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EDITORIAL

THE COMPLEX REALITY OF MALNUTRITION MANAGEMENT IN ONCOLOGY

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Impact statement: Nutritional support of cancer patients is still suboptimal. This Editorial describes the critical issues and challenges in malnutrition management in oncology.

Key words: *malnutrition; clinical nutrition; anti-cancer diet; multidisciplinary; nutritional screening*

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Clinicians often consider nutritional care as a non-essential step in patients' evaluation and treatment. This also happens in Oncology, even though it is well known that an altered nutritional status has a disastrous impact on patients' response to treatment, quality of life, and survival. Overall, it has been estimated that up to 57% of patients with stage IV cancer are malnourished or at risk of malnutrition at the time of diagnosis (1).

Many factors contribute to the development of malnutrition in cancer patients, with several differences according to cancer type and setting (2). In all patients, especially in advanced stages, the deregulation of systemic inflammation pathways leads to metabolic derangements including increase in muscle catabolism and switch to acute-phase protein synthesis. In patients with gastrointestinal cancer, mechanical (*e.g.*, bowel obstruction) or functional factors (*e.g.*, exocrine insufficiency) contribute to the impairment of nutritional status. Moreover, anticancer treatments often cause anorexia, mucositis, nausea, diarrhea, or other nutrition-related side effects. Finally, psychological effects can promote the reduction of food intake and physical activity.

A large number of clinical studies have demonstrated the deleterious effects of poor nutritional status (3). In particular, in the hospital setting malnutrition is associated with prolonged length of stay, increased post-surgical complication rates, higher susceptibility to infections, increased mortality, and higher hospital costs (4).

Nutritional support has been demonstrated to improve all the aforementioned outcomes, including survival (5, 6). It can be delivered through different modalities, depending on patients' conditions and wills. The first step in nutritional support is usually dietary counseling, which consists in individualized nutritional advice aimed at modifying eating habits to meet patient's energy and nutrient requirements. If food intake is scarce, oral nutritional supplements can be prescribed. If global oral intake is insufficient, enteral and parenteral nutrition can be used to provide a sufficient amount of calories, macronutrients and micronutrients. In particular, enteral nutrition requires a functioning gastrointestinal tract, while parenteral nutrition should be used if gastrointestinal tract is not accessible or in case of insufficient oral and enteral intake (7).

In the everyday sense of the word, malnutrition is defined as a detrimental condition resulting from deficiencies in nutrient intake or malabsorption. However, nutritional support may be needed also in patients with normal intakes and absorption, both for malnutrition prevention and for the management of associated conditions, including sarcopenia. Sarcopenia is a progressive and generalized skeletal muscle

disorder that is associated with increased likelihood of adverse outcomes including falls, fractures, physical disability, and mortality (8). Nutritional support is crucial in sarcopenia management and it should be delivered in the setting of a multidisciplinary approach that includes physical therapy. Sarcopenia is probably less recognized and treated than malnutrition, with deleterious effect that contribute worsening clinical outcomes in cancer patients (9, 10).

Despite the importance of nutrition in this setting, the proportion of cancer patients undergoing nutritional evaluation is still scarce. In particular, a nutritional consultation rate ranging from 8.4% in stage IV to 3.8% in limited-stage cancer patients was described in a recent survey (11). This probably depends on multiple factors which should be addressed at the institutional level in order to improve patients' nutritional management.

One of the most critical issues in nutritional care is the poor attention paid to early detection of malnutrition signs. The importance of weight loss is often underestimated; for example, if patients have undergone surgical procedures or exams causing temporary reduction of food intake, weight loss may be misinterpreted as a temporary "para-physiological" condition related to fasting, rather than a possible alarm sign of the deterioration of nutritional status. Moreover, due to short duration of outpatient oncological visits, an adequate nutritional evaluation is often not performed. Also, in hospitalized patients the shortage of medical and nursing personnel makes it difficult to include nutritional evaluation in the routine clinical practice, despite the usage of nutritional screening being recommended by international guidelines (7). However, screening with validated tools like the Malnutrition Universal Screening Tool (MUST) or the Nutritional Risk Screening 2002 (NRS 2002) only requires a few minutes of focused questions on weight loss, information about patient's disease and age, and detection of body weight and height (12). In particular, the NRS-2002 tool allows the rapid identification of patients at risk of malnutrition through a scoring system based on: nutritional impairment (0 points if none, 1 point if weight loss >5% in 3 months or food intake 50-75% of normal in the past week, 2 points if weight loss >5% in 2 months or food intake 25-50% of normal in the past week, 3 points if weight loss >5% in 1 month or BMI <18.5 plus impaired general conditions or food intake <25% of normal in the last week), disease severity (0 points if patient's disease is associated with normal nutritional requirements, 1 point in case of hip fracture or chronic disease, including cancer, 2 points in case of major abdominal surgery, severe pneumonia, stroke, or hematologic malignancy; 3 points in case of head injury, bone marrow transplant, ICU patients), and age (1 points if equal or greater than 70 years); patients with total score of 0-2 are at low risk of malnutrition, while patients with score equal or greater than 3 are at medium or high risk. Institutions should evaluate the need of requiring mandatory nutritional screening for all patients or, at least, for high-risk patients (*e.g.*, oncological, and gastroenterological units' patients) in order to guarantee the timely recognition of impaired nutritional status.

Ideally, all patients should be evaluated also for sarcopenia risk through the available screening tools, such as SARC-F questionnaire (13). SARC-F questionnaire could be completed by patients themselves, since it is composed by five simple questions assessing the number of falls in the last year (0 points if none, 1 point if 1-3 falls, 2 points if more than 2 falls) and difficulties in lifting and carrying a weight of 4-5 kilograms, walking across a room, transferring from a chair or bed, and climbing 10 stairs (0 points each if no difficulty, 1 point if some difficulty, 2 points if many difficulties); patients with a total score of 4 points or more are at risk of sarcopenia.

Patients at risk of malnutrition and/or sarcopenia should then undergo a complete nutritional evaluation in order to start the adequate nutritional support. The diagnosis of malnutrition should be confirmed by diagnostic criteria such as the Global Leader Initiative for Malnutrition (GLIM) criteria (14). Sarcopenia should be diagnosed by assessing the presence of low muscle mass, low muscle strength, and poor physical performance (8). In particular, muscle mass can be evaluated by simple and reproducible techniques such as bioimpedance analysis, which allows the estimation not only of appendicular skeletal mass, but also of important prognostic indexes such as phase angle (8); CT scan images can be processed by a dedicated software to calculate the skeletal mass quantity and density at L3 level; ultrasound, dual-energy x-ray absorptiometry and clinical approaches (*i.e.*, anthropometry and physical examination) could also be used. Muscle strength can be easily measured with a handgrip dynamometer or with the chair-stand test, while physical performance can be evaluated with tests such as the Short Physical Performance Battery test or the gait speed test. Unfortunately, even if malnutrition risk is identified,

it is often difficult to guarantee a specialized clinical nutritional evaluation to all patients. This is due to the lack of nutritional services in many hospitals and to the low number of clinical nutrition specialists in general. Moreover, if malnutrition is identified and nutritional support is needed, the prescription of nutritional supplements is not always available to patients, depending on the different countries' reimbursement policies. Even in the same country, the availability of nutritional support products may be heterogeneous.

The obvious consequence of the inadequate nutritional management of cancer patients is the progressive deterioration of nutritional status, leading to worse oncological outcomes, as mentioned above. The less evident but problematic consequence may be related to the patients searching for alternative anti-cancer diets, which often contribute to worsen nutritional status rather than ameliorating clinical outcomes (13, 14). In fact, in the last years nutritional advice regarding anti-cancer diets has often been delivered by social media and self-proclaimed experts, with increased risks due to a progressive detachment from the scientific bases of nutritional support. Unfortunately, in many Universities clinical nutrition courses are optional or simply inexistent, leading to scarce availability of clinical nutrition expert personnel. Inevitably, nutritional care needs to be part of the graduate and postgraduate formation programs for doctors of whichever specialty, in order to allow an adequate nutritional management by clinicians. In particular, clinical nutrition should be part of the Oncology residents' rotation plans and courses, aiming at facilitating the future multidisciplinary collaboration between clinical nutrition specialists and oncologists through a common background.

In this scenario, multidisciplinary working groups including oncologists and clinical nutritionists have been constituted both at the national/international level and in the hospital setting, in order ameliorate patients' management (15). Patients associations have greatly contributed in increasing the awareness on the importance of nutritional support in cancer care, leading to a productive collaboration with nutritional and oncological societies. On the opposite, clinicians have started involving their patients in the health care process through the so-called "patient empowerment". Empowerment has been defined as the intention to promote patients' abilities of taking greater control of their own health (16). In nutritional oncology, it may be applied for example in the self-monitoring of weight or food intake changes or in the self-administration of questionnaires or nutritional screening tools (17). Moreover, patients' associations have great merit in the creation of cooperative groups of patients, where peer to peer advice is given on the everyday cancer-related issues.

In conclusion, the awareness on the complex reality of nutritional care is slowly raising, but more focused interventions are needed in order to improve cancer patients' nutritional management. Malnutrition is still under-recognized due to reduced utilization of nutritional screening tools, defective early identification of malnutrition, poor availability of nutritional services, and inadequate university training. These conditions may lead to worsening of patients' clinical outcomes and may favor the choice of non-scientific nutritional approaches. In this setting, the collaboration among clinicians and patients is essential, and could benefit from increasing patients' empowerment. Also, the multidisciplinary teamwork is crucial to ensure adequate nutritional care to patients. For example, the inclusion of clinical nutrition specialists in oncological hospital boards could improve the timeliness of nutritional interventions and could help optimizing patients' fitness before medical and surgical treatments.

The attention paid to nutritional care is increasing, thanks to the efforts of national and international scientific societies, with the help of patients' associations. In the near future, nutritional screening will hopefully become mandatory for all cancer patients and nutritional support will be guaranteed as one of individuals' fundamental rights (18), thus being available to all patients, regardless of their conditions or disease stage.

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Authors' contributions

The Authors confirm contribution to the paper as follows: conception and supervision: RC, PP; manuscript preparation and design: VD; draft review and input to manuscript preparation: AC, EC, GP, LP, FS.

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RESEARCH ARTICLE

MOLECULAR SUBTYPES AND DIETARY PATTERNS IN BREAST CANCER PATIENTS: A LATENT CLASS ANALYSIS

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ABSTRACT: Breast cancer (BC) is the second most common cancer worldwide, with over 2,300,000 new cases estimated per year. Diet has been identified as a modifiable risk factor for BC development and prognosis. The Mediterranean diet (MD) has shown to be inversely associated with chronic diseases including BC. The aim of the present study was to assess dietary patterns according to BC molecular subtypes in a subgroup of patients at their baseline visit of a lifestyle trial conducted by our institute. A principal component analysis (PCA) was conducted to assess the best dimensional space where to summarize dietary information. An explorative unsupervised automatic clustering technique was performed to identify diet-risks groups. Final groups were analyzed as dietary patterns and comparisons made by synthetic statistics with univariable analysis. The first PCA factor was characterized mainly by vegetables (27.6%), nuts & extra-virgin olive oil (EVOO) (16.7%), and sweet & sugars (11.8%). Legumes and fats separately represented just over 10% of the first PCA factor. The second factorial axis was represented mainly by cereals (40.9%), sweet & sugars (20.2%) and nuts & EVOO (15.4%). PCA showed different behaviors between dietary variables in each molecular subtype, especially among patients with triple negative TNBC (n = 37) the strongest contribution to the first PCA factor was given by sweet & sugars (20.7%), then vegetables (17.1%), fruits (11.9%) and legumes (11.0%) while animal proteins (24.3%), nuts & EVOO (16.0%), fruits (14.1%) and fish (12.1%) determined the second factor. From k- means, three clusters of patients were found. Cluster 1 (Healthy pattern) was associated with healthier dietary habits compared with the other two groups, with approximately twice the vegetables (204 grams vs. 119 grams for cluster 2 and 95 grams for cluster 3, $p < 0.01$). Cluster 2 (Western pattern) was characterized by greater refined cereals and animal protein, sedentary behavior and higher body mass index (BMI) and central obesity (35% with ≥30 kg/m^2 compared with 13% in cluster 1 and 25% in cluster 3. Cluster 3 (Ultraprocessed pattern) was characterized by greater intakes of sweet & sugars and non-EVOO fat, cluster 2 was composed mainly of Luminal BC subtype, while TNBC were found mostly in cluster 1 and cluster 3.
Our findings revealed three main dietary risk group by BC patients at the baseline visit of a lifestyle Trial: a healthy dietary group (cluster 1), a western diet group (cluster 2) and an ultra-processed food diet group (cluster 3). The former is considered part of a healthy Mediterranean diet which is known to improve the metabolic and hormonal risk factors for BC and reduce total mortality. A concern emerged for the high-risk group of TNBC patients who tend to be younger and appeared to consume more sweets and fats which are known risk factors for chronic diseases and poor cancer prognosis.

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Impact statement: Different dietary patterns emerged according to Breast Cancer molecular subtypes through a principal component analysis (PCA) that analyzed dietary information in a lifestyle trial conducted by our institute; moreover, an explorative unsupervised automatic clustering technique was performed to identify diet-risks groups.

Key words: *breast cancer; dietary patterns; molecular subtypes; Latent class analysis; Cluster analysis.*

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INTRODUCTION

Breast cancer (BC) is the second most common cancer worldwide, with over 2,300,000 new cases estimated in 2020 (1) and the fifth cause of cancer death globally (683,100 deaths estimated in 2020). In women, it is the most common neoplasm overall and the leading cause of cancer death. Since the 1990s, a gradual and constant reduction in mortality has been observed due to the implementation of screening programs and the availability of effective diagnostic-therapeutic approaches.

In Italy, approximately 55,700 new cases of BC are estimated each year (2). The risk of developing BC increases exponentially with age, particularly in the post-menopausal period. Recent data showed an increase in BC incidence in Italy (+0.5% compared to 2020) due to a higher life expectancy and early detection due to screening (2). The incidence is higher in the Northern than in Central Italy and the Islands due to different implementation and diffusion of screening programs and distribution of risk factors. In Italy as well BC represents the leading cause of cancer death in women with 12,500 deaths in 2021 although there has been a constant reduction in mortality over the last decade (estimated -2.2% per year) (2).

BC can be classified into the following four subtypes according to the expression of estrogen receptors (ER), progesterone receptors (PgR), cellular proliferation index (Ki67) and overexpression/amplification of the human epidermal growth factor receptor 2 (HER2):

1. Luminal A-like: low grade, with high expression level of ER/PgR, HER2 negative and Ki67;
2. Luminal B-like: high grade, variable ER/PgR expression, high Ki67, further divided into HER2 positive or negative;
3. HER2 positive tumors: ER/PgR negative and HER2 positive;
4. Triple negative breast cancer (TNBC): ER, PgR and HER2 negative (none expressed).

TNBC accounts for approximately 15% of BC cases is more frequent in young and obese women, often carries BRCA1 mutations and has the worst prognosis among all BC subtypes.

The absence of a targeted therapy, the tendency to metastasize to the central nervous system and visceral organs (3, 4) and the higher risk of relapse and distant recurrence represent the main factors explaining poor prognosis of TNBC (4).

Among modifiable risk factors for BC morbidity and mortality, diet plays a key role. It has been estimated that 30-50% of BC deaths could be avoided by dietary modifications alone (5). The dietary recommendations from international cancer institutions such as the World Cancer Research Fund (WCRF) suggest for the primary and secondary prevention of BC a diet rich in whole grains, vegetables, fruits, and legumes, while limiting the consumption of fast foods and processed foods, red and processed meat, sweets, sugar-sweetened drinks, and alcohol (6). Specifically for BC survivors the guidelines recommend a diet rich in soy foods and fiber as they have been significantly inversely associated with BC outcomes (recurrence, cancer-specific mortality, and all-cause mortality) (6, 7). The Mediterranean diet is one of the healthiest dietary patterns. The Mediterranean diet is considered by high consumption of plant-based foods (*i.e.*, vegetables, fruits, whole grains, legumes, nuts, olives and olive oli as the main source of fat); low intake of red and processed meat, saturated fats, and refined sugars; low to moderate consumption of dairy products; moderate consumption of fish; and moderate intake of alcohol (mostly red wine) with meals (8). Adherence to the Mediterranean diet has been inversely associated with multiple chronic disease, including cardiovascular disease and its risk factors, diabetes, and cancer, in epidemiological investigations and clinical trial (9-13). In the Nurses' Health Study, a healthy diet reach in

fruits and vegetables has been inversely associated with decreased BC risk particularly for the most aggressive BC subtypes (14).

The aim of the present study was to assess baseline dietary patterns by principal component analysis (PCA) overall and according to molecular subtypes in BC patients participating in a lifestyle trial in Italy (15).

MATERIALS AND METHODS

Patients

This study included 223 women (age range 30-70 years) with a BC diagnosis (stages I-III) participating in an ongoing multicenter randomized controlled trial of the effect of a treatment program of dietary modification, physical activity, and vitamin D supplementation (DEDiCa Study) on BC recurrence (21). The study protocol was approved by the Italian Ministry of Health, Italian Medicine Agency (AIFA) and the Ethic Boards of each recruiting hospital (ClinicalTrials.gov NCT02786875). Participants were recruited and followed up in national cancer institutes or oncologic departments of hospitals located in Southern and Northern Italy: Istituto Nazionale Tumori IRCCS Fondazione G. Pascale (Naples), Clinica Mediterranea (Naples), Villa Betania (Naples), Ospedale dei Colli Monaldi (Naples), Cannizzaro Hospital (Catania), San Vincenzo Hospital (Taormina), Istituto Nazionale Tumori IRCCS CRO (Aviano). Eligible participants were found through surgical lists of participating hospitals. Patients were contacted by phone and offered to learn more about the study during group information sessions. Informed consent was obtained at baseline from all participants randomized in the study. The main inclusion criteria were women with primary diagnosis of histologically confirmed BC within 12 months from diagnosis and no history of any cancer except non-melanoma skin cancer. The demographic characteristics of the 223 participants are shown in **Table 1**.

Dietary variables

Dietary data were derived from 7-day food records completed by participants 7 days before their baseline study visit, reviewed by trained dietitians and analyzed with the nutritional analysis software Winfood. Food and food groups were calculated in grams per 1000 Kilo calorie (Kcal) of

Table 1. *Distribution of main BC patients' characteristics.*

CHARACTERISTICS	N = 223
Age (years)	
Mean (SD)	52 (9)
Cancer stage	
IA-2A	166 (74%)
2B	25 (11%)
3A-3B	32 (14%)
Molecular subtypes	
Luminal	145 (65%)
Her2+	41 (18%)
Triple Negative	37 (17%)
Waist circumference (cm)	
Mean (SD)	94 (14)
Body mass index, BMI (kg/m²)	
Mean (SD)	27.2 (5.8)
<25	95 (43%)
25-29	70 (31%)
≥30	58 (26%)
Education (years)	
Mean (SD)	12.7 (4.6)
PREDIMED score (out of 14 points)	
Mean (SD)	8.21 (1.92)
Potatoes (grams/1000 Kcal/day)	
Mean (SD)	14 (12)
Fish (grams/1000 Kcal/day)	
Mean (SD)	31 (24)
Vegetables (grams/1000 Kcal/day)	
Mean (SD)	131 (72)
Fats and oil dressings (grams/1000 Kcal/day)	
Mean (SD)	1.35 (1.79)
Animal protein (grams/1000 Kcal/day)	
Mean (SD)	49 (26)
Cereals and pizza (grams/1000 Kcal/day)	
Mean (SD)	110 (32)
Legumes (grams/1000 Kcal/day)	
Mean (SD)	19 (25)
Fruits, fruit drinks and jams (grams/1000 Kcal/day)	
Mean (SD)	226 (105)
Sweets and sugars (grams/1000 Kcal/day)	
Mean (SD)	32 (31)
Nuts and EVOO (grams/1000 Kcal/day)	
Mean (SD)	14 (9)

Abbreviations: EVOO: extra virgin olive oil; HER2: human epidermal receptor-2; PREDIMED: Prevencion con Dieta Mediterranea (Spanish study); SD: standard deviation.

total energy intake and included animal proteins (meat, processed meat, eggs); cereals and pizza (whole and refined grains); fats and dressings (butter, lard, vegetable oils but not olive oil); fish and seafood; fresh fruit, fruit jams and fruit juices; legumes (including soya beans); nuts and extra virgin olive oil (EVOO); potatoes; sugar and sweets (including commercial sweet beverages); and vegetables (non-starchy vegetables).

Adherence to the Mediterranean diet (MD) was also summarized by the 14-item PREDIMED (Prevencion con Dieta Mediterranea) questionnaire administered by the study staff. This questionnaire was created by the PREDIMED study group in Spain to investigate adherence to the MD of participants in the dietary intervention trial (16). The PREDIMED questionnaire consists of 14 questions in total: 12 questions on food quantities and frequency of consumption (extra-virgin olive oil, vegetables, fruit, red or processed meats, butter, soda drinks, legumes, fish, commercial sweets, nuts, wine, sofrito sauce) and 2 general questions on intake habits regarding olive oil and meat. Each question included two possible answers and scores: 1 score for "yes" answer, indicating greater adherence to the MD and 0 for "no" answer. The PREDIMED final score ranges from 0 to 14 where 14 represented the highest adherence to MD.

Statistical analysis

Main BC patients' characteristics were analyzed as means and standard deviation (SD) for numerical variables and count (with percentages) for categorical variables. A univariable analysis was performed between molecular subtypes and the main variables investigated in the present analysis by Chi-Square test.

A multiple correlation matrix was performed between food groups (heat map, **Figure 1**) and a PCA was conducted to summarize dietary information about many food groups into a small set of principal components or dietary patterns. This analysis was performed in the overall sample and by molecular subtypes with the aim of assessing potential differences in dietary patterns among subgroup (**Figure 2**). An explorative unsupervised automatic clustering technique by k-means methodology was performed to identify diet-risks groups (**Figure 3**). A Cluster tendency statistic (Hopkins' statistic) was reported as index of goodness of cluster performance for the data. A value more far than 0.5 was considered as an indication of good performances (17). This technique reduces the number of observations by classifying them into homogeneous clusters, identifying the groups without previously knowing group memberships or the number of possible groups. Final groups were analyzed as dietary profiles and characterized with main synthetic statistics comparisons by univariate analysis.

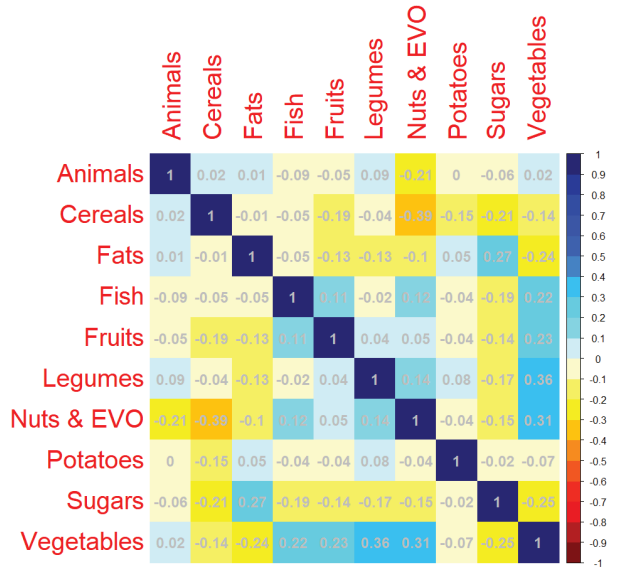

Figure 1. *Correlation matrix as heat-map of food groups.*

RESULTS

Patients' characteristics are reported in **Table 1**. Participants' mean age was 52 ± 9 years, BMI was 27.2 ± 5.8 kg/m^2, waist circumference was 94 ± 14 cm, 74% had low cancer stage (I or IIA), 65% had hormonal-dependent BC and reported medium adherence to the MD diet. **Table 2** shows patients' distribution according to BC molecular subtypes. No significant differences emerged between food groups and molecular subtypes. Pearson's correlation matrix was plotted in **Figure 2**. Statistically linear correlations were reported if the correlation coefficient ρ was greater than 0.3: vegetables with legumes (ρ = 0.36, p < 0.01) and with nuts & EVOO (ρ = 0.31, p < 0.01), nuts & EVOO with cereals (ρ = 0.39, p < 0.01). Overall PCA showed that the total variance explained 35.2% for the first two dimensions (**Figure 2**, whole data graph). The first factorial axis (or first PCA factor or dimension) was mainly built by vegetables (27.6%), nuts & EVOO (16.7%), and sweet & sugars (11.8%). Legumes and fats were just over

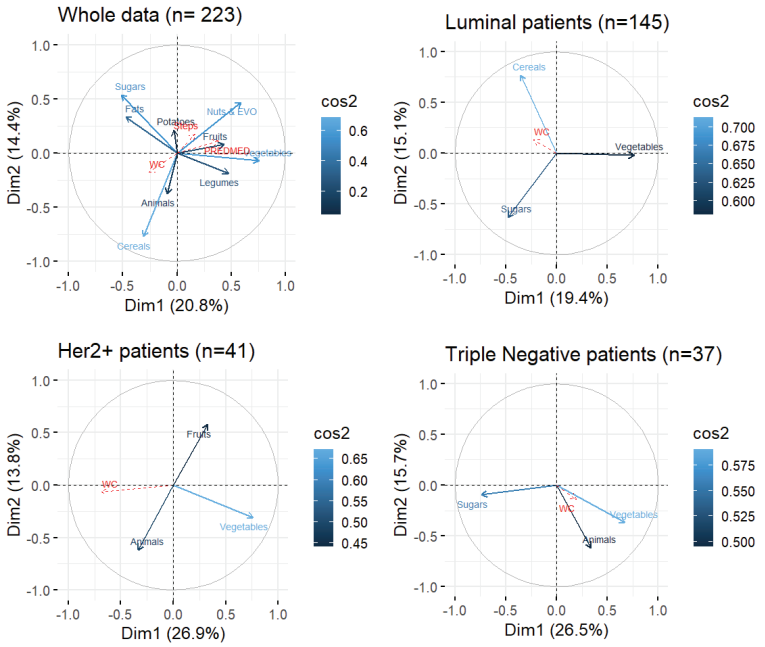

Figure 2. *Principal component analysis (PCA) results for the overall sample and by molecular subtypes.*

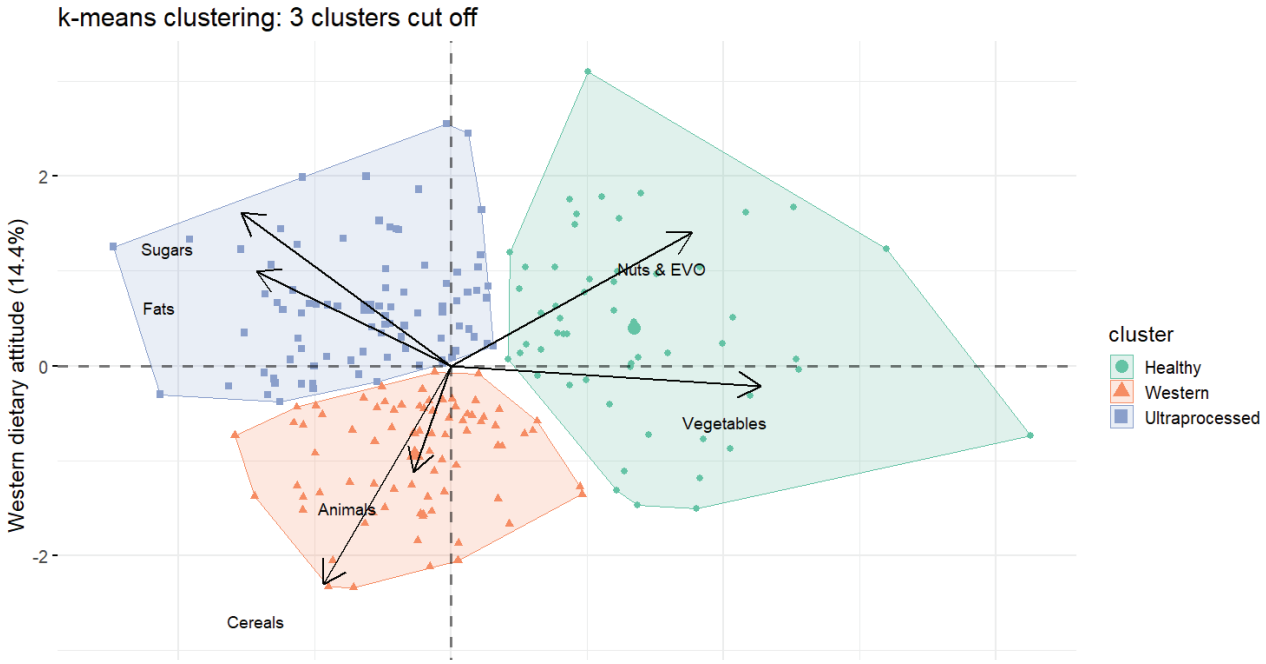

Figure 3. *K- means clusters in 2D-representation.*

10%. Similarly, cereals (40.9%), sugars & sweets (20.2%) and nuts & EVOO (15.4%) were main determinants of the second factorial axis. The third factorial axis was mainly composed of legumes (26.7%), potatoes (23.4%), animal protein (24.4%) and fish (16.7%). The fourth dimension was represented by fruits (34.5%), animal proteins (24.1%) and potatoes (19.3%), and the fifth dimension by potatoes (42.6%), fish (17.1%) and fruits (16.6%). In the analysis of molecular subtypes, the first and second dimensions explained >34% of the total variance. In the luminal subtype (**Figure 2**, Luminal graph) the main contribution was similar to that of the overall dataset. The first factorial axis was characterized mainly by vegetables (30.0%) and nuts & EVOO (17.5%); the second component by cere-

Table 2. *Distribution of patients' characteristics according to BC molecular subtypes.*

CHARACTERISTICS	LUMINAL N = 145[1]	HER2+ N = 41[1]	TRIPLE NEGATIVE N = 37[1]	P-VALUE[2]
Age (years)				0.803
Mean (SD)	52 (9)	52 (9)	51 (10)	
Cancer stage				0.203
IA-2A	108 (74%)	26 (63%)	32 (86%)	
2B	16 (11%)	6 (15%)	3 (8.1%)	
3A-3B	21 (14%)	9 (22%)	2 (5.4%)	
Waist circumference (cm)				0.636
Mean (SD)	93 (15)	95 (14)	94 (11)	
Body mass index, BMI (kg/m^2)				0.405
Mean (SD)	27.0 (5.9)	28.1 (6.1)	26.9 (5.4)	
BMI class (kg/m^2)				0.522
<25	61 (42%)	15 (37%)	19 (51%)	
25-29.9	49 (34%)	12 (29%)	9 (24%)	
≥30	35 (24%)	14 (34%)	9 (24%)	
Education (years)				0.915
Mean (SD)	12.6 (4.5)	12.6 (4.6)	12.9 (4.8)	
PREDIMED score (out of 14 points)				0.530
Mean (SD)	8.10 (2.01)	8.49 (1.57)	8.30 (1.94)	
Steps (n/day)				0.302
Mean (SD)	6,004 (2,856)	5,099 (2,514)	5,801 (2,700)	
Potatoes (grams/1000 Kcal/day)				0.459
Mean (SD)	13 (11)	15 (14)	16 (14)	
Fish (grams/1000 Kcal/day)				0.168
Mean (SD)	28 (21)	36 (28)	37 (31)	
Vegetables (grams/1000 Kcal/day)				0.242
Mean (SD)	124 (67)	140 (78)	146 (79)	
Fats and oil dressings (grams/1000 Kcal/day)				0.665
Mean (SD)	1.44 (1.95)	1.22 (1.42)	1.19 (1.50)	
Animal Proteins (grams/1000 Kcal/day)				0.354
Mean (SD)	50 (27)	46 (28)	48 (22)	
Cereals and Pizza (grams/1000 Kcal/day)				>0.999
Mean (SD)	109 (32)	111 (35)	110 (29)	
Legumes (grams/1000 Kcal/day)				0.593
Mean (SD)	18 (23)	22 (35)	17 (23)	
Fruits, fruit drinks and jams (grams/1000 Kcal/day)				0.209
Mean (SD)	219 (109)	243 (100)	233 (93)	
Sweets and sugars (grams/1000 Kcal/day)				0.805
Mean (SD)	32 (30)	33 (36)	33 (28)	
Nuts and EVOO (grams/1000 Kcal/day)				0.114
Mean (SD)	14 (10)	15 (7)	14 (6)	

[1]n (%); [2]Kruskal-Wallis rank sum test; Fisher's exact test; Pearson's Chi-squared test.

als (38.7%), sugars & sweets (26.3%), nuts & EVOO (15.9%) and fats (11.2%).

In the HER2+ subtype (n = 41) the first factorial axis was characterized mainly by nuts & EVOO (26.8%) and vegetables (22.4%), legumes (13.6%) and fish (11.7%) and the second factorial axis by animal proteins (27.6%), fruits (24.3%), legumes (18.5%) and fats (14.6%). Finally, in the TNBC subtype (n = 37) the strongest contribution to the first factorial axis was from sweet & sugars (20.7%), then vegetables (17.1%), fruits (11.9%) and legumes (11.0%) while animal proteins (24.3%), nuts & EVOO (16.0%), fruits (14.1%) and fish (12.1%) determined the second factor (**Figure 2**).

Three clusters of patients were found: cluster 1, Healthy (N = 54), cluster 2, Western (N = 79), and cluster 3 Ultra-processed (N = 85) (**Figure 3**). BC patients from cluster 1 (**Table 3**) were associated with healthier dietary habits compared to the other two groups, with approximately twice the vegetable intake compared with cluster 2 (204 grams *vs*. 119 grams for cluster 2 and 95 grams for cluster 3, $p < 0.01$). Cluster 1 also consumed more fish (44 grams *vs*. 29 grams and 24 grams, $p < 0.01$), fruits (296 grams *vs*. 208 grams and 201 grams, $p < 0.01$), legumes (34 grams *vs*. 18 grams and 10 grams, $p < 0.01$). BC patients from cluster 2 were more sedentary and with a higher BMI and central obesity (35% ≥30 kg/m^2 compared with 13% in cluster 1 and 25% in cluster 3, $p \leq 0.05$ for both comparisons), consumed more animal proteins (62 grams *vs.* 44 grams and 42 grams, $p < 0.01$) and cereals (135 grams *vs*. 91 for cluster 1 and 99 grams for cluster 3, $p < 0.01$ for both comparisons) the majority of which were refined (84%). BC patients belonging to cluster 1 were less overweight (25.2 kg/m^2 *vs*. 28.5 and 27.2, $p < 0.01$), with a higher adherence to the Mediterranean diet (PREDIMED score 9.2 *vs*. 7.9, out of 14) and with higher physical activity (8840 *vs.* 5121 steps/day for cluster 2 and 5796 steps/day for cluster 3, overall $p < 0.01$). Finally, cluster 3 consumed more than twice the amount of sweet & sugars (46 grams *vs*. 21 grams cluster 1 and 19 grams for cluster 2, $p < 0.01$) and four times the amount of fats compared with cluster 1 (2.0 grams *vs.* 0.5 grams and 1.0 grams, $p < 0.01$). BC molecular subtypes were equally distributed in cluster 1 and 3, while cluster 2 was mainly composed of luminal BC patients (72%) *vs*. 56% in cluster 1 and 64% in cluster 3, $p < 0.04$ for both while TNBC were little represented. HER2+ were distributed evenly among clusters (15-22%). Conversely, TNBC patients were found mostly in cluster 3 (51% of all TNBC) and cluster 1 (32% of all TNBC).

DISCUSSION

Our cluster analysis found three distinct dietary patterns: cluster 1-Healthy pattern characterized by vegetables, extra-virgin olive oil, tree nuts, fruits and legumes, cluster 2-Western pattern characterized by cereals and animal proteins and cluster 3-Ultraprocessed pattern characterized by sweets, sugars, and non-olive oil fats. The first cluster included more people with normal body weight and waist circumference. The second and third clusters included more people with higher body weight and waist circumference compared to cluster 1. Patients with hormone-related (luminal) BC tended to be older and were found mostly in cluster 2 while TNBC patients were found mostly in the Ultra-processed cluster 3. Considering that two thirds of our patients had hormone-related BC (luminal) it is discouraging to find that most women living in a Mediterranean country followed a Western dietary pattern. Moreover, the baseline diet is generally representative of patients' habitual diet suggesting a possible causal link with the development of this type of cancer. In a Spanish case-control study that used PCA analysis to derive three main dietary patterns, the Western dietary pattern, which is characterized by high intakes of refined cereals, animal sources of food, saturated fatty acids, and cholesterol, was associated with 46% increased risk in BC which increased to 75% in premenopausal women while the Mediterranean dietary pattern with a 44% protection (18). In this Mediterranean population the BC risk reduction with a Mediterranean diet was particularly strong in patients with TNBC (68%). This knowledge makes it even more relevant to strongly advise a Mediterranean diet to women with TNBC which is the most aggressive subtype and who tend to be younger women and high consumers of sweets and sugars and non-EVOO fats (ultra-processed pattern) in our study.

In the 2018 meta-analysis of international studies, the Mediterranean diet has been significantly and inversely associated with total mo rtality, cardiovascular and cancer mortality and with incidence of total cancer including BC (9). Case-control and cohort studies conducted in Italy after the 2018 meta-analysis indicate that a higher adherence to the Mediterranean diet was associated with 18% lower risk of BC (19) and with 63% higher 15-year surviv-

Table 3. *Distribution of main clusters.*

VARIABLE	CLUSTERS			P-VALUES	
	HEALTHY (CLUSTER 1) N = 54[1]	WESTERN (CLUSTER 2) N = 79[1]	ULTRA-PROCESSED (CLUSTER 3) N = 85[1]	CLUSTER 1 *VS.* CLUSTER 2[2]	CLUSTER 1 *VS.* CLUSTER 3[2]
Age (years)				0.781	0.162
Mean (SD)	52.4 (7.9)	52.6 (10.1)	51.4 (9.4)		
Education (years)				**0.020**	0.359
Mean (SD)	13.6 (3.9)	11.7 (4.6)	12.9 (5.0)		
Body mass index, BMI (kg/m^2)				**0.002**	**0.018**
Mean (SD)	25.2 (5.5)	28.5 (6.2)	27.2 (5.4)		
BMI class (kg/m^2)				**0.006**	0.051
<25	32 (59%)	28 (35%)	33 (39%)		
25-30	15 (28%)	23 (29%)	31 (36%)		
≥30	7 (13%)	28 (35%)	21 (25%)		
Waist circumference (cm)				**0.001**	**0.004**
Mean (SD)	87.9 (13.5)	96.7 (15.4)	94.2 (12.1)		
Waist circumference class (cm)				0.064	**0.033**
<88	28 (52%)	27 (34%)	27 (32%)		
≥88	26 (48%)	52 (66%)	57 (68%)		
PREDIMED score (out of 14 points)				**<0.001**	**<0.001**
Mean (SD)	9.2 (1.8)	7.9 (2.0)	7.9 (1.7)		
Steps (n/day)				**0.001**	0.057
Mean (SD)	6,840.1 (3,156.8)	5,121.5 (2,515.6)	5,796.0 (2,586.8)		
Molecular subtypes				**0.039**	0.448
Luminal	30 (56%)	57 (72%)	54 (64%)		
Her2+	12 (22%)	16 (20%)	12 (14%)		
Triple Negative	12 (22%)	6 (8%)	19 (22%)		
Animal proteins (meats and eggs, grams/1000 Kcal/day)				**<0.001**	0.794
Mean (SD)	43.9 (25.6)	61.5 (29.4)	42.0 (19.3)		
Cereals (grams/1000 Kcal/day)				**<0.001**	0.114
Mean (SD)	91.0 (27.3)	135.4 (23.0)	99.2 (26.7)		
Fats and oily dressings (grams/1000 Kcal/day)				**0.034**	**<0.001**
Mean (SD)	0.5 (0.6)	1.0 (1.3)	2.0 (2.0)		
Fish (grams/1000 Kcal/day)				**0.002**	**<0.001**
Mean (SD)	44.3 (28.9)	28.7 (23.5)	24.4 (18.7)		
Fruit, fruit drinks and jams (grams/1000Kcal/day)				**<0.001**	**<0.001**
Mean (SD)	296.2 (96.1)	207.8 (95.5)	200.9 (99.3)		
Median (IQR)	297.6 (224.2, 365.9)	202.7 (141.3, 270.1)	201.6 (129.1, 254.2)		
Legumes (grams/1000 Kcal/day)				**0.007**	**<0.001**
Mean (SD)	33.6 (39.5)	18.0 (21.2)	9.6 (8.5)		
Potatoes (grams/1000 Kcal/day)				0.658	0.468
Mean (SD)	13.6 (11.9)	12.0 (12.0)	15.5 (13.2)		
Nuts and EVOO (grams/1000 Kcal/day)				**<0.001**	**<0.001**
Mean (SD)	22.1 (8.8)	10.4 (4.1)	12.2 (5.7)		
Sweets and sugar (grams/1000 Kcal/day)				0.903	**<0.001**
Mean (SD)	20.5 (15.6)	19.3 (11.9)	45.9 (25.6)		
Vegetables (grams/1000 Kcal/day)				**<0.001**	**<0.001**
Mean (SD)	204.2 (77.3)	119.2 (50.3)	94.9 (46.5)		

[1]*n (%);* [2]*Wilcoxon rank sum test; Pearson's Chi-squared test.*

al (20). Oncologic treatment and side effects can be more challenging in women with overweight/obesity and metabolic diseases. Also, obesity is a known risk factor for BC, linked to excess adipose tissue which can increase circulating estrogen levels by higher aromatase activity, especially in the postmenopausal status, with consequent excessive hormonal stimulation to the mammary gland. However, interventions with the Mediterranean diet have been effective in reducing body weight (21) and cardiovascular events (22) in Mediterranean populations. Features of the Mediterranean diet (*i.e.*, high vegetable and fruit intakes) have shown beneficial effects on BC survival in the clinical trials WINS and WHEL conducted in the USA (23, 24). Furthermore, within the Mediterranean diet, a less glycemic dietary pattern that induces insulin economy, may contribute not only to lower diabetes risk but also BC risk: observational studies showed that a diabetes-risk reduction diet (DRRD) reduced BC risk by 24% in an Italian population (25) and reduced BC-specific mortality by 20% and overall mortality by 34% in an American population (26). A low glycemic index diet has shown to reduce BC risk by 6-8% in international studies (27, 28) and by 40% in an Italian population (29). Consuming a large amount of the daily caloric requirements in the form of sweets and refined carbohydrates may increase both the dietary glycemic index (GI) and the DRRD, however adhering to the traditional Mediterranean diet helps to reduce the dietary GI and the inflammatory potential of the diet (30). Furthermore, diets rich in ultra-processed foods which include processed meats, soft drinks, biscuits, and commercial sweets (our clusters 2 and 3), have been associated with higher risk of cardiometabolic diseases and cancer (31, 32). Ultra-processed foods represent 58% of the total calories consumed in a typical Western diet (33) although in our study sample it was 3-fold lower. The potential mechanisms of action of the Mediterranean diet, low GI diets, DRRD, and ultra-processed foods are several and could be distinguished into two main pathways: an oxidant/inflammation pathway and a glycemic/insulinemic pathway. A high intake of saturated fatty acids, such as red and processed meat, increase inflammatory processes and may generate more reactive oxygen species (ROS) which can damage cell membranes and increase DNA mutations (34). A diet rich in refined carbohydrates, sugars and sweets is characterized by a high GI index which may also increase inflammation, ROS and insulin levels. Insulin is an anabolic hormone with high homology for insulin-like growth factor 1 (IGF1), which stimulates cell proliferation (34). This type of diet was found directly associated with the risk of developing cancers at various sites (19) including BC (29). Foods with a protective role include: 1) whole grains, rich in fiber, phenolic compounds, minerals, vitamins and other trace elements, which in addition to increasing the sense of satiety, reduce the glycemic response and improve insulin sensitivity; 2) nuts, characterized by a rich mono/polyunsaturated fatty acid profile and by a high content of fiber and polyphenols, which contribute to the reduction of the risk of cardiovascular diseases and diabetes; 3) extra-virgin olive oil, characterized by a high content of polyphenols, mono-unsaturated fatty acids, vitamin E and chlorophyll which together concur to reduce cellular and DNA oxidation.

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CONCLUSIONS

Our findings revealed three main dietary risk group by BC patients at the baseline visit of a lifestyle Trial: a healthy dietary group (cluster 1), a western diet group (cluster 2) and an ultra-processed food diet group (cluster 3).

The largest European Epidemiologic Investigation on Diet and Cancer (EPIC) study showed that the consumption of fresh fruit and vegetables, especially green leafy vegetables (lettuce, spinach, chard) reduced the risk of developing BC (35). Part of the protective role of this type of dietary pattern comes from dietary fiber, vitamins, and minerals, flavonoids which act as antioxidants, immune enhancers, and hormone regulators.

A healthy diet, based on a Mediterranean dietary pattern and regular daily physical activity, as recommended by the international guidelines for the primary and secondary prevention of cancer (6), could improve the metabolic and hormonal profile, support immune function and limit inflammation thereby reducing the risk of BC and improving disease outcomes (6, 7).

COMPLIANCE WITH ETHICAL STANDARDS

Fundings

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Conflict of interests

The Authors have declared no conflict of interests.
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Availability of data and materials

The data presented in this study are available at: https://doi.org/10.5281/zenodo.7688957;

Authors' contributions

CA, AL: conceptualization, writing, original draft preparation; CA, AL, CS: writing, review and editing; VS, PE, AL, MC, PG, LA: performing follow-up visits; CA, CS, DGP: performing statistical analyses; PM, PG, VS, PE, AL, PR: data managing; RE, GM, CE: research support; AL: language editing; IF, CM, DLM, AA: medical and surgical support; CEg, PF: management.

Ethical approval

Human studies and subjects

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by Ethics Committee of each recruiting hospital (ClinicalTrials.gov NCT02786875; 17 March 2016). Informed Consent Statement: informed consent was obtained from all subjects involved in the study.

Animal studies

N/A.

Publications ethics

Plagiarism

The contents of the article are original and any overlaps with other articles are by the Authors themselves and appropriately cited.

Data falsification and fabrication

All the data correspond to the real.

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RESEARCH ARTICLE

L1CAM EXPRESSION IDENTIFIES DIFFERENT COLORECTAL TUMOR SUBGROUPS

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ABSTRACT: Neural cell adhesion molecule L1 (L1CAM) is a stem cell marker belonging to the immunoglobulin superfamily of cell adhesion molecules (IgCAMs). L1CAM shows multiple functions, depending on its homophilic or heterophilic interactions with other L1CAM molecules or with cell-matrix components. Previous studies have shown that L1CAM is aberrantly activated in colon cancer development and progression, being associated with metastasis. The present study was aimed to assess, using immunohistochemistry, the expression pattern of L1CAM in colorectal cancer (CRC), in order to correlate L1CAM expression with the aggressivity of tumor cells. To this end, formalin-fixed paraffin-embedded tissue samples from 51 patients affected by CRC, ranging in age from 38 to 88 years, 21 females and 30 males, were analyzed. The most important finding emerging from our work is the marked interindividual variability in L1CAM immunoreactivity in CRC, with 31 out of 51 (61%) cases expressing L1CAM. In positive cases, the expression of L1CAM in tumor cells ranged from mild immunostaining in 22 cases (score 1), to moderate reactivity in 6 (score 2) to strong and diffuse expression in 3 tumors (score 3). This is a new finding regarding L1CAM expression in CRC, which evidences that in a large cohort (39%) of CRC, tumor cells are not immunoreactive for this cell adhesion molecule. In the vast majority (21 out of 30) of tumors with budding margins and morphological features of epithelial-mesenchymal transition (EMT), L1CAM was focally expressed in 14/21 cases, and 3 out of 21 cases showed high L1CAM immunoreactivity. These properties of L1CAM suggest a major role for L1CAM in tumor cell migration in CRC and make it a potentially useful new marker for cancer progression and a candidate for anti-cancer therapy.

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Impact statement: This work highlights different expressions of L1CAM and their relative frequencies in colorectal cancer and provides new data regarding the interindividual variability in L1CAM expression in this tumor. L1CAM is here indicated as a marker associated with EMT. It is also hypothesized to may represent a new prognostic marker in CRC and a putative new anti-cancer target.

Abbreviations: L1CAM: L1 cell adhesion molecule; CRC: colorectal cancer; EMT: epithelial-mesenchymal transition; IgCAM: the superfamily of immunoglobulin-like adhesion molecules.

Key words: *L1CAM; colorectal cancer; immunohistochemistry; grading; epithelial-mesenchymal transition.*

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INTRODUCTION

The neural cell adhesion molecule L1 (L1CAM) is a stem cell marker belonging to the immunoglobulin superfamily of cell adhesion molecules (IgCAMs) (1). L1CAM is a 200-220 kDa transmembrane glycoprotein, with a long ectodomain that comprises six immunoglobulin-like (Ig) domains followed by five fibronectin type III repeats (2), a single transmembrane domain and a relatively short cytoplasmic domain (3). L1CAM is known for its role in neural development, being able to regulate processes such as neurite outgrowth, fasciculation, cell adhesion, cell migration, myelination, and cell survival, and allowing the construction of a dynamic neural network (4). L1CAM shows multiple functions, depending on its homophilic or heterophilic interactions with other L1CAM molecules (5, 6) or with cell-matrix components (7, 8), such as integrins, CD24, and other binding partners including neurocan or neuropilin-1 (5). Furthermore, the cytosolic tail of L1CAM interacts with several different binding partners, mediates interaction with the cytoskeleton proteins, and activates downstream signaling pathways (9, 10).

In physiology, L1CAM plays a fundamental role in fetal development and is highly expressed in the central (11) and peripheral nervous systems (12). Recently, L1CAM has been reported in multiple fetal organs, including the kidneys (13) and the gastrointestinal tract (14). However, L1CAM is aberrantly expressed in several types of human solid tumors, including endometrial cancer (15), ovarian cancer (16), and melanoma (17).

Previous studies have shown the association of L1CAM expression with metastasis in cancer (18). In particular, it has been hypothesized that L1CAM might represent a downstream target gene of the Wnt/β-catenin signaling pathway, one of the most basic pathways involved in intercellular communications during development, that is aberrantly activated in colon cancer development and progression (19). Moreover, a study carried out in cells in culture, identified L1CAM as the most prominent E-selectin ligand, confirming a possible major role for this adhesion molecule in carcinogenesis (20). Recent studies on L1CAM expression in colorectal cancer reported that patients displaying high levels of L1CAM in tumor cells were characterized by a higher risk for metastasis (21). According to recent hypotheses, the enteric neuronal network should be involved in tumor cell migration in colon cancer (22, 23), partly via L1CAM expression. This process, called perineural invasion, might occur along extrinsic nerves, with Schwann cells expressing L1CAM and providing physical guidance for tumor cells (24).

In recent times, L1CAM expression has been also reported in cancer-associated fibroblasts (CAFs) suggesting a further role for L1CAM in regulating cancer cell-stromal cell communications (25). Moreover, L1CAM has been identified as the most prominent ligand for E-selectin, a player in the binding of tumor cells to the endothelium, a major event in metastasis (26). These findings suggested a major role for L1CAM in the activity of tumor-associated stromal cells including the tumor-associated endothelial cells (TECs), cancer-associated fibroblasts (CAFs), and tumor-associated other inflammatory cells that produce growth factors, angiogenic factors, and proteolytic enzymes which might enhance tumor cell invasion and metastasis (27, 28). These findings allowed the establishment of a better link between L1CAM and metastasis (29).

Recently, the expression of L1CAM in colorectal cancer (CRC) has been indicated as an ancillary tool for stratifying carriers of colorectal cancer and identifying patients prone to dismal prognosis (30). The present study aimed to assess, using immunohistochemistry, the expression patterns of L1CAM in CRC, in order to correlate the immunohistochemical pattern of L1CAM with the aggressivity of tumor cells.

PATIENTS AND METHODS

We performed a retrospective study on formalin-fixed paraffin-embedded tissue samples (according to conventional techniques) of CRC, to test the immunoreactivity of tumor cells for L1CAM. The biopsy samples and tissue resections of CRC were obtained at the Division of Pathology of the University Hospital Agency of Cagliari.

In the present study, 51 patients affected by colorectal adenocarcinoma, ranging in age from 38 to 88 years, 21 females and 30 males, were analyzed. The main clinical and pathological data are reported in **Table 1**.

Three- four micron-thick sections were stained with hematoxylin and eosin (H&E) and immunostained with a mouse monoclonal antibody (Sigma-Aldrich, clone UJ127) against L1CAM (mouse IgG1 isotype). The ultra-View Universal DAB Detection Kit was used for detecting primary antibodies. The sections were automatically dewaxed and rehydrated with concentrated EZ Prep (10X) (cat. no. 950-102/05279771001)

Table 1. *Clinical, histological, and immunohistochemical data regarding 51 cases of colorectal cancer.*

CASE	AGE	GENDER	TUMOR GRADE	BUDDING MARGINS	L1CAM SCORE	CDX2	MLH-1	PMS-2	MSH-2	MSH-6
1	66	F	G1	-	0	+	+	+	+	+
2	59	M	G2	-	0	+	+	+	+	+
3	61	F	G3	-	0	+	+	+	+	+
4	61	F	G3	-	0	+	+	+	+	+
5	65	M	G2	-	0	+	+	+	+	+
6	65	M	G2	-	0	+	+	+	+	+
7	51	F	G1	-	1	+	+	+	+	+
8	43	F	G1	-	1	+	+	+	+	+
9	67	F	G2	+	1	+	+	+	+	+
10	67	M	G3	-	0	+	+	+	+	-
11	81	M	G3	+	1	+	+	+	+	+
12	83	F	G2	+	2	+	+	+	+	+
13	82	F	G3	-	0	-	-	-	+	+
14	81	F	G2	-	0	+	-	-	+	+
15	66	F	G2	++	2	+	+	+	+	+
16	82	M	G2	-	1	+	+	+	+	+
17	88	F	G2	-	0	+	+	+	+	+
18	80	M	G1	+	1	+	+	+	+	+
19	79	M	G3	-	0	+	-	-	+	+
20	49	M	G2	+	1	+	+	+	+	+
21	49	M	G2	+	3	+	+	+	+	+
22	71	M	G3	-	0	+	+	+	+	+
23	59	M	G1	-	1	+	+	+	+	+
24	38	F	G3	+	1	+	+	+	+	+
25	70	M	G1	+	1	+	+	+	+	+
26	79	F	G2	+	1	+	+	+	+	+
27	75	F	G2	-	0	+	-	-	+	+
28	55	F	G1	-	1	+	+	+	+	+
29	49	F	G2	-	1	+	+	+	+	+
30	70	M	G2	-	1	+	+	+	+	+
31	74	M	G2	-	2	+	+	+	+	+
32	84	F	G2	+	1	+	+	+	+	+
33	47	M	G2	-	0	+	+	+	-	-
34	65	M	G2	+	3	+	+	+	+	+
35	81	M	G2	-	0	+	+	+	+	+
36	40	F	G2	+	3	+	+	+	+	+
37	84	M	G1	-	0	+	+	+	+	+
38	82	F	G2	-	0	+	-	-	+	+
39	67	M	G2	+	1	+	+	+	+	+
40	78	M	G2	+	1	+	+	+	+	+
41	70	M	G2	+	1	+	+	+	+	+
42	77	M	G1	-	0	+	+	+	+	+
43	85	M	G2	-	1	+	+	+	+	+
44	81	F	G2	-	0	+	+	+	+	+
45	54	M	G1	-	1	+	+	+	+	+

CASE	AGE	GENDER	TUMOR GRADE	BUDDING MARGINS	L1CAM SCORE	CDX2	MLH-1	PMS-2	MSH-2	MSH-6
46	58	M	G2	-	0	+	-	-	+	+
47	74	M	G2	+	2	+	+	+	+	+
48	74	F	G2	+	2	+	+	+	+	+
49	69	M	G2	+	2	+	+	+	+	+
50	78	M	G2	+	1	+	+	+	+	+
51	65	M	G2	+	1	+	+	+	+	+

BM: Budding Margins; CDX2: Caudal related homeobox gene; MLH1: mutL homolog 1; MSH2: mutS homolog 2; MSH6: mutS homolog 6; PMS2: homolog 2, mismatch repair system component; L1CAM: cell adhesion molecule L1.

and pretreated with the recovery of the heat-induced epitope in ULTRA Cell Conditioning Solution (ULTRA CC1) (cat. 950-224/0524569001), for 64 minutes at 95°, to follow the slides were incubated for 20 minutes at room temperature at 1:100 dilution of the monoclonal anti-L1CAM primary antibody. Nervous structures were utilized as internal positive controls for L1CAM. As appropriate negative controls, tissue sections were processed omitting the primary antibody for L1CAM.

L1CAM expression was evaluated semi-quantitatively by two independent Authors (Cau F and Faa G), in a blinded fashion, without knowledge of clinical and pathological information. The sections were analyzed at high magnification to assess the positivity of L1CAM immunostaining in tumor cells. We regarded the staining as positive in cases with cytoplasmic, Golgian, and/or cell membrane positivity. No nuclear reactivity for L1CAM in tumor cells was detected. In cases of discrepant assessments, slides were reinvestigated by both Authors under a multi-head microscope and an agreement was obtained. The χ^2 test was used to examine the association between L1CAM expression and various clinicopathological characteristics, including age, gender, tumor location, and degree of differentiation. To obtain a semiquantitative evaluation of the degree of immunoreactivity for L1CAM, the following semiquantitative scoring system was applied: 0 = no reactivity; 1+ = <10% of immunoreactive tumor cells; 2++ = 10-50% of immunoreactive cells; 3+++ = >50% of tumor cells immunostained for L1CAM (**Table 2**) (**Figure 1**). In addition, immunohistochemistry for CDX2 and for genes involved in microsatellite instability (MSI) (MLH-1, PMS-2, MSH-2, MSH-6) was performed, in order to investigate a possible correlation between L1CAM expression and MSI. All procedures were performed according to the Ethical National standards of the responsible committee on human experimentation and approved by the Ethic Human Studies Committee of the University Medical Center of Cagliari (N. PG/2020/10912).

RESULTS

The study group included 30 males and 21 female patients ranging from 38 to 88 years of age (mean, 68 years) who underwent surgery for colorectal cancer. Pertinent clinical, histological, and immunohistochemical findings regarding L1CAM expression in colorectal cancer are presented in **Table 1**. At histology, 10 tumors were well differentiated and were classified as slow-grade adenocarcinomas (G1), 22 showed a moderate grade of differentiation (G2) and the remaining 8 cases were scarcely differentiated and were classified as high-grade adenocarcinomas (G3). In 21 out of 51 cases, we observed budding margins, with tumor cells detaching from the tumor mass and infiltrat-

Table 2. *The scoring system applied to the evaluation of immunostaining for L1CAM.*

0	No reactivity of L1CAM
1+	<10% of immunoreactivity cells in membrane and cytoplasm
2+	>10% and <50% of immunoreactivity in membrane and cytoplasm
3+	>50% of cells immunostained in membrane and cytoplasm

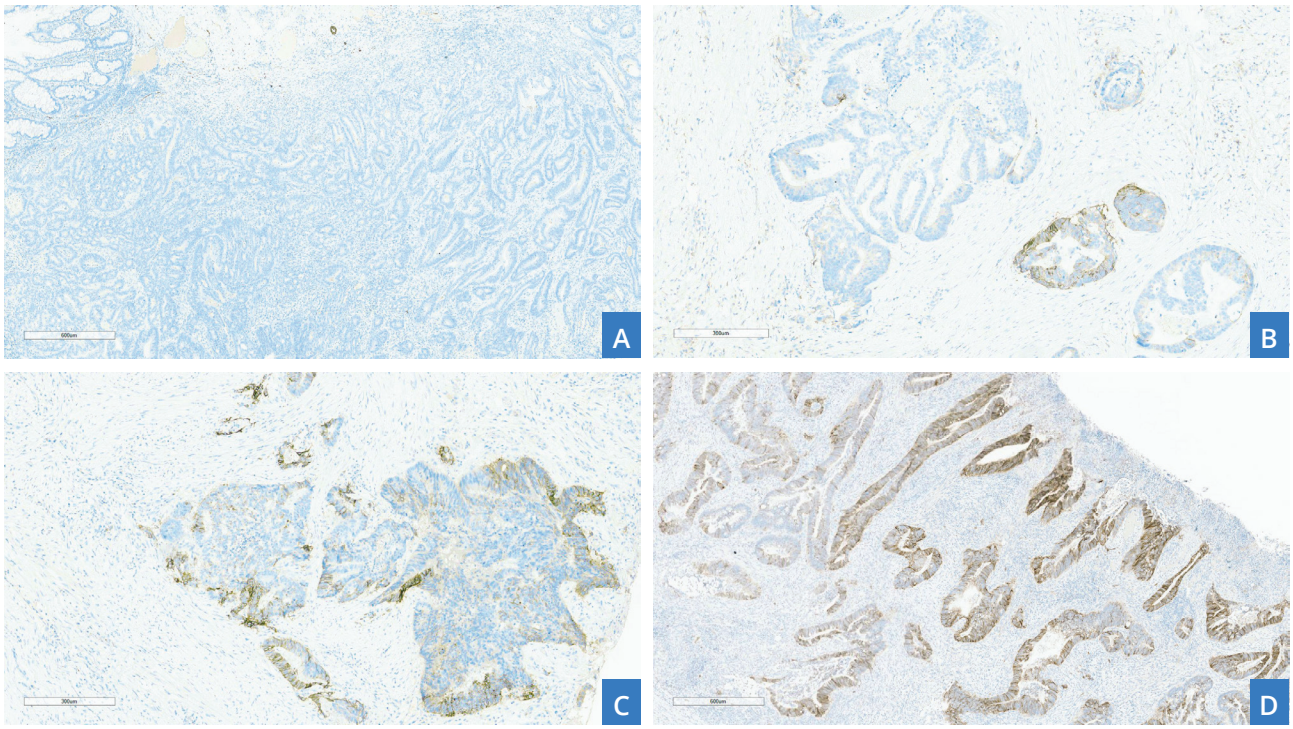

Figure 1. *Scoring system for L1CAM. (**A**) No reactivity for L1CAM (score 0); (**B**) <10% of immunoreactive tumor cells (score 1); (**C**) 10% - <50% of immunoreactivity in tumor cells (score 2); (**D**) >50% of tumor cells immunostained for L1CAM (score 3).*

ing the peritumoral microenvironment. In 50 out of 51 cases, tumor cells were immunoreactive for CDX2. Immunohistochemistry for the 4 genes involved in MSI revealed negative immunostaining for MLH-1 and PMS-2 in 6 out of 51 cases. All these 6 cases were also negative for L1CAM (**Table 1**). Immunohistochemical analyses regarding L1CAM expression in tumor cells showed immunostaining for L1CAM in 31 out of 51 cases (61%). No reactivity was detected in tumor cells in the remaining 21 tumors (39%). In positive cases, L1CAM was mainly expressed along the cell membrane, with some tumor cells expressing the adhesion molecule even in the cytoplasm. No nuclear reactivity was detected in this study in cancer cells.

A marked interindividual variability was observed regarding the degree of immunoreactivity for L1CAM in the 31 positive cases (**Table 1**): focal staining, involving less than 10% of tumor cells (score 1) was observed in 22 out of 31 positive cases (**Figure 1B**); in 6 cases, immunoreactivity was found in less than 50% of tumor cells (score 2) (**Figure 1C**); in the remaining 3 positive cases, a diffuse reactivity for L1CAM (score 3) was observed (**Figure 1D**).

A strict association was found between the presence of budding margins and the expression of L1CAM. This adhesion molecule was expressed in all 21 adenocarcinomas characterized by infiltrative margins (see **Table 1**). All 21 colorectal cancers with budding margins were characterized by L1CAM expression, with a score of 3 in three cases, a score of 2 in 6 cases, and a score of 1 in the remaining 12 cases (**Table 1**). In tumors with budding margins, L1CAM was mainly expressed in tumor cells acquiring an invasive and motile phenotype, detaching from the tumor mass, and infiltrating the peritumoral microenvironment (**Figure 2A, B**). In tumor cells with an infiltrative phenotype and with morphological features suggestive of epithelial-to-mesenchymal transition, L1CAM was strongly expressed along the cell membrane. The strong immunostaining for L1CAM observed in infiltrating cancer cells often contrasted with the absence of expression in the tumor mass (**Figure 3**).

No significant correlation was observed between the degree of differentiation of colorectal cancer cells and L1CAM expression in tumor cells. Cases with the highest scores for L1CAM expression, score 2 and score 3, were all classified as intermediate grade of differentiation (G2) (see **Table 1**).

L1CAM expression was unevenly distributed in the tumor mass. Areas with strong reactivity for L1CAM were frequently observed adjacent to areas negative for the cell adhesion molecule (**Figure 4**).

L1CAM expression was not restricted to tumor cells, being detected also in other components of the tumor microenvironment. L1CAM was occasionally ob-

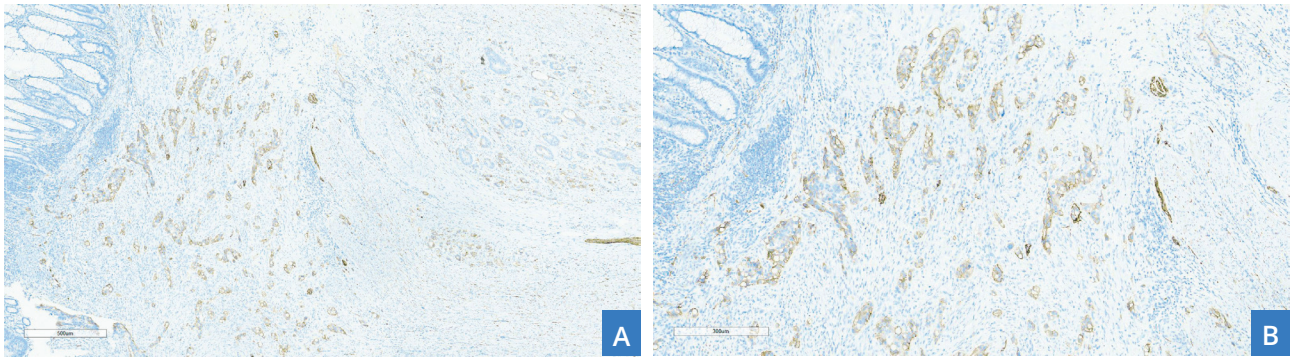

Figure 2. *(**A**) At low power, tumor cells acquiring a motile phenotype show strong reactivity for L1CAM; (**B**) at higher power, immunostaining for L1CAM appears mainly localized at the cell membrane of cancer cells.*

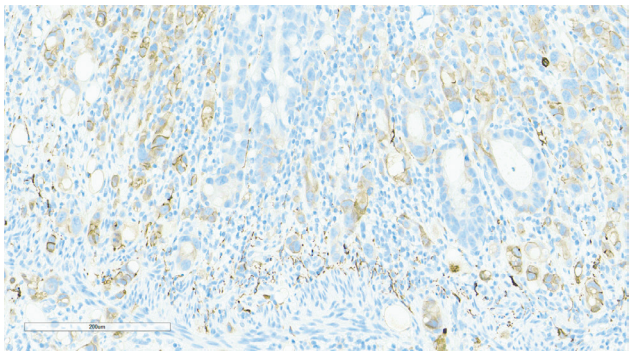

Figure 3. *Strong reactivity for L1CAM in tumor cells infiltrating the peritumoral environment contrasts with the absence of immunostaining in the tumor mass.*

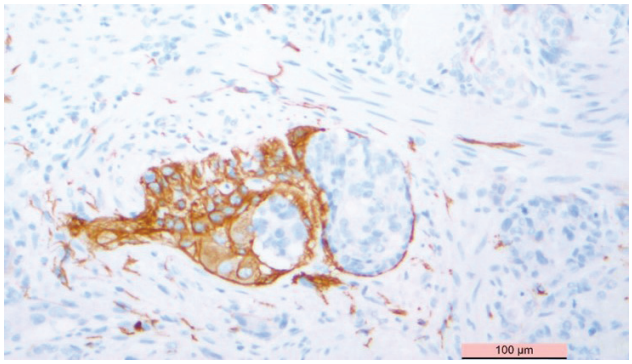

Figure 6. *The strong reactivity of nervous structures for L1CAM allows better identification of perineural invasion of cancer cells, with L1CAM-positive Schwann cells providing physical guidance for tumor cells.*

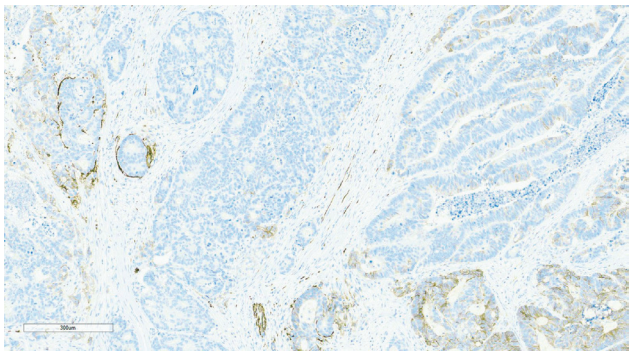

Figure 4. *This picture shows cancer cells with strong immunostaining for L1CAM adjacent to negative tumor cells.*

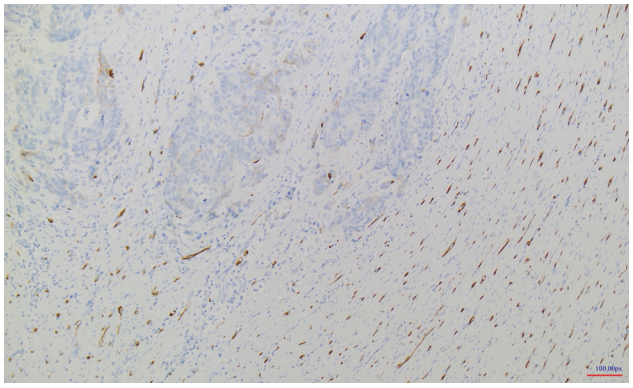

Figure 5. *L1CAM expression in cancer-associated fibroblasts.*

served in stromal cells surrounding the tumor mass, including cancer-associated fibroblasts (CAFs). In these cells, L1CAM was expressed along the cell membrane and inside the cytoplasm (**Figure 5**). Moreover, L1CAM was strongly expressed in the nerve structures and in the ganglia cells of the intestinal wall. The strong reactivity of nervous structures for L1CAM allowed a better identification of the process called perineural invasion occurring along extrinsic nerves, with Schwann cells expressing L1CAM and providing physical guidance for tumor cells (**Figure 6**).

Among the 51 cases of colorectal adenocarcinomas analyzed, 8 cases were already treated with neoadjuvant therapy, 3 of which were negative for L1CAM in tumor cells. In the 5 remaining cases, in 3 the expression for L1CAM was focal (score 1), and in 2 cases L1CAM expression was detected in 10%-50% of tumor cells (score 2).

The χ^2 statistical analysis did not highlight any significant association between L1CAM expression and the other variables analyzed in this study, both using the four classes of L1CAM expression (χ^2 (6, N = 51) = 10.4, p = .108) and the two classes approach (χ^2 (2, N = 51) = 5.96, p = .051).

DISCUSSION

CRC is currently the third leading cause of cancer death worldwide. Among the main risk factors for the disease, in addition to age, an unvaried and unbalanced diet, smoking, and random errors in the DNA occurring during cell division may be included (31).

The most common colon-rectal tumors are represented by adenocarcinomas (95%), followed by less frequent forms such as carcinoids, gastrointestinal stromal tumors, lymphomas, squamous cell carcinomas, leiomyosarcomas and melanomas (32, 33). A majority of cases of CRC develop sporadically, without a family history or hereditary genetic predispositions (34) causing uncontrolled cell growth of tumor cells themselves. The efficacy of the treatments available for the treatment of colorectal cancer has improved significantly in recent years, thanks to the identification of specific molecular targets which could improve and make more decisive the most suitable treatments for each patient (35).

In this study, we analyzed L1CAM expression in tumor cells in a large series of CRC. The most important finding emerging from our work is the marked interindividual variability of L1CAM immunoreactivity in colon cancer cells, with 31 out of 51 (61%) cases expressing L1CAM. In positive cases, the expression of L1CAM in tumor cells ranged from mild immunostaining in 22 cases (score 1), to moderate reactivity in 6 (score 2), strong and diffuse expression (score 3) being restricted to 3 tumors (see **Table 1**). This is a new finding regarding L1CAM expression in CRC, which evidences that in a large cohort of CRCs (39%) tumor cells are not immunoreactive for this cell adhesion molecule. Moreover, in 22 out of 30 positive cases, immunoreactivity was mild and focal, L1CAM being expressed in less than 10% of tumor cells. Grouping the negative cases (score 0) (**Figure 1A**) with cases with low reactivity (score 1) (**Figure 1B**), the vast majority (42/51) of CRCs were characterized by the absence or by a very low expression of L1CAM. Grouping cases with moderate immunostaining (score 2) and cases characterized by a strong expression (score 3), only 9 out of 51 CRCs analyzed in this study (18%) showed a remarkable expression of L1CAM.

In positive cases, a marked intratumoral variability among tumor cells was also observed, regarding the expression of L1CAM. Cancer cells with strong immunostaining for L1CAM were frequently observed in strict contact with negative cells (**Figure 4**). Moreover, in some tumors, the front invasion of the tumor was characterized by higher levels of L1CAM expression as compared to the superficial areas of the tumor mass. In CRC cases characterized by budding margins, it was possible to observe a strong expression of L1CAM in cells acquiring a motile and infiltrative phenotype with morphological features suggestive for epithelial-to-mesenchymal transition (EMT). The high expression of L1CAM in infiltrating cells often contrasted with the absence or low reactivity for L1CAM in the adjacent tumor cells (**Figure 3**).

Focusing on the 9 cases with medium-high L1CAM expression, their score ranging from 2 to 3, all cases were characterized by budding margins with histological evidence of tumor cells detaching from the tumor mass. These findings reinforce the hypothesis of a strong association between high levels of L1CAM expression and a motile phenotype in cancer cells.

In previous studies, it has been suggested that L1CAM might play a dualistic role: i) a static function, acting as an intercellular adhesion molecule that functions as a glue between cells forming zipper-like structures along the intercellular boundaries (36); ii) a motility-promoting function, driving cell migration during development and allowing tumor cell invasion (37). According to this hypothesis, the switch in the functional mode of L1CAM might be triggered by the following factors: i) the cleavage from the cell surface of the long ectodomain of L1CAM by membrane proximal proteolysis (38); ii) the ability to change binding partners, moving from the homophilic binding typical of the static function to the heterophilic bindings, including integrin binding, in the motility-promoting mode (2, 39). By augmenting its interactions with Beta-1-integrins, L1CAM might drive cell migration both in physiology and in pathological settings (40). In oncology, L1CAM has been previously reported to be overexpressed in multiple cancers, including ovarian cancer, endometrial cancer, pancreatic adenocarcinoma, melanoma, and glioblastoma (41, 42). In the tumor setting, L1CAM has been associated with the induction of an invasive and motile phenotype in tumor cells, supporting an aggressive clinical behavior and favoring vascular invasion and metastases (43). In experiments carried out on colon cancer cells *in vitro*, L1CAM was able to increase cell motility and growth, whereas its suppression in colon cancer cells decreased motility (44). Moreover, the up-regulation of L1CAM has been associated with the up-regulation of

epithelial-mesenchymal transition (9-45). On one hand, our findings confirm these previous studies on the relevance of L1CAM in favoring an invasive and motile phenotype in tumor cells in colon cancer (41). In 9 out of 9 cases characterized by medium-high expression of L1CAM in tumor cells, L1CAM expression was higher in tumors with budding margins, and in cells showing an infiltrative phenotype as compared to tumor cells of the tumor mass (**Figure 3**).

Moreover, in cases with a low L1CAM score, we occasionally observed tumor cells with invasive behavior negative for L1CAM. The interpretation of these findings appears complex and evidences that the molecular bases of invasion in colorectal cancer have not been completely elucidated yet. We may hypothesize that different molecular pathways might be involved in the induction of invasion and metastasis in CRC. On one hand, our findings confirm the existence of an L1CAM-dependent signaling pathway in which L1CAM promotes a motile phenotype in cancer cells (9, 46). In this pathway, up-regulation of L1CAM might be induced by TGF-Beta-1 (47). On the other hand, the finding of fields in which the acquirance of a motile phenotype in tumor cells was associated with the absence of immunoreactivity for L1CAM, clearly indicates that other molecules and other molecular pathways should be involved in tumor cell invasion.

In previous studies from our group, Thymosin Beta-4 was demonstrated at the invasion front of CRC, being mainly expressed in tumor cells undergoing EMT (48). The immunohistochemical data were subsequently confirmed by a proteomic study (49). The expression of TB4 might represent an alternative molecular pathway involved in EMT in colon cancer and might explain the absence of L1CAM found in a subset of tumor cells undergoing EMT.

In this study, as highlighted by the statistical analysis, we have not observed any significant association between L1CAM expression and the histological tumor grade. Among the 8 cancers with G3 grading, 6 were negative for L1CAM whereas the remaining 2 cases showed a mild expression (score 1) of the cell adhesion molecule. These findings indicate that L1CAM might play different roles in cancer insurgence and progression and that L1CAM expression is not related to the degree of differentiation of cancer cells.

The absence of L1CAM expression in 6 cases characterized by negative immunostaining for two genes involved in microsatellite instability, MLH-1, and PMS-2, remains, to the best of our knowledge, unexplained and deserves further studies on a larger series of colorectal cancers. Nevertheless, there could still be a weak or moderate association between the variables that was not detected due to limitations in the sample size or measurement methods. Therefore, further research may be needed to confirm these conclusions and explore any potential practical implications of the reported results.

A recent study on L1CAM expression in ovarian cancer suggested the hypothesis that L1CAM might induce stemness in cancer cells, indicating that this adhesion molecule could be involved in cancer stem cell induction and regulation (16). This study is in agreement with a previous study from our group, which identified the stem cell marker CD44 in colon cancer cells, this molecule being associated with an unfavorable prognosis (50). Another interesting finding emerging from this study is the ability of L1CAM to evidence marked changes in the neurons of the local enteric nervous system (SEL). The expression of L1CAM was present in tumor cells spreading along the nerve fibers. This finding may indicate the involvement of the peritumoral SEL which could promote, through specific receptors, the proliferation of tumor cells by the expression of pro-survival signaling pathways (22).

All these data taken together, L1CAM appears as a fascinating molecule with multiple functions both in physiology and pathology. Given the role played by L1CAM in the induction of a motile and infiltrative phenotype in tumor cells, we suggest its introduction in clinical practice, in order to better characterize all CRCs. Further studies should be carried out to better evidence the linkage between L1CAM expression and the clinical behavior of patients affected by CRC. These properties of L1CAM, in addition to its cell surface localization, make it a potentially useful diagnostic marker for cancer progression and a candidate for personalized anti-cancer therapy.

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REVIEW

A BRIEF OVERVIEW OF SEVERAL RECENT ADVANCEMENTS OF TARGETED-THERAPIES AND ANTIBODY-CONJUGATE DRUGS FOR ADVANCED TRIPLE-NEGATIVE BREAST CANCER

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ABSTRACT: Breast cancer (BC) is among the most prevalent and aggressive cancers affecting women. One of the main subtypes of BC, triple-negative breast cancer (TNBC), is considered the most aggressive and it is associated with high mortality, poor prognosis, and early and frequent recurrence, especially in premenopausal women. Unlike other subtypes, hormone receptor (HR) positive and human epidermal growth factor receptor 2 (HER2) positive, TNBC does not have specific cellular receptor markers, which would favor response targeted treatments. For this reason, the conventional standard-of-care (SOC) for early onset TNBC consists of neoadjuvant/adjuvant chemotherapy, alone or in combination with surgery and/or radiotherapy despite its toxic and off-target side effects. In recent years considerable efforts have been made to identify specific predictive biomarkers for TNBC to open a window for more targeted and precise therapy to improve overall survival and quality of life. Along with immunotherapy immune checkpoint inhibitors, targeted-therapies with poly (ADP-ribose) polymerase (PARP) inhibitors and mammalian target of rapamycin (mTOR) inhibitors have emerged and show promising results. One of the most recent targeted therapies approved by the FDA and EMA is an antibody-conjugate drug (ACD or ADC) called sacituzumab govitecan (SG) (Trodelvy). The results of clinical trials point to Trodelvy as a potential novel targeted therapy for TNBC.

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Impact statement: Recent advancements in drug development have led to an expanded list of FDA and EMA approved drugs against triple-negative breast cancer.

Key words: *antibody-drug conjugate (ADC); breast cancer; triple-negative breast cancer (TNBC); sacituzumab govitecan (SG), targeted-therapy.*

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INTRODUCTION

Breast cancer (BC) is a genetically and clinically heterogeneous disease with different biological, clinical, and molecular characteristics (1). Molecular classifications divide breast cancer into six sub-groups: luminal A, luminal B, HER-2, basal, normal breast like and claudin-low (2). According to immunohistochemistry (IHC) or a combination of IHC and microarray expression methods (gene signatures), there are three main subtypes of BC: Hormone receptor positive – estrogen receptor (ER+) or progesterone receptor (PR+), human epidermal growth factor receptor 2 (HER2) positive, and triple-negative (low or absence of ER, PR, and HER2 amplification) (3).

More recent data on molecular classification of BC indicate prognostic associations which include intrinsic subtypes, integrative cluster subtypes, triple-negative sub-classification and mutation-based profiling (4). Triple-negative breast cancer (TNBC) accounts for 15-20% of all invasive breast cancers (5). Among the subtypes, TNBC is associated with high mortality, early and frequent recurrence and poor treatment response. Unfortunately, TNBC cases in premenopausal women and in women of African descent are more frequent compared to other subtypes (6). Additionally, there is a significant overlap of the *BRCA* (BReast CAncer) gene,

BRCA-associated TNBC phenotypes which may further contribute to a poor prognosis (7). However, it is important to mention that not all patients with TNBC harbor *BRCA* mutations.

Lehmann *et al*. (8) genetically profiled 587 TNBC patient tumor samples identifying different groups: basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM) and luminal androgen receptor (LAR) (8). Their study determined the expression of cell-cycle regulating and specific DNA repair-related genes, abnormal activation of signaling pathways involved in cell migration, extracellular matrix-receptor interactions, and differentiation in order to subdivide the samples into various subtypes (8). Through the genomic classification of TNBC, there may be gradual advancements to more precise treatments and therapeutic targeting based on the specific subclassifications.

The latest clinical trials are mainly focusing on testing the efficacy of antibody-drug conjugates (ADCs). ADCs are biopharmaceutical drugs composed of highly selective monoclonal antibodies (mAb), a cytotoxic drug, and a chemical linker (see **Figure 1**). The mAbs are designed for tumor-associated antigens expressed at lower levels in normal (healthy) cells (9, 10). The cytotoxic drug will induce targeted cell death whereas the chemical linker is processed with the release of the cytotoxic agent in target cells. The first successful ADC administered ina clinical trials was used in patients with advanced metastatic carcinoma, colorectal and ovarian cancers in 1983 (11). Almost four decades later, after numerous clinical trials, ADCs are emerging as a promising targeted therapy for cancer (9).

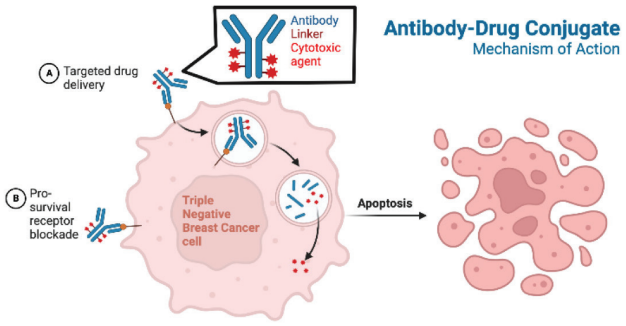

Figure 1. *Graphic represantiation of an antibody-drug conjugate (ADC) mechanism of action an antibody-drug conjugate (ADC) is composed of three components: monoclonal antibody (mAb), cytotoxic agent/drug, and a linker. The antibody will recognize a specific antigen target primarily expressed on the surface of the cancerous cells at higher levels compared to normal (healthy) cells. In this way, delivery is efficient and precise, reducing toxity to normal cells. An example of and ADC used in clinical trials in TNBC is Sacituzumab govitacan (Trodelvy). The antibody is designer to revognize the surface expression of Trop-2 and is connected to chemotherapeutic SN-38. Adapted from "Antibody-Drug Conjugate Mechanism of Action," by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.*

STANDARD-OF-CARE FOR EARLY ONSET TRIPLE-NEGATIVE BREAST CANCER

Due to the molecular signatures of TNBC, patients do not benefit from therapies designed to target hormone receptors or HER2 (7). The conventional standard-of-care (SOC) for early onset TNBC, as is the case with other malignancies, consists of neoadjuvant/adjuvant chemotherapy, alone or in combination with surgery and/or radiotherapy (12). Neoadjuvant therapy has proven advantageous for early-stage TNBC based on the results of various trials including KEYNOTE-172 Phase 1b (NCT02622074), I-SPY2 Phase II (NCT01042379), KEYNOTE-522 Phase III (NCT03036488), and NeoTRIPaPDL1 Phase III (NCT02620280) which explored the effects of neoadjuvant chemotherapy with or without pembrolizumab or atezolizumab, immune checkpoint inhibitors that bind to protein PD-1 or PD-L1, respectively (52, 53, 57, 40).

In addition to the cytotoxic chemotherapy agents, platinum agents, which interfere with DNA repair mechanisms, and the use of antimetabolite adjuvant capecitabine, which inhibits DNA and RNA synthesis, have proven to be advantageous for the treatment of TNBC (15, 16). The role of the antimetabolite oral prodrug, capecitabine, has been tested in the adjuvant setting for early and metastatic BC (62). Several randomized controlled trials (RCTs), such as the FinXX trial, investigated the role of capecitabine standard adjuvant or neoadjuvant therapies in combination with docetaxel, epirubicin and cyclophosphamide and these trials demonstrated no clinical benefits (63). The CREATE-X trial evaluated adjuvant capecitabine in patients with HER2-negative BC who had not achieved a pathological complete response (pCR) after standard neoadjuvant chemotherapy (64). Both disease-free survival and overall survival were significantly improved in the capecitabine group, and the effect was more prominent in the subgroup of patients with TNBC (62, 64).

A downside to these agents are the off-target effects resulting in toxicity and severe side effects lowering quality of life for the individual patient (17). A way to manage the off-target effects is through surgical excision, a method to locally control the tumor; however, not all patients can

be candidates for surgical removal. Furthermore, surgery does not eliminate the possibility of future local or regional recurrence for TNBC (13).

Along with surgery and chemotherapy, radiation therapy is considered a SOC for early onset TNBC. However, similar to the former, the latter presents the drawback of off-target effects. To avoid major side effects and irritation to normal tissues, radiation doses are carefully determined by the radiotherapist or radiation oncologist (18). Radiation administered on the chest wall, nodal and non-nodal irradiation after mastectomy or breast-conserving surgery (BCS) in TNBC benefits patients and improve survival (19). On the other hand, partial-breast radiation therapy for TNBC is not beneficial (20). Furthermore, a study conducted by Wang *et al*. (2019) investigated the benefits of BCS in combination with adjuvant radiation therapy and concluded that for early staged TNBC patients there was a better prognosis with BCS and radiation rather than mastectomy alone (51). There is currently no SOC chemotherapy regimen for patients with relapsed/refractory TNBC (40). Patients with advanced TNBC are treated with anti-metabolites capecitabine and gemcitabine, non-taxane microtubule inhibitor eribulin, and DNA cross-linker platinums (40). These available treatments for advanced TNBC are not SOC and research is being conducted to develop new treatment options for patients, especially if surgery is not an option (40).

THE NEED FOR TARGETED THERAPIES FOR EARLY AND ADVANCED TNBC

In contrast to hormone receptor (ER/PR)-positive and HER2-positive breast cancers, TNBC does not respond to hormonal or anti-HER2 monoclonal antibody trastuzumab-based targeted therapies (21). For this reason, there is great effort aiming at developing targeted therapies specific for TNBC. In response to this unmet clinical need, the U.S. Food and Drug Administration (FDA), after numerous clinical trials, has approved several targeted therapies. Nonetheless, there is still an urgent demand to develop and test additional targeted therapies. Such targeted therapeutic drugs based on the specific TNBC subtype include PARP inhibitors, genotoxic agents for the BL1 subtype, mTOR inhibitors and growth factor inhibitors (lapatinib, gefitinib, and cetuximab) for the BL2 and M subtypes, phosphoinositide-3 kinase (PI3K) inhibitors, Src antagonists or antiangiogenic drugs for the MSL subtype, immune checkpoint inhibitors for the IM subtype, or anti-androgen receptor (AR) therapy for the LAR subtype (23). Continued research is being conducted to better characterize the molecular signature of TNBC and identify novel targeted therapies based on gene expression profiles.

TARGETED THERAPIES AND IMMUNOTHERAPIES

PARP inhibitors, mTOR inhibitors, immune checkpoint inhibitors

Through sophisticated analytical technologies in recent years researchers have acquired insight into different possible molecular biomarkers and targets for TNBC treatment and therapies. The targeted therapies that have emerged in recent years are promising. Despite significant efforts to find novel molecular biomarkers, only a few potentials have been identified such as noncoding RNAs (ncRNA), microRNAs (miRNA) and long noncoding RNAs (lncRNAs) (24). There is a relevant lack of predictive biomarkers. However, *BRCA1* and *BRCA2* mutations have been beneficial markers for targeted therapy. These genes encode for proteins involved in regulating cell growth and division, aiding in suppressing tumor growth through homologous recombination (HR) repair pathway. Approximately 10-30% of TNBC patients have a *BRCA* germline (*BRCA*g) mutation (25). BRCA1 and BRCA2-deficient tumors exhibit impaired homologous recombination repair (HRR) and synthetic lethality with PARP inhibitors (26).

PARP1 is a chromatin-associated enzyme involved in cell proliferation, DNA repair, maintenance of genome stability and pro-inflammatory signals while PARP2 regulates DNA damage response (28). Thus, PARP inhibitors target underlying defects in DNA repair causing a block in cancer cell division (29). The limitation of this therapy is that PARP inhibitors are often associated with resistance developed by tumor cells (30). Additionally, PARP inhibitors can only be used in specific patient subsets defined by their DNA repair biomarker signatures (28).

Numerous studies have been conducted utilizing PARP inhibitors on TNBC with *BRCA* mutations, and the results indicate promising response and outcome for patients (27). In 2018, the FDA approved olaparib and talazoparib to treat advanced-stage HER2-negative BC in patients with *BRCA1* or *BRCA2* mutation (*BRCA*g mutation) (40). The results from OlympiAD Phase III (NCT02000622) and EMBRACA

Phase III (NCT01945775) lead to the approval of olaparib and talazoparib, respectively (54-56). These PARP inhibitors are effective and improve patient survival compared to other physician choice standard chemotherapeutic agents, such as capecitabine, vinorelbine, eribulin, or gemcitabine (40).

Several other signaling pathways have been analyzed and tested for TNBC treatment. The PI3K/protein kinase B (AKT) signaling pathway, which is involved in angiogenesis, tumor proliferation and inhibition of apoptosis, is an important target for BC treatments. Due to the potential of the PI3K/AKT pathway to cause resistance to immunotherapy and chemotherapy, various inhibitors targeting the pathway components have been evaluated in multiple clinical trials (40). For patients with advanced TNBC, various PI3K/AKT inhibitors have been studied in combination with other therapies such as paclitaxel and immunotherapies. The EPIK-B3 Phase III trial (NCT04251533) plans to assess the effect of alpelisib, an oral PI3K inhibitor, with nab-paclitaxel (40). In LOTUS Phase II trial, ipatasertib, a pan-AKT inhibitor, was assessed with first-line paclitaxel and improved Progression Free Survival (PFS) in locally advanced or metastatic TNBC (60). The IPATunity130 Phase III trial (NCT03337724) is assessing the efficacy of ipatasertib + paclitaxel for phosphatase and tensin homolog (PTEN)/PI3K/AKT-altered advanced TNBC or HR+, HER2-negative breast cancers, to corroborate the results from the LOTUS trial (61). When investigating the possible role of the PI3K pathway and the mTOR pathway for TNBC treatment, it remains unclear if the inhibition of these pathways has a significant effect on tumor growth and for this reason must be further investigated (31). In recent years, a combination of the mTOR inhibitor, Everolimus, with tyrosine kinase inhibitors (TKIs) has been effective for treatment of TNBC carrying activating mutations of the PI3K (32). Additional clinically investigated drugs include stimulator of interferon genes (STING) agonists involved in activation of the transmembrane protein, STING, utilized in the innate immune response, immune checkpoint inhibitor, Maternal Embryonic Leucine Zipper Kinase (MELK) inhibitors, which inhibit the mitotically regulated kinase MELK overexpressed in TNBC, and many other agents based on the genetic profile of the individual TNBC patient (33, 34). A characteristic of TNBC that may prove beneficial for treatment is the fact that TNBC cells are more immunogenic compared to the other BC subtypes (35). Since TNBC cells may exhibit high levels of programmed cell death-ligand 1 (PD-L1), a regulatory molecule expressed in T cells with immunoregulatory function, immunotherapies have been developed, such as atezolizumab and pembrolizumab, anti-PD-L1 antibodies (36-38). Based on the IMpassion130 trial (NCT02425891), the immunochemotherapy approach of utilizing atezolizumab in combination with nanoparticle albumin-bound (nab)-paclitaxel has become SOC for patients with PD-L1^{+}, unresectable, locally advanced, or metastatic TNBC (40). These immune checkpoint inhibitors (ICIs) are quite promising novel therapies specifically for TNBC leading to durable tumor remission and prolonged anti-tumor immunity (39). Despite the development of novel agents for specific subtypes of TNBC, only a fraction of patients responds to immune checkpoint or PARP inhibitors and often develop resistance and relapse (40). For this reason, clinical studies have been conducted and are underway to further assess the synergy and crosstalk that exists between PARP inhibition and the PD-L1/PD-1 immune checkpoints (58). Such clinical trials include MEDIOLA Phase I/II trial (NCT02734004) in which the combination of olaparib and durvalumab, an immunotherapeutic that binds to PD-L1, is studied in patients with *BRCA*g mutation metastatic BC (59) as well as the DORA Phase II trial (NCT03167619) evaluating olaparib with or without durvalumab in patients with advanced TNBC (40).

Due to the limited range of scope of current immunotherapy targeted treatments, further investigation is needed in the networks of DNA damage response (DDR), cell surface or intracellular receptors, cell surface markers, and signaling pathways for selective drug delivery and ADCs. This need is widely recognized and has contributed to the development and ultimate approval of therapeutic drugs for TNBC. However, the list of approved FDA drugs for treating TNBC is limited in number (**Table 1**).

CLINICAL TRIALS FOR EMERGING TARGETED THERAPY

In recent years, several clinical trials have been conducted to study the effects of numerous ADC as potential BC targeted therapies. The results of ADC clinical trials have been presented at major oncology conferences, such as ESMO and ASCO. Although most ADC clinical trials have focused on the subtype HER2-positive breast cancer, there is growing interest in investigating the potential efficacy of ADCs for treating TNBC.

Table 1. *Table of Therapeutic Drugs for Triple-Negative Breast Cancer (TNBC) as of September 2021. Drugs that have been approved by the FDA for patients with TNBC include Paclitaxel, Doxorubicin, Ixabepilone, Pembrolizumab, Atezolizumab, and Trodelvy.*

DRUG NAME	TARGET	DRUG TYPE	DOSAGE FORM	FDA APPROVAL DATE
Liposomal Doxorubicin (Doxil)	Anthracycline – Top2, Topoisomerase II DNA intercalation	Chemical	IV infusion	February 1999
Paclitaxel protein-bound particles for injectable suspension (Abraxane)	Taxane – microtubule target	Chemical	IV infusion	January 2005
Ixabepilone (Ixempra)	Taxane – microtubule target	Chemical	IV infusion	October 2007
Atezolizumab (Tecentriq) in combination with nab-paclitaxel (Abraxane)	PD-L1	Monoclonal antibody	IV infusion	March 2019
Pembrolizumab (Keytruda) in combination with chemotherapy	PD-1	Monoclonal antibody	IV infusion	November 2020
Sacituzumab govitecan-hziy (Trodelvy)	Trop2, Topoisomerase I	ADC	IV infusion	April 2020

ADC: antibody-drug conjugate; PARP: poly (ADP-ribose) polymerase; PD-1: programmed cell death protein 1; Trop2: trophoblast cell surface antigen 2; PD-L1: programmed cell death ligand 1.
Source: Mandapati 2022 "Triple negative breast cancer: approved treatment options and their mechanisms of action" and FDA.gov drugsatfda. https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approved-drugs-breast-cancer.

Antibody-drug conjugate: trastuzumab deruxetcan

Another subclassification of BC is HER2-low BC. HER2-low advanced BC is characterized by low levels of HER2 receptor protein and may include both hormone receptor-positive and hormone receptor-negative breast cancers (15). Promising results have emerged from the clinical trial DESTINY-Breast04 (NCT03734029) testing the ADC, trastuzumab deruxetcan, and its effects on HER2-low advanced BC (15). Patients with HER2-low metastatic BC who were treated with trastuzumab deruxetcan resulted in longer progression-free and overall survival compared to the physician's choice of chemotherapy (15). Of the 557 patients, 63 (11.3%) were hormone receptor-negative, a small proportion of patients. The median progression-free survival for the hormone receptor-negative cohort was 8.5 months (95% CI, 4.3 to 11.7) in the trastuzumab deruxetcan group and 2.9 months (95% CI, 1.4 to 5.1) in the physician's choice group while the median overall survival was 18.2 months (95% CI, 13.6 to not evaluable) in the trastuzumab deruxetcan group and 8.3 months (95% CI 5.6 to 20.6) in the physician's choice group (15). These results not only point to a possible targeted therapy for HER2-low advanced BC but may provide further evidence for a tailored treatment for TNBC.

Antibody-drug conjugate: sacituzumab govitecan

A handful of ADC has been approved by the European Medicine Agency (EMA) and the FDA. The ADC called Sacituzumab govitecan (SG) (Trodelvy) was approved by the FDA in April 2021 and by EMA in November 2021 (41, 42). SG consists of a monoclonal antibody designed against human trophoblast cell-surface antigen 2 (TROP-2) linked to a cytotoxic drug called SN-38. TROP-2 is a protein expressed on the surface of TNBC cells as well as other epithelial and metastatic breast cancers. SN-38, an active metabolite of irinotecan, is a topoisomerase inhibitor, which blocks the enzyme topoisomerase I, involved in copying DNA of the cell (42). The mechanism of this ADC action is initially mediated by anti-TROP-2 monoclonal antibody binding to the TROP-2 protein on the breast cancer cell surface. Then, the cytotoxic agent, SN-38, is delivered into the cancerous cells where it becomes active and inhibits cancer cells proliferation (43). Thus, SG is considered a promising new targeted therapy for locally advanced or metastatic TNBC (mTNBC) (41). Several clinical trials have been conducted since the development of ADC, SG (**Table 2**). A phase I/II single group study was done to evaluate the activity of SG in a cohort of 108 TNBC patients who had undergone two prior treatment methods (NCT01631552). The

drug was administered intravenously with a concentration of 10 mg/kg on days 1 and 8 of a 21-day cycle. The Overall Response Rate (ORR) was 33% and the median duration of response (DOR) was 7.7 months. The median progression-free survival (PFS) was 5.5 months, and the median overall survival (OS) was 13 months. The confirmatory ASCENT Phase III study of a cohort of 529 patients evaluated SG compared to physician's choice of chemotherapy, *e.g.*, eribulin, gemcitabine, capecitabine or vinorelbine. The ASCENT Phase III study showed great promise and was granted accelerated approval by the FDA based on the results of the IMMU-132-01 Phase II clinical trial treatment of adult mTNBC. Thus, SG is the first ADC approved by the FDA specifically for relapsed or refractory mTNBC (40). However, this targeted therapy has some relevant side effects, including anemia, neutropenia, and gastroenteritis (44).

DISCUSSION

The five pillars of cancer treatment consist of surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy. While great progress has been made in certain BC subtypes to have more than one option as standard-of-care (SOC) therapies, TNBC still remains treated with SOC consisting of neoadjuvant/adjuvant chemotherapy alone or in combination with surgery and/or radiation. Thus, there is urgent need to move beyond these treatments and uncover possible predictive biomarkers or immune checkpoint markers that could help in developing novel targeted therapies and immunotherapies for TNBC patients.

The technology and principle of ADC is very revolutionary since it links a monoclonal antibody to a cytotoxic agent, thereby allowing for precise targeted treatment against tumor cells. Emerging evidence shows that SG is one of the most effective ADCs for TNBC (43). Although SG has been approved by EMA and FDA for metastatic TNBC, further investigations must be conducted with the goal of comparing this ADC with multiple standard-of-care chemotherapies (43).

CONCLUSIONS AND FUTURE DIRECTION

In conclusion, studies of heterogeneity and subtypes of TNBC have opened the doors to many possible therapeutics that will one day overtake the SOC of neoadjuvant/adjuvant chemotherapy, alone or in combination with surgery and/or radiotherapy. Taxane- and anthracycline-based combination chemotherapy remains the standard-of-care for early-stage TNBC while advanced stage usually consists of chemotherapy, targeted therapy and immunotherapy (14). The treatment options available for patients are dictated by the stage of the tumor, local or metastasized (7). However, the estimated five-year TNBC survival rates greatly decrease depending on the status of metastasis. The five-year survival rate for a patient with localized TNBC is 91.3%. For patients with regional spread to lymph nodes, the five-year survival is 65.8% whereas for patients with distant metastasis to bones, liver, or lungs is 12.0%, as shown in **Table 3** (Surveillance, Epidemiology, and End Results (SEER) 2020 (46)). These estimates establish a baseline for the likelihood that a treatment will be successful. When comparing the five-year relative survival percentages in **Table 3**, it is evident that TNBC, labeled ('HR-/HER2-'), has the lowest survival percentage compared to all other subtypes. There are many factors contributing to this outcome including stage at diagnosis, environmental factors, age, race, standard-of-care, and availability of treatments. For this reason, the development of pharmaceutical drugs aimed at targeting TNBC is imperative.

With the recent EMA and FDA approval of SG, the potential future treatments for TNBC appear very promising. With the use of this ADC, there is limited off-target toxicity to normal cells since the monoclonal antibody is acting against an antigen or receptor expressed at low levels on the healthy cells, reducing therefore the level of toxicity usually associated with chemotherapy. Since the antibody portion of the ADC can be modified for specific cell surface antigens or receptors, this targeted therapy can be widely adapted (47). Thus, further investigation is needed to identify potential cell surface antigens and receptors specific to TNBC.

The future aims for breast cancer research and pharmaceutical drug development, specifically with regards to TNBC, should be centered on the understanding of the molecular complexity of the disease, improving the efficacy of current treatments, discovering reliable predictive biomarkers, determining mechanisms and pathways to overcome resistance to treatments and continuing to develop and test novel targeted treatments to improve survival rate and quality of life for patients.

Table 2. *Partial list of Clinical Trials for Sacituzumab Govitecan (SG) in Triple Negative Breast Cancer taken from clinicaltrials.gov as of September 2022.*

STATUS	STUDY TITLE	CONDITIONS	INTERVENTIONS
Recruiting	Trilaciclib in patients receiving Sacituzumab Govitecan-hziy for Triple Negative Breast Cancer	Triple negative breast cancer	Drug: Trilaciclib Drug: Sacituzumab Govitecan-hziy
Recruiting	Study of Sacituzumab Govitecan-hziy (SG) in Japanese Participants with advanced solid tumors or Triple-negative Breast Cancer	Advanced Solid tumors or Triple-negative Breast Cancer	Drug: Sacituzumab Govitecan-hziy
Recruiting	Study of Sacituzumab Govitecan-hziy versus treatment of Physician's choice in patients with previously untreated metastatic Triple-Negative Breast Cancer	Triple Negative Breast Cancer PD-L1 Negative	Drug: Sacituzumab Govitecan-hziy Drug: Paclitaxel Drug: nab-Paclitaxel Drug: Gemcitabine Drug: Carboplatin
Active, not recruiting	Sacituzumab Govitecan in Chinese Patients with mTNBC of at least 2 prior treatments	Metastatic Triple-negative Breast Cancer	Biological: Sacituzumab Govitecan
Recruiting	Study of Sacituzumab Govitecan-hziy and Pembrolizumab versus treatment of Physician's Choice and Pembrolizumab in Patients with Previously untreated, locally advanced inoperable or metastatic Triple-Negative Breast Cancer	Triple Negative Breast Cancer PD-L1 positive	Drug: Sacituzumab Govitecan-hziy Drug: Pembrolizumab Drug: Paclitaxel Drug: nab-Paclitaxel Drug: Gemcitabine Drug: Carboplatin
Not recruiting	Preventive stRategy for IMMU132-relatED AEs in TNBC	Triple Negative Breast Cancer Breast Cancer	Drug: Sacituzumab Govitecan Drug: Loperamide Drug: Granulocyte Colony-Stimulating Factor
Recruiting	Sacituzumab Govitecan +/- Pembrolizumab in metastatic TNBC	Breast Cancer Triple Negative Breast Cancer PD-L1 Negative	Drug: Sacituzumab Govitecan Drug: Pembrolizumab
Active, not recruiting	A study of Sacituzumab with chemoimmunotherapy to treat advanced Triple-Negative Breast Cancer after prior therapies	Advanced Triple Negative Breast Cancer	Biological: N-803 Biological: PD-L1 t-haNK Drug: Sacituzumab Govitecan-hizy Drug: Cyclophosphamide
Not yet recruiting	Safety and efficacy analysis of an antibody associated with a chemotherapy for Patients with a Triple Negative Metastatic Breast Cancer	Triple Negative Breast Cancer Metastatic Breast Cancer	Drug: Sacituzumab Govitecan
Recruiting	Sacituzumab Govitecan in Primary HER2-negative Breast Cancer	HER2-negative Breast Cancer Triple Negative Breast Cancer	Drug: Capecitabine Drug: Carboplatin Drug: Cisplatin Drug: Sacituzumab Govitecan
Completed Has results	Trial of Sacituzumab Govitecan inn Participants with refractory/relapsed metastatic triple-negative breast cancer (TNBC)	Breast Cancer	Drug: Sacituzumab Govitecan Drug: Eribulin Drug: Capecitabine Drug: Gemcitabine Drug: Vinorelbine

STATUS	STUDY TITLE	CONDITIONS	INTERVENTIONS
Active, not recruiting	Sacituzumab Govitecan in TNBC	Invasive Breast Cancer Triple Negative Breast Cancer ER-negative Breast Cancer PR-negative breast cancer HER2-negative breast cancer	Drug: Sacituzumab Govitecan Drug: Pembrolizumab
Recruiting	Atezolizumab + Sacituzumab Govitecan to prevent recurrence in TNBC (ASPRIA)	Breast Cancer Triple Negative Breast Cancer Residual Cancer Circulating Tumor DNA	Drug: Atezolizumab Drug: Sacituzumab Govitecan
Recruiting	Study to Evaluate the Safety and Efficacy of Magrolimab Combination Therapy in Adults with unresectable, locally advanced or metastatic triple-negative breast cancer	Triple-Negative Breast Cancer	Biological: Magrolimab Drug: Nab-paclitaxel Drug: Paclitaxel Drug: Sacituzumab Govitecan
Recruiting	Avelumab with Binimetinib, Sacituzumab Govitecan, or Liposomal Doxorubicin in treating patients with Stage IV or unresectable, recurrent triple negative breast cancer	Stage III Breast Cancer Stage IIIA Breast Cancer Stage IIIB Breast Cancer Stage IIIC Breast Cancer Stage IV Breast Cancer Invasive Breast Carcinoma Recurrent Breast Carcinoma Triple-Negative Breast Carcinoma Unresectable Breast Carcinoma	Biological: Anti-OX40 Antibody PF- 04518600 Drug: Avelumab Drug: Binimetinib Biological: Utomilumab Drug: Liposomal Doxorubicin Drug: Sacituzumab Govitecan
Recruiting	A study evaluating the efficacy and safety of multiple immunotherapy-based treatment combinations in patients with metastatic or inoperable locally advanced triple-negative breast cancer	Triple Negative Breast Cancer	Drug: Capecitabine Drug: Atezolizumab Drug: Ipatasertib Drug: SGN-LIV1A Drug: Bevacizumab Drug: Chemotherapy (Gemcitabine + Carboplatin or Eribulin) Drug: Selicrelumab Drug: Tocilizumab Drug: Nab-Paclitaxel Drug: Sacituzumab Govitecan
Completed Has results	Study of Sacituzumab Govitecan-hziy (IMMU132) in adults with epithelial cancer	Gastric Adenocarcinoma Small Cell Lung Cancer Carcinoma Breast Stage IV Triple Negative Breast Cancer	Drug: Sacituzumab Govitecan-hziy (SG)

Based on the various filters applied (Female, not yet recruiting, recruiting, enrolled by invitation, completed, clinical trial), there are seventeen clinical trials worldwide which seek or have sought to better understand the function and mechanism of the antibody-drug conjugate (ADC), Sacituzumab govitecan (SG) either in combination with other drugs or alone. There have been two completed clinical trials for SG. These results do not include Scopus.gov clinical trial results, thus eliminating grant clinical trials. Source: clinicaltrials.gov.

Table 3. *5-year relative survival percent, female breast subtypes by SEER.*

SUBTYPE	LOCALIZED	REGIONAL	DISTANT
HR+/HER2-	100.0%	90.1%	31.9%
HR-/HER2-	91.3%	65.8%	12.0%
HR+/HER2+	98.8%	89.3%	46.0%
HR-/HER2+	97.3%	82.8%	38.8%
Unknown	96.1%	76.4%	15.6%
Total	99.1%	86.1%	30.0%

The 5-year survival rate presents the percentage of survival of patients after five years. In this table, TNBC is identified as subtype 'HR-/HER2'. Data was collected over the span of 6 years in women of all ages, races living in the 22 registered areas of the United States (see list below). Table taken from National Cancer Institute: Surveillance, Epidemiology, and End Results (SEER) Program, 2020. Data source: SEER 22 areas (San Francisco, Connecticut, 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, Massachusetts, Illinois, and Texas).

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Authors' contributions

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

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This is a review article and all original studies are cited as appropriate.

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BRIEF REPORT

ERBB2 VARIANTS DISTRIBUTION IN NON-SMALL CELL LUNG CANCER: AN ITALIAN REAL-WORLD EXPERIENCE

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ABSTRACT: Lung cancer still remains the leading cause of cancer-related mortality worldwide. Among the emerging biomarkers predictive for target therapy, Erythroblastic oncogene B (ERBB2) is recommended by the main international guidelines to be investigate, according to several novel therapeutic approaches. Aim of this retrospective study was to analyze the distribution of ERBB2 mutations and their location along the gene in a set of 331 consecutive lung cancer tissues analyzed by next generation sequencing (NGS) technology in the Structure of Oncological Molecular Pathology, Azienda USL Toscana Centro, Italy. ERBB2 variants were detected in 95 out 331 samples (28.7%) and statistical analysis showed a 3% distribution of oncogenic variants, in line with literature data. Moreover, NGS technology allowed to identify a substantial amount of transmembrane domain mutations, not detectable by single hot spot assay: they should represent future clinical target in lung cancer, since nowadays many trials are investigating their clinical benefit.

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Impact statement: This report examined the distribution of entire ERBB2 variants along the protein structure and the frequency of oncogenic ones in a set of lung cancer samples.

Key words: *NSCLC; ERBB2; NGS.*

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INTRODUCTION

As of today, lung cancer still remains the leading cause of cancer-related mortality for both men and women worldwide (1). Lung cancer is subdivided into two major histological types: small cell lung carcinoma (SCLC) and non-small-cell lung cancer (NSCLC). The latter has a much higher incidence, comprising approximately 80-85% of all lung cancer cases. The highly complex and heterogeneous molecular nature of NSCLC, as well as late diagnosis (2) is a major factor responsible for the statistically low survival rates of these patients. Ever since the implementation of multiplex molecular panels in clinical practice and the consequent identification of new actionable driver mutations, the treatment scenario for these patients has gradually evolved from chemotherapy-based approaches to molecularly targeted therapies. Currently, the National Comprehensive Cancer Network (NCCN), the American Society of Clinical Oncology (ASCO), and the European Society of Medical Oncology (ESMO) guidelines recom-

mend that all NSCLC tumors containing an adenocarcinoma component be tested for the following actionable biomarkers: Epidermal Growth Factor Receptor (EGFR), V-Raf Murine Sarcoma Viral Oncogene Homolog B (BRAF), Mesenchymal Epithelial Transition exon14 skipping (METex14), Anaplastic Lymphoma Kinase (ALK), ROS proto-oncogene 1 receptor tyrosine kinase (ROS1), REarranged during Transfection (RET), and Neurotrophic Tyrosine Receptor Kinase (NTRK) 1, 2, 3 (3-5). In addition, they also recommended that many other genes, classified as "emerging biomarkers", be tested not as routine stand-alone assays but as part of a larger testing panels. Unsurprisingly, the ESMO has recently recommended the use of Next Generation Sequencing (NGS), especially in the presence of scant starting material (3). Among these newly emerging biomarkers is Erythroblastic oncogene B (ERBB2), also known as Human Epidermal growth factor Receptor 2 (HER2) or Cluster of Differentiation 340 (CD340).

The ERBB2 gene is 28,515 base pairs in length: its transcript encodes the plasma membrane-bound receptor tyrosine kinase ERBB2 protein comprising a ligand-bound extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular domain (ICD). This latter subunit is divided by a juxtamembrane domain (JMD), a tyrosine kinase domain (TKD), and a carboxy terminal tail domain (CTD) (6).

In NSCLC, ERBB2 alterations are commonly amplified (2-23%) and/or overexpressed (11-32%), and only seldom mutated (1.6-4%) (1, 7). Mutations in ERBB2 lead to constitutive activation of the receptor and are considered poor prognostic predictors: fortunately, these mutations are sensitive to small-molecule tyrosine kinase inhibitors (TKIs). Although previous studies have identified the majority mutations in the TKD, more recent research using NGS has described oncogenic mutations even in TMD, ICD and ECD. Preclinical studies indicate these mutations constitute promising candidates for targeted anti-ERBB2 therapies: small molecule inhibitors and anti-ERBB2 antibodies have also been shown to be highly effective against non-TKD oncogenic mutations such as exon 17 mutations p.V659E and p.G660D in the TMD and exon 8 mutation p.S310F in the ECD (8).

The rationale of this study was to provide clinicians with grounded information regarding the plentitude of mutations potentially occurring outside cancer-type specific domains in the hope of offering patients' better NSCLC treatments once new targeted drugs become available.

Thus, the purpose of this retrospective study was to analyze the distribution of ERBB2 mutations in a set of 331 consecutive NSCLC tissues by using NGS. We first verified whether the frequency of oncogenic activating mutations was in line with literature data. Then, we examined the frequency of mutations occurring outside the activating domains and their distribution along the protein structure.

MATERIALS AND METHODS

A total of 398 NSCLC tissue samples underwent NGS analysis from June 2019 to October 2021 were retrieved from the electronic archive of the Structure of Oncological Molecular Pathology, S. Stefano Hospital, Oncological Department, Azienda USL Toscana Centro, Italy.

In brief, NGS was performed on genomic DNA isolated from formalin-fixed paraffin-embedded (FFPE) NSCLC tumor tissues. Prior to molecular analysis, an experienced pathologist selected representative tumor tissue areas (>20% neoplastic cells) from Hematoxylin-Eosin stained slide. DNA extraction was performed with the QIAamp FFPE Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and resuspended it in 20 µL of ATE buffer (Qiagen). DNA quantification was assessed by Qubit dsDNA HS assay (Life Technologies, Carlsbad, CA, USA) on Qubit™ Fluorometer (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA).

Samples with adequate DNA content were then analyzed by NGS performed on the GeneReader platform (QIAGEN), according to the manufacturer's protocol. Briefly, 40 ng of DNA was used to generate libraries with the GeneReadQIAact Actionable Insight Tumor Panel (QIAGEN). This panel covers 773 unique variant positions in 12 genes (KRAS, NRAS, KIT, BRAF, PDGFRA, ALK, EGFR, ERBB2, PIK3CA, ERBB3, ESR1 and RAF1). Data was analyzed by the QIAGEN Clinical Insight (QCI™) Analyze software (QIAGEN). A 200X minimum read coverage cutoff was used for variant calls at any position in the panel, whereas a cutoff of 5% sensitivity criteria was chosen for variant calls at the clinical level. All variants were detected with >99% confidence based on allele-frequency and amplicon coverage.

RESULTS

Out of 398 samples, 331 (83%) had adequate DNA content for subsequent NGS analysis. Within the 331 adequate NSCLC tissues, 95 (28.7%) ERBB2 mutations were detected in 89 samples, histologically divided in adenocarcinoma (73, 82%), squamous (4, 4.5%) and not otherwise specified (12, 13.5%) subtypes, respectively. The distribution denotes previous works reporting that ERBB2 mutations predominantly occur in adenocarcinoma compared with other histological subtypes (9).

Most of ERBB2 mutations (85, 89.5%) were located in the TMD and comprised germinal like mutations (p.I654V and p.I655V, exon 17). The remaining 10 (10.5%) were located in the ECD and in the TKD (exon 8 and 20, respectively) and classified as activating mutations. Of the 10 non-TMD mutations, 7 (70.0%) were exon 20 insertions located in the TKD, whereas 3 (30.0%) were p.S310F exon 8 point mutations located in the ECD (**Table 1**).

Regarding the distribution of the mutations in the TMD, 91.8% (78/85) were p.I655V and 8.2% (7/85) were p.I654V. Six samples harbored a double TMD mutation, namely, p.I654V and p.I655V. Within the ERBB2 variants, 7.4% (7/95) and 92.6% (88/95) constituted classical activating mutations and rare non-TKD mutations, respectively (**Figure 1**). As for the recurrent variants, exon 17 p.I655V was the most frequent one (82.1%, 78/95), followed by exon 17 p.I654V (7.4%, 7/95), exon 20 p.Y772_A775dup (5.3%, 5/95), exon 8 p.S310F (3.2%, 3/95), exon 20 p.G778_P780dup (1%, 1/95), and exon 20 p.A775_G776insV (1%, 1/95). Clinically, ERBB2 mutations are classified as oncogenic, benign, and uncertain significance. Oncogenic function was significantly stronger in TKD mutations than in non-TKD mutations. Intriguingly, though up to 37.5% of ERBB2 oncogenic mutations were within the non-TKD. Based on the OncoKB classification, our results displayed 10/95 (10.5%) oncogenic variants (exon 20 insertion and exon 8 point mutation p.S310F) and 85/95 (89.5%) benign/uncertain significant variants (exon 17 p.I654V and p.I655V).

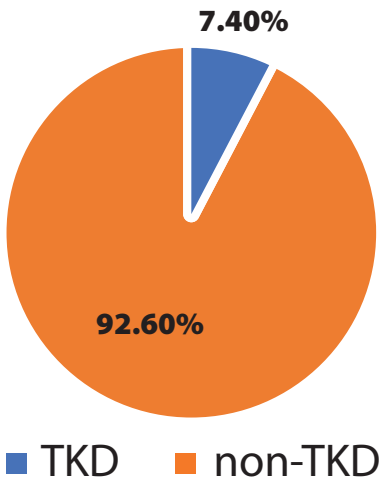

Figure 1. *TKD and non-TKD ERBB2 variants distribution detected in 331 consecutive NSCLC tissues.*

DISCUSSION

As the ERBB2 gene has recently emerged as a promising biomarker in NSCLC patients, the main international guidelines strongly recommend the routine use of NGS (10) in clinical practice to identify the highest possible number of aberrations present in ERBB2 mutations. The reason for this major endeavor is to find potentially actionable mutations and thus improve targeted treatments for NSCLC patients. Indeed, as

Table 1. *Oncogenic ERBB2 variants detected in 331 consecutive NSCLC tissues.*

SAMPLE	MUTATION	EXON	DOMAIN
1	G778_P780dup	20	TKD
2	S310F	8	ECD
3	Y772_A775dup	20	TKD
4	A775_G776insV	20	TKD
5	S310F	8	ECD
6	Y772_A775dup	20	TKD
7	Y772_A775dup	20	TKD
8	Y772_A775dup	20	TKD
9	Y772_A775dup	20	TKD
10	S310F	8	ECD

of today, traditional chemotherapy for ERBB2 in NSCLC is far from satisfactory. However, in recent years, several lines of investigation have demonstrated the clinical benefits of several therapeutic approaches in ERBB2-positive NSCLC patients (11).

These therapies, which are based on small molecule, second generation irreversible TKIs, include, for instance, afatinib, neratinib, tucatinib, and pyrotinib.

Clinical benefits of afatinib in ERBB2 ex20ins patients have been demonstrated by De Grève and colleagues. The Authors observed that patients harboring G778_P780dup and G776delinsVC mutations derived favorable outcomes from this treatment (12).

In addition, an *in vivo* preclinical study assessing the activity of various drugs against ERBB2 mutation variants found that neratinib, as well as afatinib, was more effective than other inhibitors in patients harboring the ERBB2^{YVMA} mut ation subtype (13). An equally interesting molecule is pyrotinib*, i.e*., an oral, irreversible pan-HER TKI. A phase II clinical study has shown the high efficacy of this drug in patients with advanced ERBB2 mutant lung cancer. Noteworthy, the use of NGS to detect ERBB2 alterations enabled the research team to identify mutations outside exon 20. Another clinical trial demonstrated that ERBB2-positive NSCLC patients harboring non-exon 20 aberrations achieved an ORR similar to that of patients with exon 20 mutations (14).

Finally, the most recently developed oral TKI is tucatinib: in the human lung cancer cell line NCI- H1781 inhibits ERBB2 phosphorylation. Lastly, an ongoing basket trial is also evaluating the clinical activity of tucatinib in combination with trastuzumab (NCT04579380) in patients with solid tumors harboring ERBB2 alterations, including a cohort for NSCLC (15).

Based on the above-mentioned druggable scenario ERBB2 testing showed a great potential in NSCLC clinical administration. Peters *et al.* identified a positive Objective Response Rate (ORR 20%, ranging from 5.7%-43.7%, CI 95%) in a series of NSCLC patients exhibiting 3+ score by testing ERBB2 with immunohistochemistry (IHC) under Trastuzumab Emtansine (T-DM1) administration. Moreover, an improving in response duration was also observed in same experimental arm (10.8 months). IHC is sometime afflicted by some technical and interpretation limitations, so we opted to analyze ERBB2 alterations by NGS (16).

The principal aim of this report was to investigate whether the distribution of ERBB2 oncogenic mutations in NSCLC samples in our real-world experience was consistent with the literature data. To this aim, we used the GeneReadQIAact Actionable Insight Tumor Panel (QIAGEN) for our NGS analysis. Such system investigated 489 missense mutations along exons 2-6, 8, 10-26, 28 of the ERBB2 gene and encompassed all three domains, namely, ECD, TMD, and TKD.

Interestingly, in our cohort of 331 samples, the prevalence of ERBB2 was higher than that reported in previous studies (28.7% *vs*. 2-4%) (17). We reasoned that this discrepancy was most likely due to the fact that the previous studies detected only TKD mutations, particularly exon 20 mutations. In fact, ERBB2 mutations are clinically categorized as oncogenic, benign, and uncertain significance. Studies have shown that whereas oncogenic function is significantly stronger in TKD mutations than in non-TKD mutation, up to 37.5% of ERBB2 oncogenic mutations are present within the non-TKD (11). Based on the OncoKB classification, ERBB2 variants located at exons 20 and 8, are considered oncogenic. Indeed, in our samples, we found seven mutations at exon 20 (represented by Y772_A775dup, A775_G776insV, G778_P780dup), and three at exon 8 (represented by S310F), as shown in **Table 1**. In our experience, oncogenic variants made up 3% (10/331) of the total samples analyzed, which is consistent with the literature data.

The secondary aim of our study was to investigate the presence and distribution of activating mutations along the ERBB2 gene. We found a significant fraction of TMD mutation: 89.5% (85/95) germinal-like mutations at exon 17, precisely at aminoacidic positions 654 and 655. However, since they are considered non-oncogenic germline alterations, their association with increased risk of developing cancer is still controversial (18) . Previous data indicate that p.I655V is more frequent than p.I654V: consistently, in our experience distribution was 91.8% (78/85) *vs*. 8.2% (7/85), respectively. Although today these mutations do not represent clinical targets in lung cancer, many recent clinical trials and case reports have instead highlighted the clinical benefits of targeted treatments for NSCLC patients harboring ERBB2 TMD mutations (19). Furthermore, we also found mutations both in the TKD (exon 20) and in the ECD (exon 18), with frequencies up to 7.4% (7/95) and 3.1% (3/95), respectively. Variants located in the TKD and in the ECD are classified as oncogenic: in our experience 10.5% of the total mutations were oncogenic and consistent with the literature data.

CONCLUSIONS

ERBB2 gene has recently emerged as a promising biomarker in NSCLC patients, regarding both oncogenic variants located at TKD or ECD, and germinal-like mutations at TMD. For the oncogenic targets, drugs are nowadays available, so the importance to fast identified them. Even though many more studies are warranted to establish the best therapeutic option for patients with non-TKD ERBB2 mutations, identifying and monitoring these alterations are crucial steps toward developing more efficient therapeutic strategies for NSCLC patients.

The identification of TKD and non-TKD ERBB2 mutations in a real-world cohort of NSCLC patients would not have been possible without the implementation of NGS. This amazing technology provided several advantages. First, it allowed us to analyze multiple druggable targets simultaneously, thereby reducing the time needed to obtain a complete diagnostic molecular classification of our patients' tumors. Second, it enabled us to examine multiple biomarkers in scant specimens. Finally, it allowed us to analyze non canonical alterations, which are generally undetectable by single hot spot technologies. The technical efficiency of our approach was indeed confirmed by the fact that the distribution of the oncogenic alterations we detected in our patients was consistent with that of previous literature. Finally, we hope that our present findings, together with the burgeoning literature in the field, may help clinicians improve the overall clinical and therapeutic framework of their patients by relying on NGS technologies to detect NSCLC targetable mutations.

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COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of interests

Umberto Malapelle has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientifics, Eli Lilly, Diaceutics, GSK, Merck and AstraZeneca, Janssen, Diatech, Novartis and Hedera unrelated to the current work. Pasquale Pisapia has received personal fees as speaker bureau from Novartis, for work performed outside of the current study. The other Authors have nothing to disclose.

Availability of data and materials

N/A.

Authors' contributions

SB, UM, MB: conceptualization; SB, MO, PP, FP: formal analysis; MO: software analysis; SB: writing-original draft preparation; UM, PP, FP: writing-review and editing; MB, GT: supervision.

Ethical approval

Human studies and subjects

The study was conducted in accordance with the ethical standards established in Declaration of Helsinki; informed consent was obtained from all subjects involved.

Animal study

N/A.

Publication ethics

Plagiarism

The contents of the article are original and any overlaps with other articles are by the Authors themselves and appropriately cited.

Data falsification and fabrication

All the data correspond to the real.

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