigating the combination of PARPi (especially Olaparib) with inhibitors of the replication stress, particularly ATR inhibitors (86), in order to elicit additive or synergistic effects and possibly overcome PARPi resistance. Given the complexity of the HR pathway and its interaction with cell cycle regulation, response to stress replication, and other DNA damage repair pathways it is unlike that one single biomarker will suffice: in all likelihood, composite HRD scores involving two or more biomarkers would be required to define "HRDness" and to predict response to PARPi alone or in combination regimens.

CONCLUSIONS

The need of predictive markers of response to PARPi is raising alongside with the increasing use of PARPi in clinical practice and the emerging of resistance to these agents. However, the current available biomarkers to infer the presence of HRD, including multigene panel testing, genomic scar and functional assays, are not able to accurately predict clinical sensitivity to PARPi. In the next future, the implementation of composite HRD scores involving multiple biomarkers identified on tumor samples from liquid biopsy will be challenging.

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Availability of data and materials

The data underlying this article can be shared just before a reasonable request to the corresponding author.

Authors' contribution

All the authors contributed equally to conception, data collection, analysis and writing of this paper.

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REVIEW

HEDGEHOG SIGNALING PATHWAYS IN MULTIPLE MYELOMA

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ABSTRACT

Multiple myeloma (MM) is a hematological disease characterized by the uncontrolled proliferation of bone marrow malignant plasma cells. Localization and survival of malignant cells relies on bone marrow niche, in turn determined by the interaction between MM cells and mesenchymal stromal cells (MSCs). Several reports suggest that Hedgehog (Hh) pathway plays an outstanding role in tumor microenvironment maintenance. Hh signaling orchestrates the transformation of the myeloma bone marrow microenvironment supporting the proliferation of malignant plasma cells by affecting NF-kB signaling. To date, different clinical approaches are currently undergoing to evaluate the role of Hh modulators as efficient MM therapy. In this review article, we discuss the recent advances in the understanding of Hh signaling pathway in MM microenvironment.

KEY WORDS

Hedgehog; myeloma multiple; tumor microenvironment.

IMPACT STATEMENT

Several reports investigated the role of Hedgehog signaling in multiple myeloma progression. In this review we discuss the recent advancements in this field, also considering the new drugs currently in clinical trial.

INTRODUCTION

Multiple myeloma (MM) is a hematological disease characterized by bone marrow malignant plasma cells enhanced proliferation (1), usually resulting into hypercalcemia, renal impairment, anemia and bone pain (2). Myeloma bone disease is a devastating complication of MM observed in more than 80% of patients (3). The pathophysiology characterizing this outcome include a series of complex biochemical and cellular processes involving osteoclasts and osteoblasts activity, orchestrated by osteocytes. These cells act as mechano-sensors mediating the bone remodeling process by secreting cytokines such as Osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL) (4). In the MM context, several studies reported an increased RANKL/OPG ratio resulting into osteoclasts activation and disruption of bone marrow homeostasis (5-9). In physiological conditions, osteocytes inhibit osteoblasts differentiation by blockage of the canonical Wingless-type (Wnt) signaling, mediated by sclerostin and Dickkopf-1 (Dkk-1) secretion (10). Elevated amounts of DKK1 in MM patients correlated with the presence of focal bone lesions (11)**.** Moreover, the bone resorption is enhanced by malignant plasma cells, acting by i) releasing macrophage inflammatory protein-1a and b (MIP-1α-β), ii) inducing mature osteoblasts apoptosis and iii) inhibiting the differentiation of their precursors (12-14). As a result, bone matrix degradation releases the growth factors and cytokines boosting MM cells survival (15). For this reason, targeting the osteocytes-osteoblasts axis may represent a promising strategy counteracting MM progression.

The Hedgehog (Hh) signaling pathway holds a critical role for intercellular communication during the development of many organs, while its aberrant activation has been reported in several cancers (16). Hh signaling mostly relies on primary cilium, a microtubule-based organelle in the surface of vertebrate cells serving as mechano-sensory structure towards microenvironment stimuli (17). Consistently, primary cilium may act as a communication hub during organ and embryonic development, immune response, and tissue homeostasis, eventually triggering different cascade, including Wnt signaling (18).

Hh mammalian proteins have been grouped into three classes: Sonic Hedgehog (Shh), Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh), in turn explicating different duties within the cellular context. The latter has been reported to play a major role in endochondral ossification during skeletal development, while Dhh expression has been described in pre-Sertoli cells leading male sexual differentiation, and Shh is secreted to mediate epithelial invagination, limbs patterning and nervous system commitment (19-21). Interestingly, activation of the Hh pathway has been reported to rely on two distinct mechanisms, namely canonical- and non-canonical- Hh activation (16). In the canonical pathway (**figure 1*A***), one of the Hh proteins binds to the hedgehog protein receptor Patched (Ptch), which is eventually internalized and degraded. Repression of the Ptch, occurring upon Hh binding, triggers 7-transmembrane protein Smoothened (Smo) activity, in turn promoting, downstream, GLI family zinc finger (Gli) nuclear traslocation. As a result, Gli modulates a plethora of genes widely identified as Hh targets (17), involved in cell cycle regulation, apoptosis, proliferation, angiogenesis, self-renewal, and epithelial-to-mesenchymal transition (22).

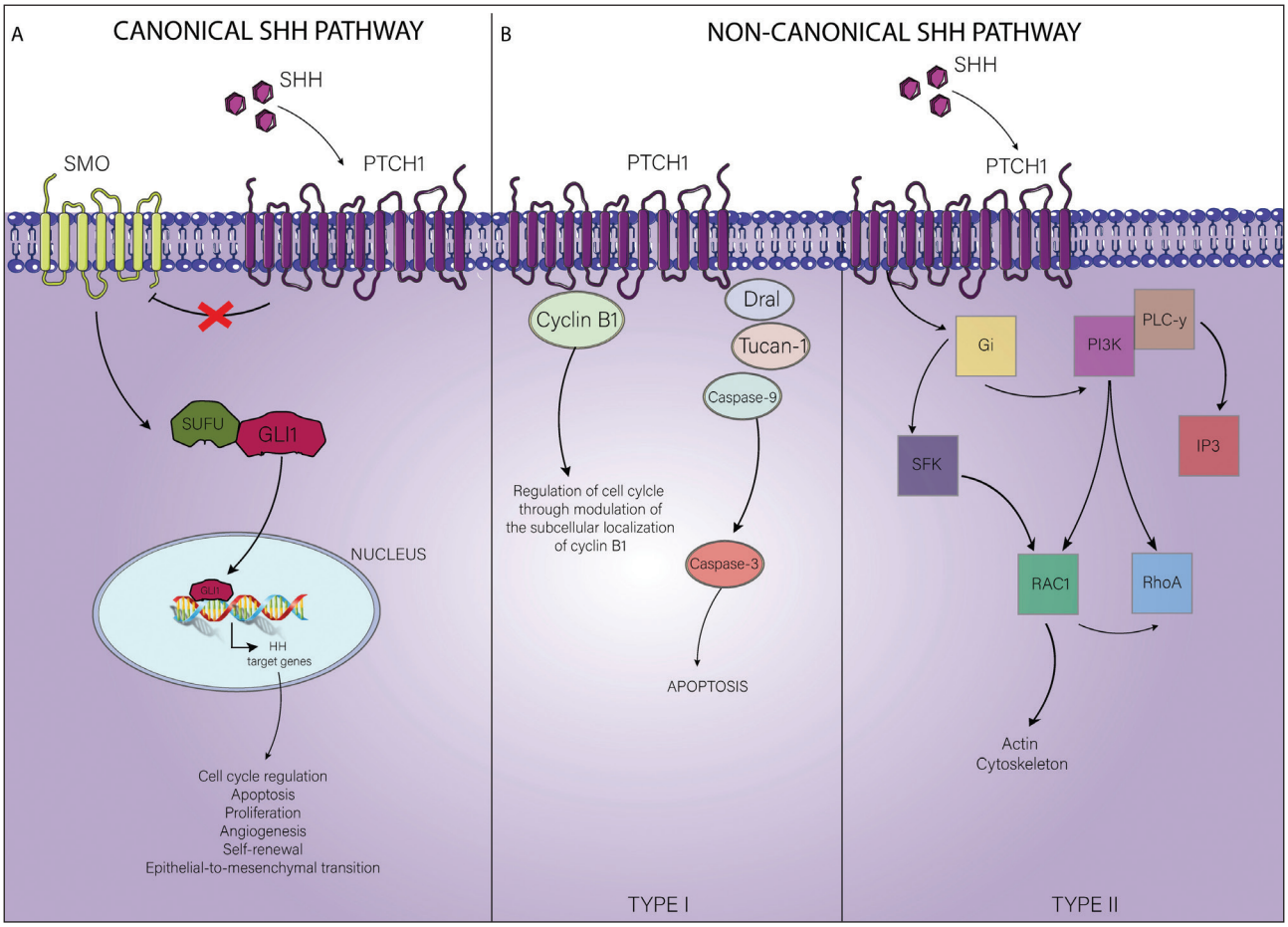
Besides, Gli are also regulated by a family of tumor suppressor proteins, namely Suppressor of Fused (SUFU) (23). When Ptch ligands are missing, Gli proteins are recruited by Sufu, which are in charge of inhibiting their nuclear translocation (24). For this reason, the full-length Gli proteins are converted to a C-terminal shorten repressed form (Gli-R). This structure is phosphorylated by glycogen synthase kinase 3 beta (GSK3β), casein kinase I (CK1), and

protein kinase A (PKA) (25). Gli proteins retained at the cytoplasm by Sufu are then degraded or processed, overall triggering Hh inhibition. However, how these last steps are operated in mammals are elusive still (23).

Non-canonical Hh activation (**figure 1 *B***), on the other hand, has been characterized to be orchestrated by two separate pathways. Type I non-canonical Hh activation relies on Ptch1-activity when Ptch ligands are missing. This noncanonical signaling activity regulates the cell cycle through modulation of the subcellular localization of cyclin B1 (26). Type II non-canonical Hh activation is Smo-dependent Gli-independent. Small GTPases RhoA and Rac1 are the main players in charge for triggering this pathway, in a cellular-context manner (27-29). In carcinogenic processes these mechanisms have been reported to be profoundly affected as a result of Hh signaling misregulation (30). Given the role of Hh pathways in cell development, its aberrant activation might thus contribute to hematological malignancies progression, overall representing a promising strategy to target for developing novel drug-based approaches (31).

HEDGEHOG SIGNALING IN MULTIPLE MYELOMA

MM cells-mesenchymal stromal cells (MSCs) interactions have been described to play an outstanding role in MM pathogenesis, eventually contributing to MM cell survival, proliferation and chemoresistance (32). Shh produced by the stromal cells supports proliferation of hematopoietic stem cells, prompts germinal-center B cells survival and antibody production (33-35). In tumor context, MSC-induced Shh signaling is important in protecting my-



Figures 1. *A.* *Canonical activation of Shh pathway. Canonical pathway is triggered by interaction between Shh and Ptch1. In response to this binding, Ptch1 no longer inhibits Smo, which in turn promotes downstream Gli nuclear translocation and target genes activation;* ***B.*** *Non-canonical Shh pathway. Non-canonical activation can be orchestrated by two separate pathways. Type I Smo-independent activation relies on Ptch interaction with cyclin B1, leading to cell cycle regulation. Type II is Smo-dependent Gli-independent. When Shh binds Ptch1, Smo activates Gi protein and small GTPases RhoA and Rac1, as well as calcium release stimulation from endoplasmic reticulum and PLC-y- catalyzed the opening of IP3-depedent channels by the generation of IP3.*

elodysplastic syndrome (MDS) cells from apoptosis (36) Despite accumulation of a plethora of genetic lesions, myeloma PCs lose their dependency on BM microenvironment only in the latest stages of disease and therefore long-term culture of primary MM cells without stromal support is rarely possible in vitro (37). Among the main MSC-released soluble factors contributing to myeloma cells survival, Shh allow survival and growth of MM cells. Indeed, its proliferative effect is inhibited by cyclopamine, an alkaloid which binds to SMO stabilizing its inactive conformation (37).

CD138+ cells from MM patients exhibit overexpression of Hh signaling components, such as *PTCH*, *GLI1* and *GLI2* through the activation of non-canonical Smo-independent pathway (16). Moreover, a significative down-regulation of Hh repressor gene *GLI3* has been described in malignant plasma cells compared to the healthy counterpart (16). MM is characterized by two distinct populations: CD138-CD19+ stem cells, resembling memory B cells, and malignant CD138+CD19- terminally differentiated plasma cells (38). Peacock et. al (32) demonstrated a marked down-regulation of *PTCH1* in CD138-CD19+ stem cell compartment, together with an increase of *SMO* and *GLI1* expression. On the other hand, CD138+CD19- differentiated plasma cells showed increased *PTCH1* levels. Therefore, CD138-CD19+ stem cell populations are more sensitive to Hh ligand than malignant CD138+CD19-terminally differentiated plasma cells (32).

In addition to the stromally induced Hh signaling, MM cells are able to produce and secrete themselves the Hh ligands. Autocrine Shh signaling enhances tumor proliferation and protects CD138+ cells from spontaneous and stress-induced apoptosis increasing BCL-2 expression levels (39). This evidence correlates with an independent study reporting that an Hh-gene signature is able to cluster MM patients in two subgroups characterized by the opposite Hh pathway expression in mature PCs and their precursors. In particular, patients with Hh hyperactivation in MM cells, but not in their B cells, show higher genomic instability associated to shorter progression-free survival and overall survival (40).

Hh signaling is also associated with the nuclear transcription factor-kB (NF-kB) pathway in several tumors such as liver cancer, breast cancer, prostate cancer, pancreatic cancer, diffuse large B-cell lymphoma (DLBCL) (41-44). NF-kB is a heterodimeric complex consisting of a p50 (NF-kB1) and p65 (RelA) subunits, which form an inactive cytoplasmic ternary complex with the inhibitory protein IKBa. In response to an extracellular stimulus, IKBa may be degraded and NF-kB can translocate into the nucleus to activate the expression of genes involved in the immune and inflammatory responses, such as interleukin 2 receptor alpha chain gene, interleukin 6, granulocyte colony-stimulating factor, interferon-beta (IFN-b) (45). Interestingly, it has been reported that canonical pathway activation of Sonic Hedgehog is responsible for the enhancement of NF-KB activity in MM cells, preventing their apoptosis (46).

Tumor-derived Hh signaling can favor the production of receptor activator of nuclear factor-kB ligand (RANKL) in osteoblasts, stimulating osteoblastogenesis and increasing bone resorption (47). Hh signaling has also been found to stimulate MSCs differentiation on osteoblasts regulating expression of Runt-related transcription factor (RUNX2) and Osterix (OSX) expression (48). Activation of osteoblastogenesis is directly modulated by SMO and GLIs-induced signaling (48). Indeed, inhibition of Shh signaling by using cyclopamine strongly reduces osteoblastogenesis (49). Myeloma PCs acts as GLI1 suppressor on MSCs, thus, reducing the potential of MSCs to differentiate in osteoblasts (50).

TARGETING HH PATHWAY

Given the crucial role played by Hh pathway in MM progression, recent reports focused on developing new therapeutic strategies aiming to its inhibition. One of them targets the Smo receptor using cyclopamine, eventually resulting in the inhibition of Hh signaling (51). Since Hh signaling regulates NF-κB through both its classical pathway (SHh/PTCH1/SMO/GLI1) and non-classical pathway by SMO recruitment of TRAF6 to ubiquitination, the SMO inhibitor cyclopamine in combination with bortezomib enhance the proteasome inhibitor-induced cytotoxic effects (46) These results enforce the hypothesis describing a proteasome-Hh axis which may be targeted in the feature studies. Despite the promising results, cyclopamine showed teratogenic potential, toxicity and poor bioavailability, overall discouraging further application aiming to clinical outcomes (52). However, cyclopamine opened the path towards further drug development aiming to target Hh pathway for MM treatment.

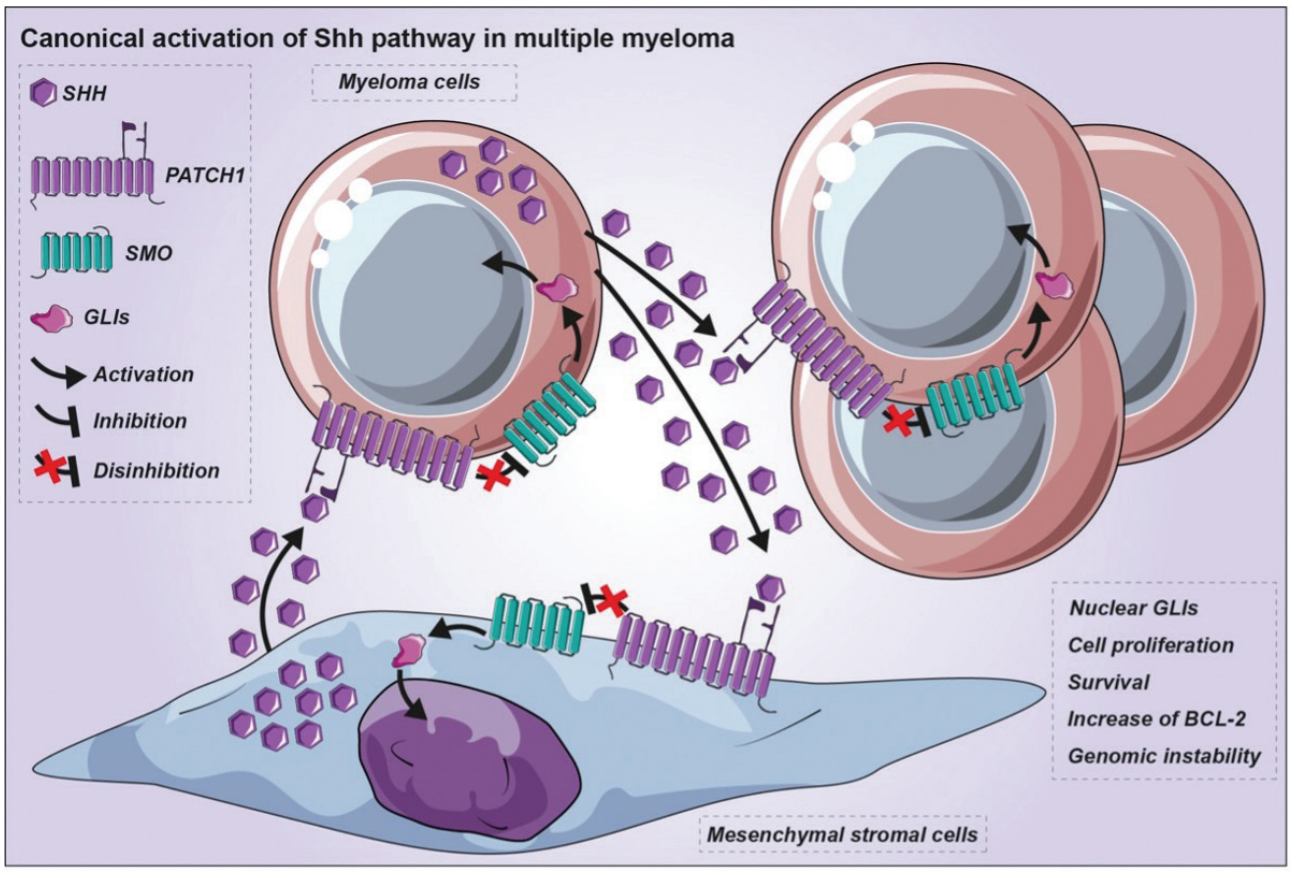


Figure 2. *Crosstalk of mesenchymal stroma cells and myeloma cells: the role of Sonic Hedgehog pathway. Stromal compartment is the most important source of Shh in bone marrow, mediating proliferation of hematopoietic stem cells, prompting germinal-center B cells survival and antibody production.*

Currently, a newer drug namely Vismodegib acting through Hh pathway inhibition has been approved by US Food and Drug Administration's (US FDA) priority review program on January 30th, 2012 for the treatment of advanced basal-cell carcinoma (BCC) (53). Since Vismodegib was found to have an acceptable safety profile and antitumor activity in patients with BCC and medulloblastoma (54, 55), new clinical trials are being planned in other malignances, including MM (**table I**). However, patients undergoing Vismodegib treatment against BCC showed bone toxicities, with premature fusion of the epiphyses reported in pediatric patients (56). Moreover, cramps or dysgeusia over the course of the therapy appeared in several patients, requiring interruption of the standard therapy, eventually shifting to an intermittent Vismodegib schedule (57).

In parallel, Sonidegib (Odomzo™), a SMO receptor antagonist, has being developed by Novartis for the treatment of BCC (58). This drug was reported to hamper cell viability, neurosphere formation, and Gli transcriptional activity, triggering the apoptotic cascade by activation of caspase-3 and cleavage of poly (ADP-ribose) polymerase *in vitro* (59). In a transgenic mouse model of islet cell neoplasms Sonidegib significantly reduced tumour volume by 95% compared with untreated littermates by inhibition of Hh signaling (59). Given the efficacy and tolerability of a topical formulation of Sonidegib in BCC patients, phase I/II investigation are underway on other malignancies including medulloblastoma, small cell lung cancer, breast cancer, myelofibrosis, chronic myeloid leukaemia, and MM (table I) (60-64). As for Vismodegib, clinical trials displayed a set of typical side effects associated with Sonidegib administration. Muscle spasms, alopecia, dysgeusia, nausea, increased Creatin Kinase, fatigue, decreased weight, diarrhea, decreased appetite, myalgia, and vomiting were frequent in patients, eventually undergoing dose interruptions, reductions, or treatment discontinuation (65). For this reason, further studies are needed to evaluate the usage and dosage of both of these Hh inhibitor for clinical approaches.

DRUG	TRIAL REGISTRATION NUMBER	LOCATION	YEARS
ATO (Arsenic Trioxide)	NCT00469209	U.T.M.D Anderson Cancer Center Huston, Texas, US	2006-2008
	NCT00258245	Barbara Ann Karmanos Cancer Institute Detroit, Michigan, US	2005-2008
	NCT00661544	U.T.M.D Anderson Cancer Center Huston, Texas, US	2004-2007
	NCT00201695	Ohio State University Columbus, Ohio, US	2004-2008
	NCT00006021	Mount Sinai Comprehensive Cancer Center at Mount Sinai Medical Center Miami Beach, Florida, US	2000-2007
	NCT00017069	Arizona Clinical Research Center Tucson, Arizona, US	2001-2005
	NCT000193544	CTI BioPharma Seattle, Washington, US	2002-2009
	NCT000193544	City of Hope Duarte, California, US	2005-2009
	NCT00003395	Memorial Sloan Kettering Cancer Center New York, New York, US	1998-2000
Vismodegib (GDC-0449)	NCT02465060	University of Alabama at Birmingham Cancer Center Birmingham, Alabama, US	Recruiting
	NCT03297606	Cross Cancer Institute Edmonton, Alberta, CA, US	Recruiting
	NCT03878524	OHSU Knight Cancer Institute Portland, Oregon, US	Recruiting
Sonidegib (LDE-225)	NCT02254551	Colorado Blood Cancer Institute Denver, Colorado, US	2015
	NCT02086552	Mayo Clinic Rochester, Minnesota, US	2014-2021

Table I. *Clinical trials. The table reports registered clinical trial on https://clinicaltrials.gov focused on Hh inhibitors for MM treatment.*

Because the GLI proteins are the final effectors of Shh pathway, the development of a GLI-targeted approach might be useful to inhibit tumor growth and therapy resistance. Among GLI antagonists, there are GANT58 and GANT61 (GLI-ANTagonist) (66) GANT61 is more specific toward GLI proteins and effectively reduces GLI1 and GLI2 DNA-binding ability. Arsenic Trioxide (ATO) (a Food and Drug Administration (FDA)-approved drug with sub-micromolar potency against GLI1/267 (67) was shown to inhibit GLI1 directly inhibiting its transcriptional activity (68). MM cells treated with ATO also show inhibition of NF-kB, hampered adhesion to MSCs with consequent disruption of tumor growth and survival (69). A first phase II study of ATO in a MM cohort was designed to assess the response to therapy of patients with relapsed or resistant MM, previously treated with autologous stem cell (70). Eligible patients (n = 10) received a 2-hour daily infusion of ATO 0.15 mg/kg for 60 days. The treatment was supplemented for 30 days more in patients showing a response, defined as a reduction in myeloma paraprotein at days 30 and 60. Three out of ten patients who completed more than 30 days of ATO infusion were characterized by >50% reduction in serum paraprotein levels (n = 2), a more stable disease (n = 1). Furthermore, one out of ten progressed. Surprisingly, five patients belonging to the initial cohort, displayed stable disease (n = 2) and progressed (n = 3), already upon < 30 days treatment. Table 1 lists completed clinical trials, providing a strong basis for the use of ATO in MM patients. Interestingly, ATO found an important clinical path in counteracting relapsed or refractory acute promyelocytic leukemia. However, ATO usage has been discouraged as a consequence of its side effects on healthy tissues, eventually resulting in cardiotoxicity (71). Notably, QT prolongation, torsades de pointes and sudden cardiac death have been reported upon ATO administration. The main reason behind ATO-related cardiac toxicity is related to the large amount of ROS produced following ATO treatment, which in cardiac cells, as a consequence of the low amount of antioxidants, it is enhanced (71, 72).

Interestingly, two parallel phase II trials aim to assess the safety and efficacy of Sonidegib in combination with bortezomib and lenalidomide, in patient with relapsed/refractory MM (NCT02254551) or as maintenance therapy following autologous stem cell transplantation of refractory multiple myeloma (NCT02086552), respectively.

Further approaches to enhance Hh inhibitors efficiency include synergic strategies with molecules targeting the Hh signaling cascade at multiple levels. With this regard such ATO has been recently tested together with Itraconazole, Vismodegib or Sonidegib (73).

For this reason, it should not be surprising if multiple combinations of Hh-targeting agents will be disclosed soon. For this purpose, we listed the literature currently available investigating the crosstalk between Hh and multiple myeloma in **table II**.

CONCLUSION AND FUTURE PERSPECTIVES

MM is a hematological disease characterized by an aberrant activation of several molecular mechanisms, eventually reshaping the bone microenvironment, and resulting in MM progression. In this landscape, researchers are aiming to identify novel therapeutic targets to improve patients' prognosis. With this regards, Hh activation has been reported to cover an underestimated role in bone marrow development, thus prompting different groups to target this cascade in different hematological diseases (74). In the MM context, Hh aberrant signaling, in turn mediated by Shh release, affects bone marrow microenvironment transformation, supporting the proliferation of malignant plasma cells by enhancing NF-kB signaling, also resulting in chemotherapy resistance (75). In this context, targeting the Hh pathway may represent a valuable strategy. To date, the main strategies are represented by three drugs (ATO, Vismodegib, and Sonidegib) which are currently being tested in different clinical trials (**table I**). However, the usage of drugs targeting Hh cascade may not be useful enough to hamper MM progression. For this reason, a further effort could be done to design more powerful Hh modulators. In alternative, the ones currently available may be tested together with molecules targeting Hh cascade on different levels to enhance Hh modulators' effect. Ultimately, an outstanding strategy may also be represented by supplementation of currently clinical-available drugs in combination with Hh modulators, aiming to obtain a more beneficial treatment. In this regard administration of Ixazomib reshapes the MM microenvironment also stimulating Hh cascade. However, further studies are needed to fully understand the regulatory mechanisms underlying Hh signaling pathway and how PIs affect them, towards the development of new treatment to efficiently hamper MM progression.

REFERENCE	TITLE	JOURNAL
3	A novel Bruton's Tyrosine Kinase inhibitor CC-292 in combination with the proteasome inhibitor carfizomib impacts the bone microenvironment in a multiple myeloma model with the resultant antimyeloma activity.	Leukemia, **2014**
16	Canonical and noncanonical Hedgehog pathway in the pathogenesis of multiple myeloma.	Blood, **2012**
31	Aberrant activation of the Hedgehog signaling pathway in malignant hematological neoplasm.	The American Journal of Pathology, **2012**
32	Hedgehog signaling maintains a tumor stem cell compartment in multiple myeloma.	Proceedings of the National Academy of Sciences, **2007**
34	Sonic Hedgehog is produced by follicular dendritic and protects germinal center B cells from apoptosis.	Journal of Immunology, **2005**
37	Essential role of stromally induced Hedgehog signaling in human CD138+ myeloma cell survival and drug resistance.	Nature Medicine, **2007**
39	A critical role of stromally induced Hedgehog signaling in B-cell malignancies.	Blood, **2014**
40	Opposite activation of the Hedgehog pathway in CD138+ plasma cells and CD138- 19+ B cells identifies two subgroups of patients with multiple myeloma and different prognosis.	Leukemia, **2016**
46	Targeting the cross-talk between the Hedgehog and NF-kappaB signaling pathways in multiple myeloma.	Leukemia & Lymphoma, **2019**
47	The role of Hedgehog signaling in tumor induced bone disease.	Cancers (Basel), **2015**
50	Ixazomib improves bone remodeling and counteracts Sonic Hedgehog signaling inhibition mediated by myeloma cells.	Cancers (Basel), **2020**
70	Ascorbic acid enhances arsenic trioxide-induced cytotoxicity in multiple myeloma.	Blood, **2001**
75	Effect of Hedgehog pathway abnormality on chemotherapeutic resistance of multiple myeloma.	Zhongguo Shi Yan Xue Ye Xue Za Zhi, **2017**

Table II. *Currently available literature investigating Hh-MM crosstalk. The table reports the available works investigating the role played by Hh in MM progression as they are cited along the main body of the text.*

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ETHICS

Conflicts of interests

The authors have declared no conflict of interests.

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Authors' contribution

Conceptualization: ID, DT, CG, AR, SG, NV, FDR, GAP; validation: AR, GS, AB, LL, ID; writing-original draft preparation: ID, DT, CG, SG, GAP, ELS, NV, TZ, FDR, GLV, RP, RP; supervision: DT, GAP, DLF, FDR, NV, DC, RP, GLV, RA, ID, GLV. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

No new data were generated or analysed in this research.

Ethical approval

N/A.

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BRIEF REPORT

BALANCE BETWEEN THE STEM CELL MARKER CD44 AND CDX2 EXPRESSION IN COLORECTAL CANCER

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ABSTRACT

CDX2 (Caudal-type homeobox transcription factor 2) is a biomarker of differentiated colon enterocytes, whose expression has been associated with a favorable prognosis in colon cancer. The absence of CDX2 has been associated with an aggressive outcome, including an higher risk of relapse. CD44 (Cluster of Differentiation 44) is a transmembrane glycoprotein involved in cell growth, survival, differentiation and migration. It is considered a typical marker of cancer stem cells, with a role in colorectal cancer progression. The aim of this study was to analyze the expression of the stem cell marker CD44 and its relation to CDX2 expression in colorectal cancer.

To this end, 65 consecutive colorectal cancers were immunostained with anti-human CD44 Rabbit monoclonal antibody (clone SP37) and anti-human CDX2 Rabbit monoclonal antibody (clone EPR2764Y). 59 cases were positive for CDX2 and 47 were positive for CD44. Regarding cases positive for CDX2, 49 were positive for CD44. Our findings show the existence of a wide spectrum, ranging from cases CDX2-/CD44- to tumors expressing both markers. Multiple further combinations of the two markers were also found. CD44 immunoreactive tumors showed an high stage at diagnosis, suggesting a possible association of CD44 expression with an aggressive outcome of colorectal cancer.

KEY WORDS

Colorectal cancer; CD44; CDX2; prognostic biomarker; cancer stem cells.

IMPACT STATEMENT

The expression of CD44 in CDX-2 negative tumors could indicate a possible target-therapy targeted to CD44.

INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer and the second in terms of worldwide mortality (1).

The regional incidence of CRC varies worldwide. The variability seems related to differences in environmental exposures and eating habits acting on a background of genetic susceptibility. The areas with a higher incidence rate are Europe, New Zealand, Australia and North America. The areas with a lower rate are Africa and South-Central Asia (2).

The conventional adenoma-carcinoma pathway is responsible for most colorectal cancers, while 10-20% of CRCs result from serrated lesions (3).

There are many risk factors, genetic and epigenetic, involved in the development of colon cancer. The most potentially preventable risk factors are smoking, high alcohol consumption, unhealthy diet, excess body weight and physical inactivity (4, 5).

The stratification of patients affected by colorectal cancer is a key factor for the identification of patients who require adjuvant chemotherapy after tumor resection. In the absence of simple reliable criteria for the stratification of CRC patients at higher risk of relapse, decision making for adjuvant chemotherapy often represents a dilemma for oncologists (6). To address this problem, many studies explored the possibility of stratifying CRC patients according with the tumor gene expression profiling (7), metastasis-associated gene expression changes (8), the molecular profile of tumor cells (9), the cancer stem cell signature (10, 11) and the correlation with epithelial-mesenchymal transition-related gene expression (5). Given the difficulty of utilizing gene-expression signatures in clinical practice (7), in recent times researchers focused on the immunohistochemical expression of multiple markers with the aim of identifying a signature that could be used to identify the more aggressive forms of CRC. Researchers focused on the identification of immunohistochemical markers possibly associated with an aggressive behaviour of CRC. A project from our group in this field, aimed to identify markers associated with CRC aggressivity, identified Thymosin beta-4 (TB4) (12) at the invasion front of a subset of CRCs (13, 14). In these studies, TB4 was highly expressed in tumor cells undergoing epithelial-to-mesenchymal transition, suggesting a role for this peptide in invasion and metastasis.

Dalerba and coworkers focused on the caudal-type homeobox transcription factor 2 (CDX2), as a biomarker of well differentiated colon enterocyte. The analysis of 466 CRC patients showed that CDX2 expression is associated with an higher disease-free survival as compared with the CDX2-negative patients. Conclusively, this study evidenced that lack of CDX2 expression identifies a group of patients at high risk of relapse, who may benefit from adjuvant chemotherapy, irrespectively of the tumor stage (15). Furthermore, patients with colon cancer without CDX2 expression were more likely to have aggressive features: high grade tumor, mucinous tumors, lymph node involvement and advanced overall pathological staging (16).

Considering that many studies produced evidence on the presence in CRC of self-renewing stem progenitor tumor cells, the so-called cancer stem cells (CSCs), we initiated a search for a biomarker that might better characterize CDX2-negative undifferentiated tumors, focusing on cancer stem cell markers previously described in human colon, including CD44, CD133, CD90, SOX2, SOX9, ALDH1A1 and EpCAm (17).

Our aim was to find, by means of immunohistochemistry, a simple marker of immature colon cancer cell which, joined with CDX2, might be used in clinical practice for identifying the less differentiated and possibly more aggressive forms of CRC.

MATERIALS AND METHODS

We examined 65 cases of colorectal adenocarcinoma diagnosed between 2008 and 2021, ranging in age from 49 up to 85 years, 37 males and 28 females**.** Ethics Committee approval was obtained

for the study (Protocol number 2020/10912 – code: EMIBIOCCOR) and written informed consent was obtained from all participants for their tissues to be utilized for this work.

Tissue samples were routinely processed for histological observation and stained with hematoxylin-eosin (H.E). For immunohistochemical analysis, 3 µm thick sections were obtained from the paraffin block. All reagents were purchased from *Ventana Medical Systems Inc. 1910 E. Innovation Park Drive Tucson, Arizona 85755 USA.* The sections were automatically dewaxed and rehydrated with EZ Prep 1X (Ref. 950-102) and pre-treated with heat-induced epitope retrieval in Ultra CC1 (Ref. 950-224), following Dealer's instructions. Slides were then incubated at room temperature with anti-human CD44 Rabbit monoclonal antibody – clone SP37 – (Ref. 790-4537) and with anti-human CDX2 Rabbit monoclonal antibody – clone EPR2764Y – (Ref. 760-4380). All immunostaining procedures were performed using the UltraView Universal DAB Detection Kit (Ref. 760-5000) on the BenchMark Ultra (*Ventana Medical Systems Inc. 1910 E. Innovation Park Drive Tucson, Arizona 85755 USA*) instrument, according to the manufacturer's instructions. For CD44 interpretation, we used the following grading score system, based on HER2/neu scheme (**table I** and **figure 1**). For CDX2 evaluation we utilized the scoring system shown in **table II**.

Statistical analysis was performed with the MedCalc Statistical Software Version 14.10.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014). The association between categorical variables was estimated by the Fisher exact test for categorical binomial variables or by the chi-square test in all other instances.

RESULTS

The clinicopathological features of patients here analyzed are reported in **table III**. In our study cohort, the median age was 66 years (range 49-85), 37 patients (57%) were men and 28 (43%) were women. 17 (26.1%) tumors were located in the right colon, 3 in the transverse colon (4.6%), 21 (32.4%) were found in descending colon, 5 (7.7%) in sigma, 2 in sigma-rectum (3.1%), 15 (23.1%) were in the rectum, 1 case affected rectum and right colon (1.5%) and 1 case cecum and transverse colon (1.5%).

CD44 negative or weak membrane staining in less than 10% of tumor cells (score 0) was observed in 18 (27.7%) patients, 15 (23.1%) showed weak membrane staining in at least 10% of tumor cells or moderate in less than 10% of tumor cells (score 1+), 18 (27.7%) moderate membrane staining in at least 10% of tumor cells or intense in less than 10% of tumor cells (score 2+) and 14 (21.5%) intense membrane staining in at least 10% of tumor cells (score 3+) (**table IV**). In this series, CD44 expression was more frequent in cancers of the sigma and rectum (80-100%) *versus* 70% in the colon.

CD44 EXPRESSION	SCORE
Negative or weak membrane staining in less than 10% of tumor cells	0
Weak membrane staining in at least 10% of tumor cells or moderate membrane staining in less than 10% of tumor cells	1+
Moderate membrane staining in at least 10% of tumor cells or intense membrane staining in less than 10% of tumor cells	2+
Intense membrane staining in at least 10% of tumor cells	3+

Table I. *CD44 scoring system*

CDX2 EXPRESSION	SCORE
Negative or nuclear staining less than 5% of tumor cells	0
Nuclear staining in 6%-33% of tumor cells	1+
Nuclear staining in 34%-66% of tumor cells	2+
Nuclear staining in more than 66% of tumor cells	3+

Table II. *CDX2 scoring system.*

		CDX2 +	CD44 +
Age	49-85 years (avg 66 y)	67 years	65 years
Sex			
Male	37 (57%)	33 (89,2%)	27 (73%)
Female	28 (43%)	26 (92.9%)	20 (71.4%)
Site			
Right colon	17 (26,1%)	15 (88.2%)	12 (70.6%)
Transverse colon	3 (4.6%)	3 (100%)	1 (33.3%)
Descending colon	21 (32.4%)	20 (95.2%)	15 (71.4%)
Sigma	5 (7.7%)	5 (100%)	5 (100%)
Sigma-rectum	2 (3.1%)	2 (100%)	1 (50%)
Rectum	15 (23.1%)	12 (80%)	12 (80%)
Rectum and right colon	1 (1.5%)	1 (100%)	1 (100%)
Cecum and transverse colon	1 (1.5%)	1 (100%)	1 (100%)

Table III. *The clinicopathological features of 65 patients with CRC.*

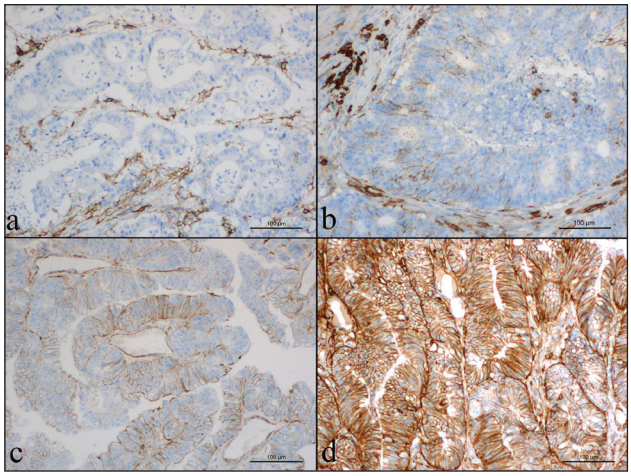

Figure 1. *CD44 scoring system:* ***a****. Score 0: negative or weak membrane staining in less than 10% of tumor cells;* ***b****. Score 1+: weak membrane staining in at least 10% of tumor cells or moderate membrane staining in less than 10% of tumor cells;* ***c****. Score 2+: moderate membrane staining in at least 10% of tumor cells or intense membrane staining in less than 10% of tumor cells;* ***d****. Score 3+: intense membrane staining in at least 10% of tumor cells.*

		CDX 2			
		0	**1+**	**2+**	**3+**
CD44	**0**	2	1	2	13
	1+	1	0	2	12
	2+	0	2	0	16
	3+	3	0	0	11

Table IV. *Immunoreactivity for CD44 and CDX2.*

CDX2 total loss of expression (score 0) was observed in 6 (9.2%) patients, 3 (4.6%) showed nuclear staining in 6%-33% of tumor cells (score 1+), 4 (6.2%) stained 34%-66% of tumor cells (score 2+) and 52 (80%) nuclear staining in more than 66% of tumor cells (score 3+) (**table IV**). In this series, CDX2 loss of expression was more common in males (M:F ratio = 2:1).

Following our scoring systems for CDX2 and CD44, we reached 16 groups of patients: 2 CD44 0/CDX2 0; 1 CD44 0/CDX2 1+; 2 CD44 0/CDX2 2+; 13 CD44 0/CDX2 3+; 1 CD44 1+/CDX2 0; 0 CD44 1+/CDX2 1+; 2 CD44 1+/CDX2 2+; 12 CD44 1+/CDX2 3+; 0 CD44 2+/CDX2 0; 2 CD44 2+/CDX2 1+; 0 CD44 2+/CDX2 2+; 16 CD44 2+/CDX2 3+; 3 CD44 3+/CDX2 0; 0 CD44 3+/CDX2 1+; 0 CD44 3+/CDX2 2+; 11 CD44 3+/CDX2 3+.

The data regarding immunoreactivity for CD44 and CDX2 are summarized in **table IV**.

In short, according with the different degree of reactivity for CDX2 and CD44, the cases of colon cancer analyzed were differentiated into 16 groups. At the extremes of the spectrum we found 4 cases CDX2 negative and CD44 positive and 16 cases CDX2 positive and CD44 negative. All the other cases showed a more complex co-expression of the two markers (**figure 2**).

CD44 and CDX2 expression did not show a significant correlation with any of the mutational analysis carried out. There was no correlation between CD44 expression and BRAF mutations. BRAF mutations were found in 14.3% of CD44-negative patients *versus* 12.2% of CD44-positive patients (p =

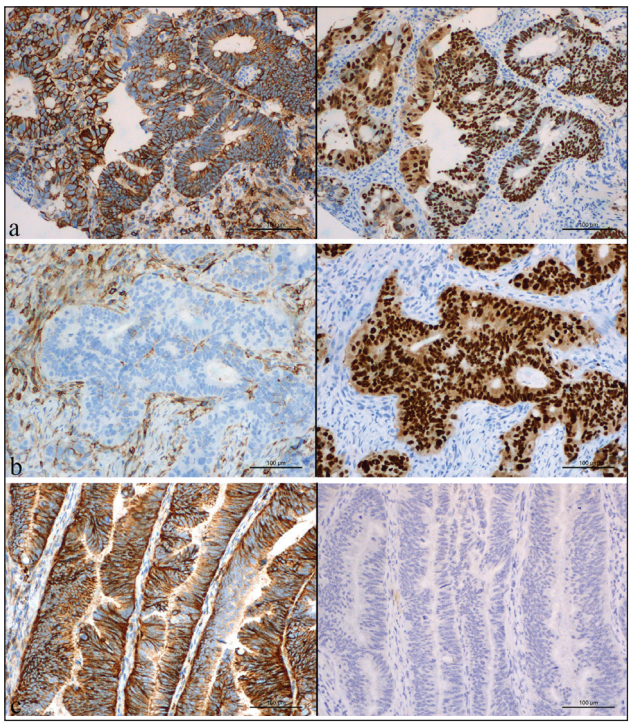

Figure 2. *At the extremes of the spectrum:* ***a****. Case 1: CD44 3+ (on the left) and CDX2 3+ (on the right);* ***b****. Case 2: CD44 0 (on the left) and CDX2 3+ (on the right);* ***c****. Case 3: CD44 3+ (on the left) and CDX2 0 (on the right).*

0.84). Specifically, relating to the CD44 score: BRAF was mutated in 14.3 % of CD44 negative, in no CD44 1+, in 12.5% of CD44 2 + *versus* 25% of CD44 3+ patients (p = 0.3).

There was a non-statistically significant higher occurrence of mutated BRAF in CDX2- negative patients than in CDX2 positive patients (33.3% *versus* 12%, p = 0.3). Relating to the CDX2 score: BRAF was mutated in 33.3% of CDX2 negative patients, 50% of CDX2 1+, in no CDX2 2+ and 11.4 % of CDX2 3+ (p = 0.2).

There was no correlation between CDX2 expression and RAS mutations. RAS mutated was found in 66.7% of CDX2-negative *versus* 56.9% of CDX2-positive patients (p = 0.74). Specifically, relating to the CDX2 score: RAS was mutated in 66.7% of CDX2 negative, in 50% of CDX2 1 +, in no CDX2 2+, and in 56.8% of CDX2 3+ (p = 0.7).

There was no correlation between CD44 expression and RAS mutation. RAS was mutated in 53.3% of CD44-negative patients and in 61% of CD44-positive patients (p = 0.6). Specifically, relating to CD44 score: RAS was mutated in 53.3% of CD44-negative, in 69.2% of CD44 1+, in 56.2% of CD44 2+, and in 58.3% of CD4 3+ patients (p = 0.9).

Furthermore, there was a non-statistically significant higher percentage of patients with a high degree of differentiation in CD44 positive patients. 90% of patients with CD44 positive had a high degree of differentiation (G2-G3) compared to 77.8% of patients with CD44 negative (p = 0.3). Mainly all CD44 3 + had a grading of 2-3.

DISCUSSION

Colon cancer is a major problem for the oncologists also because it affects middle aged as well as younger patients. Therefore it is important to study and search for new markers that can allow to stratify patients, to understand which characteristics give the tumor greater aggressivity or less response to therapy. Starting from the article on the New England Journal of Medicine (15) and from our observation of a patient with colon cancer with complete loss of CDX2 expression, we began to study CD44 in a cohort of CRC patients.

CD44 is a multifunctional transmembrane glycoprotein encoded by a single gene on chromosome locus 11p13 expressed ubiquitously throughout the body (18). It is involved in cellular processes such as survival, adhesion, cell division and migration (17). There are multiple CD44 isoforms based on presence of alternative exons at specific site in the extracellular domain (3). Several studies have shown that the expression of different CD44 isoforms seem to play a key role in tumor progression (19). Moreover, CD44 has been shown to transform a non-metastatic cell line into a more metastatic line (19). The different effects of CD44 on cellular processes depend on its binding to different ligands, such as hyaluronic acid (HA), collagens, osteopontin (OPN) and matrix metalloproteinases (MMPs) (17). CD44 has been studied in several organs: it is considered a cancer stem cell marker in colon cancer but CD44 is expressed in other organs, such as breast, lung, prostate and bladder (3). There is evidence showing that high expression of the CD44 variant 2 in CRC patients is associated with a poorer prognosis than other CD44 variants (20).

In our study, at first, we did not find a simple relationship between CDX2 negativity and CD44 positivity. In fact we found that CDX2 and CD44 may be combined in many ways, ranging from the expression of both CDX2 and CD44 up to the absence of both markers. We divided the patients into 16 group. The most represented group is

CD44 2+/CDX2 3+. We found no correlation between expression of CDX2/CD44 and the site of lesions, age or sex.
Further studies are required to clarify the binding between CD44 expression and clinicopathological features of colon cancer.

CONCLUSIONS

Given the complexity, more than expected, regarding the relationship between CDX2 and CD44 expression in CRC, on the basis of our preliminary results, CD44 cannot be simply identified as a marker of undifferentiated CRC. Our initial hypothesis that CD44 expression might be restricted to CDX2-negative tumors has not been confirmed in this study. Relationships between CD44 and CDX2 expression in CRC tumor cells are much more complex than hypothesized. The spectrum is broad, ranging from a modest amount of cases CDX2+ and CD44- (16 out of 65.25%) up to few cases characterized by CD44 reactivity and absence of CDX2 expression (4 out of 65.6%). In the middle, we found the majority of cases analyzed including 39 patients with CDX2 3+ and positivity for CD44, with no significant difference between CD44 1+ (12 cases), CD44 2+ (16 cases) and CD44 3+ (11 cases). The meaning and the clinical significance of the expression of CD44 in CRC has to be clarified, especially with regard to the co-expression with CDX-2.
Our work should be considered as a contribution to assessing the role of CD44 with regard to the ability to metastasis, local infiltration and response to chemotherapy. Consequently, the expression of CD44 in CDX-2 negative tumors could indicate a possible target-therapy targeted to CD44.

ETHICS

Fundings

There were no institutional or private fundings for this article.

Conflict of interests

Prof. Mario Scartozzi has had a role as consultant, advisory board and speakers' bureau for the following companies: Amgen, Sanofi, MSD, CISAI, Merck, Bayer. The remaining authors have declared no conflict of interests.

Availability of data and materials

All the data supporting the findings of this study are available within the article and can be shared upon request to the corresponding author.

Authors' contribution

All authors contributed to manuscript writing and approving the final version.

Ethical approval

Ethics Committee approval was obtained for the study (Protocol number 2020/10912 – code: EMIBIOCCOR).

Consent to participate

Written informed consent was obtained from all participants.

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