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Genomic molecular markers to monitor minimal residual disease with a non invasive liquid biopsy in breast cancer patients

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INTRODUCTION

Breast Cancer

Epidemiology

Incidence

Breast cancer is the most common neoplasia in women worldwide. One woman out of eight in the United States will develop breast cancer during her life[1]. In Italy, in 2018, about 52,800 new cases were diagnosed[2].

Mortality

Breast cancer is not only the most common malignancy in women, but also the leading cause of death by cancer. It represents the 29% of the causes of oncological death before the age of 50, 21% between 50 and 69 years and 14% after 70 years[2].

Survival

In Italy the relative survival, is 87% at 5 years and 80% at 10 years. The 5-year survival difference between age groups is relatively limited: 91% in women between 15 and 44 years, 92% between 45 and 54 years, 91% between 55 and 64, 89% between 65 and 74 years, 79% in women over 75 years old[2].

Geographical variability

The incidence of breast cancer presents a wide national and international variability, with a non-homogeneous distribution worldwide, maybe due to inhomogeneity of risk factors and diagnostic modalities[3].

Risk Factors

The literature concerning breast cancer risk factors is still debated about the understanding of the underlying biological mechanisms. Furthermore in many cases breast cancer do not have specific and therefore potentially predictable risk factors[4].

The best known are:

AGE: The risk of developing breast cancer progressively increases with age, reaching a peak around 50 years; starting from this age it continues to grow even if more slowly. The probability of developing breast cancer within the age of 49 is 2.4% (1 out of 42 women), 5.5% between 50 and 69 (1 out of 18 women) and 4.7% among 70 and 84 years (1 woman out of 21). This trend seems to be due to the continuous endocrine-proliferative stimulus that the mammary gland receives, combined with the progressive damage to the DNA and to the accumulation of epigenetic alterations that modify the expression of oncogenes and tumor suppressor genes.

SEX: The vast majority (99-99.5%) of breast cancer affects women. Male breast cancer is rare[5].

FAMILIARITY AND INHERITANCE: Most breast cancers are sporadic. A woman with a family of first degree positive for breast cancer has a doubled chance of developing breast cancer[6]. Only in 7-8% of cases the tumor is defined as hereditary[3]. The mainly involved genes are BRCA1 and BRCA2 (genes with autosomal dominant transmission), which are normally responsible for DNA repair[7, 8]. These genes are also associated with ovarian cancer. Other high penetrance genes associated with breast cancer are TP53, PTEN CHD1, STK1113. Then there are intermediate penetrance genes, such as CHEK2, ATM, PALB214, which represent 2-3% of breast cancer cases. Today the research activities are focus on the role of Single Nucleotide Polymorphisms (SNPs)[9].

MENSTRUAL AND REPRODUCTIVE FACTORS: The larger the fertility window, the higher the probability of developing breast cancer. In this sense a late menarche and an early menopause had a protective effect[10, 11]. Nulliparity, a first full-term pregnancy in late age and failure to breastfeed carry an increased risk[11]. In particular, the relative risk of breast cancer is reduced by 7% for each full-term pregnancy and 4.3% for each year of breastfeeding.

HORMONAL FACTORS: The higher the levels of circulating estrogen and the longer the glandular epithelium of the breast is exposed, the greater the risk of developing breast cancer[3]. The literature of the last 20 years on the association between hormone replacement therapy (HRT) and breast cancer is very extensive and articulated[18-14]. Users of hormonal contraceptives have a mild increase in the likelihood of illness compared to non-users, this risk returns to be equal to that of non-users about 10 years after the suspension[13].

DIETARY AND METABOLIC FACTORS: A high consumption of animal fats and alcohol seem to be associated with the development of breast cancer[3]. Obesity and the metabolic syndrome are a recognized risk factor, probably linked to the excessive presence of adipose tissue, the main source of estrogen in menopause[15]. Protective role is instead played by physical education[16].

RADIATION EXPOSURE: Women who have been exposed to radiation therapy in the thoracic district, especially if at a young age, have a higher frequency of breast cancer[17]. The risk of disease increases in proportion to the amount of radiation received and the earliness of the exposure[18].

PRECANCEROUS LESIONS: A condition of epithelial hyperplasia without atypia is associated with a 1.5 to 2 times greater risk of developing a carcinoma. In atypical ductal hyperplasia and in atypical lobular hyperplasia this risk becomes 4-5 times greater. Atypical hyperplasia, in fact, is a clonal proliferation with the same histological features of carcinoma in situ, but smaller or equal in size to 2mm.

Natural History

The origin of breast cancer is a clone of cells with multiple genetic aberrations, which cause a defect in the mechanisms of proliferation, differentiation and apoptosis. The acquisition of these mutations is conditioned by the inheritance of susceptibility genes or by environmental factors. Most carcinomas arise in terminal duct lobular units (TDLU). Subsequently they produce tumors that differ in morphology and biological behavior. The role of the tumor microenvironment in progression disease is now widely recognized[19,20]. The distinction between carcinoma in situ and invasive carcinoma is fundamental.

We must also consider the metastatic diffusion, both by lymphatic or hematogenous pathways. Among the lymph node groups that may be affected in case of breast cancer we find the axillary lymph nodes, the supraclavicular lymph nodes and the lymph nodes along the internal mammary chain.

Breast cancer tends to metastasize to bones, lungs, central nervous system and liver. In the 1970s, Bernard Fisher defined breast cancer as a systemic disease[21].

Diagnosis

Mammography and Tomosynthesis

Mammography is nowadays the gold standard for the breast evaluation, both in symptomatic and asymptomatic women[22–24].

The types of isolated or variously associated anomalies searched are: nodular lesions, architectural distortions and calcifications. Mammography is the only technique able to recognize microcalcifications and distortions of the mammary architecture, these type of lesions can represent the only "spy" of an asymptomatic tumor. Sensitivity increases with the increase of the adipose component: if under the age of 50 it does not exceed 83%, in women over fifty it exceeds 98% [25]. In dense breasts sensitivity is reduced up to 70% and in these cases it can be very useful to integrate with ultrasound[26] analysis.

Tomosynthesis is a new method, which produces images of organ sections. It therefore allows an increase in diagnostic sensitivity and specificity compared to mammoghaphy[27].

Breast Ultrasound

Ultrasonography is generally used in association with mammography, causing an increase in sensitivity on both palpable and non-palpable lesions, especially in the presence of dense breasts. Instead, it represents the gold standard technique for young women under 35 years[28, 29].

Breast ultrasound allows the differentiation of cystic from solid nodules, analyzing the content of cysts, assessing the presence of axillary lymph nodes. Last but not least, offers the possibility of ultrasound-guided biopsies for pathological diagnosis both on breast and lymph node.

Magnetic resonance (MRI)

Magnetic resonance has gained an increasingly important role in recent years both for diagnosis and for loco-regional staging of breast cancer[30].

The applications of magnetic resonance are: define the exact extension of the tumor in case of discrepancy between mammography and ultrasound, identify a multifocality or a multicentricity, lobular histological type, identify periprosthetic nodules, evalue the efficacy of neoadjuvant chemotherapy, management of occult carcinoma of the breast; it can finally be used as a screening test in high risk patients of breast cancer (carriers of BRCA1 or BRCA2 mutations or of women with a strong family positivity) [31]. Its routine use is still much debated today. In particular, too high cost and too many false positives are attributed to MRI [32,33].

Cytology and Histology

The diagnosis of certainty of breast cancer can be reach only with cytological and / or histological sampling. The difference between the two procedures is given by the quantity of tissue that is taken[34,5].

A recent needle biopsy method, but now widely established in clinical practice is the Core Needle Biopsy (CNB); it involves the taking of a small frustule of tissue from the suspect area. The degree of reliability of the method exceeds 96% [36].

The Vacuum-Assisted Breast Biopsy (VABB), better known by the name of the device used to perform it, the Mammotome, allows the collection of tissue samples from very small lesions through a sterile probe. This method is indicated for radiologically dubious or suspected mammary microcalcifications and for the evaluation of small parenchymal distortions, nodules or opacity[37].

Histological classification, grading and staging

Histological classification

The classification of breast bening or malignant neoplasm that is used today is the one of the World Health Organization of 2012 (Table 1).

Malignant lesions of the breast are generally differentiated between epithelial tumors and non epithelial tumors, starting from the surrounding stroma. Malignant epithelial tumors can be distinguished from anatomo-pathological point of view in carcinomas in situ and invasive carcinomas[38].

The “non-special type infiltrating carcinoma” (NST) represents the majority of invasive forms (from 44 to 75%)[3]. Invasive lobular carcinoma is at second place by frequency (about 15%) [39]. It is characterized by the proliferation of small, poorly cohesive cells arranged in a fibrous stroma. This variant generally is difficult to be identified at screening mammography because it tends to be camouflaged in the glandular parenchyma, often multifocal or multicentric and bilateral (20-40%)[39–43]. Other special types occur less frequently[38,44].

Type	Classification
Invasive carcinoma of no special type (NST)	8500/3
Pleomorphic carcinoma	8522/3
Carcinoma with osteoclast-like stromal giant cells	8035/3
Carcinoma with choriocarcinomatous features	
Carcinoma with melanotic features	
Invasive lobular carcinoma	8520/3
Classic lobular carcinoma	
Solid lobular carcinoma	
Alveolar lobular carcinoma	
Pleomorphic lobular carcinoma	
Tubulolobular carcinoma	
Mixed lobular carcinoma	
Tubular carcinoma	8211/3
Cribriform carcinoma	8201/3
Mucinous carcinoma	8480/3
Carcinoma with medullary features	
Medullary carcinoma	8510/3
Atypical medullary carcinoma	8513/3
Invasive carcinoma NST with medullary features	8500/3
Carcinoma with apocrine differentiation	
Carcinoma with signet-ring-cell differentiation	
Invasive micropapillary carcinoma	8507/3
Metaplastic carcinoma of no special type	8575/3
Low-grade adenosquamous carcinoma	8570/3
Fibromatosis-like metaplastic carcinoma	8572/3
Squamous cell carcinoma	8070/3
Spindle cell carcinoma	8032/3
Metaplastic carcinoma with mesenchymal differentiation	
Chondroid differentiation	8571/3
Osseous differentiation	8571/3
Other types of mesenchymal differentiation	8575/3
Mixed metaplastic carcinoma	8575/3
Myoepithelial carcinoma	8982/3
Epithelial-myoepithelial tumors	
Adenomyoepithelioma with carcinoma	8983/3
Adenoid cystic carcinoma	8200/3
Rare types	
Carcinoma with neuroendocrine features	
Neuroendocrine tumor, well-differentiated	8246/3
Neuroendocrine carcinoma poorly differentiated (small cell carcinoma)	8041/3
Carcinoma with neuroendocrine differentiation	8574/3
Secretory carcinoma	8502/3
Invasive papillary carcinoma	8503/3
Acinic cell carcinoma	8550/3
Mucoepidermoid carcinoma	8430/3
Polymorphous carcinoma	8525/3
Oncocytic carcinoma	8290/3
Lipid-rich carcinoma	8314/3
Glycogen-rich clear cell carcinoma	8315/3
Sebaceous carcinoma	8410/3

Table 1. Invasive breast carcinomas (without microinvasive carcinoma and invasive papillary lesions)

Grading

The histological grading of an invasive carcinoma is important to determine the characteristics of breast cancer and its prognosis[45]. Several schemes have been proposed for the gradation of neoplasms, but the one most frequently used is the scheme proposed by Elston and Ellis[46]:

- Grade 1: well differentiated tumor;
- Grade 2: discretly differentiated tumor;
- Grade 3: poorly differentiated tumor.

Staging

Staging of breast cancer is initially clinical, formulated on the basis of physical examination and radiological examinations, and subsequently histopathological. Staging is essentially based on three parameters: tumor size (T), number of regional lymph nodes involved (N) and distant metastases (M).

In addition to the clinical examination to complete the evaluation of the T parameter is essential to carry out a bilateral mammography and an ultrasound of the breast; in selected cases it may be useful to use a nuclear magnetic resonance (see above). The N parameter (evaluation of regional lymph nodes) is done by an axillary ultrasound and, in case of doubt, a needle aspiration citology. Finally, to evalue the M parameter (distant metastases) the mostly used tests are[47]:

Chest X-ray: to highlight the presence of metastatic lung lesions

Abdominal ultrasound: exclude the presence of metastases in the liver and for the evaluation of the genital apparatus;

Bone scintigraphy: to detect bone metastases[48];

Laboratory analisis: some are part of the normal preoperative routine, others are useful for staging. In this second category we find the tumor markers CA 15-3, CEA, CA125[49-50].

Molecular classification

The immunohistochemical analysis performed on the specimen allows an evaluation of the expression of:

Hormonal receptors: estrogen receptors (ER), and progesterone receptors (PgR).

Ki67: nuclear protein encoded by the MKI67 gene. It is used to estimate the growth fraction of the neoplasm.

HER2: receptor tyrosine kinase responsible for the control of cell growth, survival and proliferation. Its activation therefore promotes tumor growth.

According to the San Gallen Consensus Conference of 2013 breast cancers can be divided into five molecular subtypes according to the receptor status and the gene expression profile. Each subtype is characterized by a specific prognosis and response to therapy:

Luminal A (40-55%): usually associated with a better prognosis. These tumors express hormone receptors, but not HER2. These tumors express ER, PgR> 20% and Ki67 <20% [51], generally well-differentiated, with a low proliferative index and strongly responsive to hormonotherapy.

Luminal B HER2-negative (6-10%): although they express ER, these carcinomas express PgR <20% or Ki67> 20%, less differentiated; they may benefit from chemotherapy as well as hormone therapy.

Luminal B HER2-positive (15-20%): positive hormone receptors, over expressing HER2, regardless of the proliferation index, medium-high grading; they also benefit from monoclonal antibody therapy.

HER2-positive (non-luminal, 7-12%): over-express HER2, but do not express hormone receptors, generally poorly differentiated, highly proliferating and high risk of metastasis.

Triple negative (13-25%): do not express either hormone receptors or HER2. They generally have a high proliferative index (> 50%), a good response to chemotherapy.

Therapy

Breast cancer treatment is multidisciplinary. Only the cooperation between each member of the core-team is foundamental to plan the correct and personalized strategy for the patients.

Surgical Therapy

Conservative breast surgery (lumpectomy)

Lumpectomy is a surgical technique which allow to remove the cancer and some normal tissue around it, but not the breast itself guaranteeing a radical oncology and a natural respect for cosmetics. Its oncological legitimacy has been demonstrated; included in a multidisciplinary treatment, it guarantees survival rates identical to mastectomy[52].

The indications are extended to all the cases in which the relations between the diameter of the neoplastic lesion and the breast volume allows an oncologically correct resection and a good aesthetic result. It is not recommended in case of multicentricity, the impossibility to perform adjuvant radiotherapy and previous radiotherapy or a state of pregnancy in the first or second trimester.

Radiotherapy is to be considered complementary to conservative surgery and necessary for a complete loco-regional control of the disease[53].

In the last decade, conservative surgery has undergone further evolution with the oncoplastic technique. Oncoplastic surgery is a new surgical technique combining oncological radicalism with the use of plastic surgery for remodeling and rebuilding the breast[54-56].

Mastectomy

Mastectomy is the complete removal of the mammary gland, which can be performed with different techniques.

Halsted radical mastectomy involves the removal of the mammary gland with a large portion of skin including the nipple-areola complex, large and small pectoral muscles, and axillary lymph nodes of I,

II and III level[57]. Nowadays it is performed only in very rare and selected cases of massive muscular infiltration by the neoplasia.

In the radical mastectomy modified according to Patey, the gland and skin are removed with only the small pectoral muscle and the axillary lymph nodes up to the II or III level.

The Madden-modified radical mastectomy differs from the latter in saving both pectoral muscles.

Simple mastectomy involves the removal of gland and skin without an axillary dissection.

In recent years it has become increasingly widespread the so-called "conservative" mastectomy technique[58]. They guarantee surgical radicality and a better reconstructive aesthetic result.

Axillary lymph node treatment

The study of axillary lymph nodes has a therapeutic and prognostic utility. Lymph node status strongly affects the prognosis of breast cancer patients[59]. The axillary lymphadenectomy of I, II and III level is currently the standard treatment in the presence of metastatic lymph nodes.

The gold standard surgical technique for invasive breast cancer with non metastatic lymph nodes is the sentinel node technique (LNS). The sentinel node is defined as the first lymph node that receives lymph directly from the bed of the primary neoplasia[60,61]. The diameter of the sentinel lymph node metastasis influences the possible involvement of the other axillary lymph nodes: in the presence of isolated tumor cells (ITC) or micrometastasis (<0.2 cm) axillary dissection is avoided, as it has no impact on prognosis[62]. Furthermore, clinical studies are currently on going concerning the actual need for axillary lymphadenectomy in the case of macrometastases, the results of which will be available in the near future[63–65]. Different substances and techniques can be used to identify the sentinel node; the use of a radiometabolic tracer (albumin macromolecules with 99mTc) is the most widespread technique.

The removal of a single lymph node avoids acute and chronic complications compared to lymphadenectomy such as: pain, paresthesia, lymphoceles, lymphedema, functional impotence of the omolateral upper limb[66].

Radiotherapy

The association between surgery and radiotherapy is the gold standard in conservative treatment of invasive breast cancer to eliminate any residual tumor cells after surgery. Indeed, radiotherapy after conservative surgery strongly impacts the risk of local recurrence[67,68].The irradiation of the chest wall, after mastectomy, is indicated for pT3 and pT4 tumors, in the case of at least 4 positive axillary lymph nodes or extracapsular lymph node diffusion[68,69]. Radiation therapy involves the administration of 50.0-50.4 Gy with a conventional fractionation (1.8-2 Gy / day, in 5 fractions per week) on the residual breast parenchyma or on the chest wall, followed by an overdose (boost) on the operating bed (10-16 Gy) [70,71].

Radiation therapy can lead to acute toxicity (asthenia, skin erythema, epidemiolysis) or late (hardening and retraction of the breast, cutaneous dystrophy, telangiectasia, lymphedema of the arm) [72,73].

Medical Therapy

The adjuvant pharmacological approach of breast breast cancer is very complex. The therapy for each patient is personalized and must be taken after a multidisciplinary evaluation.

Hormonal therapy:

Hormonal therapy may be used for patients with hormone-responsive breast cancer (ie with ER expression ≥ 1% or PgR ≥ 1%) [74]. This tumors (Luminal A and B) are the most frequent form in all age groups, particularly in older women[75]. The side effects of these drugs include sudden hot flushes, fluid retention and mood changes; less frequently can be observed thromboembolic phenomena or an increased incidence of endometrial tumor[76].

Chemotherapy:

Chemotherapy treatment can be neoadjuvant or adjuvant. Neoadjuvant chemotherapy precedes surgery. It is generally used in the case of locally advanced carcinoma (Locally Advanced Breast Cancer, LABC). The primary objectives of neoadjuvant chemotherapy are: obtain a downstaging of

the neoplasia and control distant micrometastases through an early "sterilization" of the whole organism. The treatment aims to achieve a complete response, the only event related to improving the patient's prognosis.

Adjuvant chemotherapy, on the other hand, aims to reduce the risk of loco-regional recurrence. Generally, a drug association (polychemotherapy) is used for 6 months[74]. The most widely used drugs are anthracyclines and taxanes.

In case of metastatic disease, is generally more appropriate to proceed immediately with chemotherapy and to rely on surgical therapy only in selected cases when a full remission of the lesion is obtained for at least 6-12 months with permanence of the primitive carcinoma.

Among the toxicities related to cytotoxic chemotherapy particularly important is leuko-neutropenia and chemo-induced amenorrhea (CIA) [76,77].

Target therpy:

More than 20% of breast carcinomas express the HER2 protein[78]. Although this characteristic is generally associated with an unfavorable immunohistochemical characteristics it has a further specific therapeutical perspective represented by trastuzumab: monoclonal antibody drug able to act selectively on HER2 receptors[79-81].

Prognosis

The prognosis of breast cancer depends on the biological profile of the tumor and on the stage of the disease. The information necessary for an accurate prognostic evaluation comes from the immunohistochemical profile of the neoplasia. The presence of inflammatory carcinoma and distant metastases have a high impact on survival and consequently on prognosis[82,83].

The factors that have the greatest impact on the risk of recurrence and death are[84]:

- ***Age (<35 years):*** Under the age of 35, breast cancer has a higher risk of relapse and a reduced overall survival[85,86]

- ***Distinction between infiltrating and in situ carcinoma:*** Carcinoma in situ, by definition, has no metastatic potential. The mortality in the 20 years following the diagnosis is 3.3%. The risk of developing an infiltrating recurrence in the same breast within the next 20 years is 6.2%[87].

- ***Status of axillary lymph nodes:*** In the absence of distant metastases, it represents the most important variable for prognosis evaluation. For non metastatic lymph nodes the disease-free survival at 10 years is about 70-80%; for 1-3 positive lymph nodes the index drops to 35-40% and 10-15% in the presence of more than 10 positive lymph nodes[88].

- ***Histological subtype:*** Special histotype carcinomas, such as tubular, mucinous, medullary and papillary carcinomas have a more favorable outcome than non-special invasive carcinoma (NST) [44,89].

- ***ER:*** Estrogen receptors are expressed in 75-80% of breast cancers. Even low levels of ER expression may allow a response to hormone therapy (≥1%) [90]. However, there is a direct correlation between the level of expression and the level of response[91].

- ***PgR:*** Progesterone receptors are expressed in 60-70% of cases. Their expression is positively influenced by the expression of ER[92]. The percentage of expression affects the response to endocrinotherapy with an independent impact on overall survival[93].

- ***HER2:*** Expressed by 20-30% of tumors. HER2 overexpression represents at the same time a consolidated negative prognostic factor and a positive predictor of a response to Trastuzumab therapy[78–80].

- ***Ki67:*** Estimates the growth of the neoplasia. Although the Ki67 expression cut-off is still under discussion it represents an independent factor to determine the prognosis of breast cancer[51,94].

- ***Histological grade***: A G3 neoplasm (poorly differentiated) has a worse prognosis than a G1 neoplasm (well differentiated)[95].

- ***Lymphovascular invasion:*** Defined as the presence of tumor cells inside endothelial cells belonging to blood or lymphatic vessels of the breast. Many studies in the literature observe an association between lymphovascular invasion, increasing of local recurrence risk and reducing of overall survival [96].

- ***Multifocality / multicentricity:*** Multifocality and multicentricity are correlated with the presence of lymph node metastasis, causing a reduction in disease-free survival (DFS) and in overall survival (OS) [97–99].

- ***Margins status:*** The role of surgical resection margins is still widely discussed in the literature. An involvement of the margins by the tumor represents a risk factor for recurrence.

Homologous Recombination Deficency (HRD) in Breast Cancer

DNA repair pathways represent a tightly controlled network protecting cells from exogenous and endogenous DNA damage. These pathways are frequently aberrant in cancer cells, leading to the accumulation of DNA damage and genomic instability. BRCA1 and BRCA2 genes are the most known and are associated with hereditary breast and ovarian cancer; they play an important role in the process of DNA repair.

This highly conserved pathway is involved in double-strand DNA break repair by the process of homologous recombination (HR). The base excision repair pathway is a second highly conserved repair process involved in single-strand DNA breaks. Poly (ADP-ribose) polymerase (PARP) enzymes play an important role in this pathway.

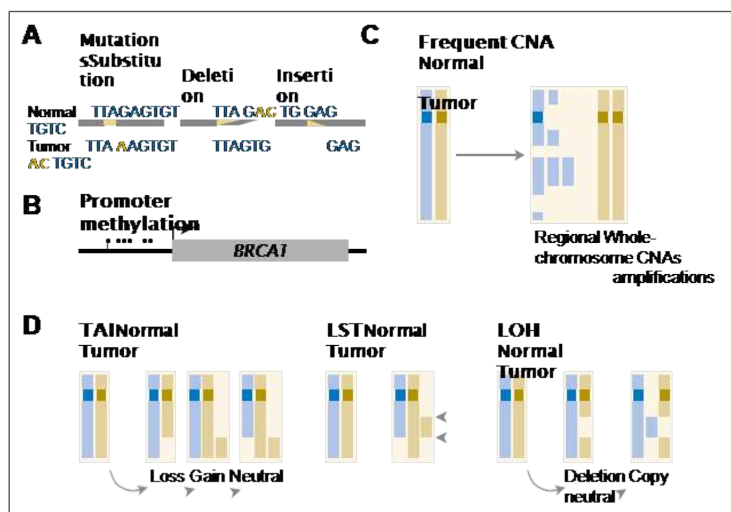
PARP is important for resolving stalled replication forks, and its inhibition during base excision repair requires BRCA-dependent HR to resolve[100,101]. Targeting DNA damage response pathways has emerged as an attractive strategy to destabilize tumor genomic integrity and trigger genomic catastrophe and cell death in a tumor-specific fashion.

The role of *BRCA1*/*BRCA2* in double-strand DNA repair via HR has been well documented[102], and there is mounting evidence that breast cancers arising in *BRCA1*/*BRCA2* germline mutation carriers respond favorably to therapies that target DNA repair pathways, such as platinum salts and PARP inhibitors (PARPis)[103-105].

Some sporadic breast cancers also harbor defects involving the HR pathway and respond similarly to platinum salts. It is also hypothesized that some sporadic breast cancers with defects in the HR pathway may benefit from the addition of PARPis to standard therapies[106]. These sporadic breast tumors are commonly referred to as being BRCA-like and are often associated with TNBC. It is estimated that up to 40%[107]of familial and sporadic breast cancers are HR deficient (HRD).

Current research has focused on the development of a companion diagnostic to identify sporadic BRCA-like tumors that would allow clinicians to identify those patients who may benefit from drugs targeting DNA repair pathways and to spare those who are unlikely to benefit.

Tests of HRD focus on either the detection of the underlying driver mutations responsible for the HR defect or the resultant mutational landscape of deficient HR inferred by nonspecific collateral damage to the genome Fig. 1.



Driver germline (inherited) or somatic (acquired) mutations may take the form of sequence or structural variants that generally result in loss of function or aberrant functioning of *BRCA1*/ *BRCA2* or other genes encoding members of the HR pathway.

TESTS OF DRIVER MUTATIONS

Sequence variants (or mutations) include substitutions, deletions, or insertions of nucleotides, 1 kb (Fig 1A). Those that occur within genes may result in pathogenic protein abnormalities. The genes and their protein products involved in HR are numerous, and their interactions are complex.

Germline Mutations in HR-Associated Genes

In addition to germline *BRCA1*/*BRCA2* mutations, clinical genetic testing panels now include a number of proposed breast cancer predisposition genes, although not all of these genes have definitively been shown to increase breast cancer risk. Other hereditary predisposition genes involved in HR that are proven to be moderate to high risk include *PALB2*, *ATM*, and *CHEK2*. More recently, *BARD1* and *RAD51D* have been shown to increase breast cancer risk, whereas some genes (*NBN*, *MRE11A*, *RAD50*, *RAD51C*, *BRIP1*) are unlikely or confirmed not to increase breast cancer risk[108].

It is not clear if breast cancers arising from these germline mutations are as sensitive to DNA-damaging therapies as *BRCA1/BRCA2*-mutated breast cancers[109].

Somatic Mutations in HR Genes

Somatic mutations may also arise in genes involved in HR. Somatic mutations in *BRCA1*/ *BRCA2* occur in approximately 2.5% of all sporadic breast cancers[110]. It is hypothesized that somatic *BRCA1/BRCA2*-mutated breast cancers will respond similarly to DNA-damaging therapies, but it is not definitively known if germline and somatic *BRCA1/BRCA2* mutations are biologically equivalent. Next-generation sequencing studies[110-113]continue to expand the list of genes involved in breast cancer, and this list includes HR genes. The extent to which these HR genes drive tumor genesis continues to be explored.

Looking beyond germline *BRCA1/BRCA2* mutations has implications in terms of choosing patients who will benefit from DNA-damaging therapies, most notably PARPis. How this can be achieved is not certain. It is possible that HRD status will be a better predictor of PARPi response in breast cancer.

Copy Number Aberrations

Copy number aberration/alteration (CNA) refers to acquired changes in copy number of genes in tissue, such as tumor, whereas copy number variant (CNV) refers to changes in copy number of genes

in the germline, affecting all cells in an individual. CNVs may also be reported in cancer, although they are usually qualified with the term acquired as compared with constitutional, so as to differentiate between the somatic and germline settings. In contrast to entire chromosome number gains or losses, CNA/CNVs are on a much smaller, generally submicroscopic, scale, with the size of DNA copy-number alterations (gain or loss) being >1 kb in length (Fig1C). Although the extent to which CNAs contribute to tumorigenesis is not entirely known, some of the well-established driver events in cancer are CNAs (eg, Myc, HER2, Cyclin D1). Furthermore, an increased burden of CNAs is associated with higher genomic instability and subsequent malignant transformation[114].

Structural Rearrangements

Inversions, translocations, and recombination change the location or orientation of a DNA sequence[115]. Translocations result in the exchange of DNA between non homologous regions of DNA. Inversions result in the change of orientation of a segment of DNA. Recombination results in exchange of DNA between homologous regions of DNA, and this structural rearrangement may lead to loss of heterozygosity (LOH), a gross chromosomal event that results in the loss of entire genes (eg, *BRCA1*, *BRCA2*). Two types of acquired LOH are important to note: deletion LOH, where there is a copy number loss; and copy number–neutral LOH, where the absolute copy number remains the same (Fig 1D). Both deletion and copy number–neutral LOH, as with CNAs, lead to allelic imbalance that can be inferred by studying single-nucleotide common variation across the genome (single-nucleotide polymorphism [SNP] analysis); this may be in the form of other types of DNA microarrays that may be fully or partially based on SNP probes across the genome.

Three tests of structural rearrangements have come to the forefront: telomeric allelic imbalance (TAI), large-scale transition (LST), and LOH.

TAI was developed using a SNP genotype array platform[116] to detect the number of chromosomal regions with allelic imbalance extending to the subtelomere, a common genomic abnormality that leads to an unequal contribution of maternal and paternal DNA sequence but does not necessarily

change overall DNA copy number. Break points of TAI regions were nonrandom and enriched for CNVs, which results in an imbalance and then leads to HRD, which may result in platinum sensitivity in the way that *BRCA1* associated cancer responds. Allelic imbalance was the best predictor of cisplatin sensitivity after identifying associations between a variety of subchromosomal abnormalities and cisplatin sensitivity.

LST measures chromosomal breaks between adjacent chromosomal regions of at least 10 Mb.

LOH measures the number of LOH regions > 15 Mb and less than a whole chromosome and was recognized as a discriminatory assay in two independent data sets of ovarian tumors[117]. A composite index of all three markers called the homologous recombination deficiency (HRD) assay was developed which has been shown to have better sensitivity than the individual scores in predicting HR deficiency[118].

While Triple Negative Breast Cancer (TNBC) is heterogeneous, a significant portion of TNBC is characterized by defective homologous recombination (HR)[109,119]. Homologous recombination is a high-fidelity DNA repair mechanism that is critical for efficient repair of double-strand DNA breaks. While this is a sentient feature of BRCA1/2 mutated breast cancer, defects in HR are common in a larger group of TNBCs as well. While Germline BRCA1 and BRCA2 mutations are present in approximately 14–20% of TNBCs, a significantly larger percentage of patients have been reported to harbor HR defects[109,120].

Clinical identification of HR deficiency at the time of diagnosis is currently being actively investigated for its potential to guide optimal therapy selection in TNBC patients.

Although the primary focus is on TNBC, the role of HRD in many other tumor types is emerging as an important target. *BRCA1* and *BRCA2* mutations are the archetype of HR-deficient tumors and have provided insight into other causes of HRD. Developing a reliable biomarker will be the key to identifying patients with HRD tumors who may benefit from HRD-targeted therapies.

In contrast to genomic tests that characterize the mutations in HR pathway genes or characterize the mutational landscapes of HRD tumors, functional measures of HR pathway deficiency provide the most direct evidence of an HR pathway defect.

Breast Cancer Genes

Family linkage studies have identified high-penetrance genes, BRCA1, BRCA2, PTEN and TP53, that are responsible for inherited syndromes. Moreover, a combination of family-based and population-based approaches indicated that genes involved in DNA repair, such as CHEK2, ATM, BRIP1 (FANCJ), PALB2 (FANCN) and RAD51C (FANCO), are associated with moderate BC risk.

Breast cancer predisposition today can be attributed to several levels of genetic susceptibility: rare high-risk alleles, conferring a risk more than five and up to 20 times as high as the risk among the general population; rare moderate-risk alleles with a relative risk greater than 1.5 and lower than 5, and common low-risk alleles conferring risks between 1.01 and 1.5 of the general population.

Whereas high-risk genes may be identified by traditional linkage analysis of genetic markers in BC families, discovering moderate and low risk BC genes requires a different approach.

Most of these variants occur with high frequency and they have a significant impact on the BC risk.

High-risk inherited syndrome genes

Hereditary breast and ovarian cancer syndrome (HBOC) is a highly penetrant autosomal dominant disorder. It is caused by an inherited germline mutation in cancer susceptibility genes, BRCA1 or BRCA2. BRCA1 was cloned in 1994 following a long search for the gene using linkage analysis and maps on chromosome 17q21[121]. This was closely followed by discovery of BRCA2 on chromosome 13q12–13 in 1995[122]. They are both classic tumour suppressor genes, which are involved in the maintenance of genomic stability by facilitating DNA repair, primarily executing DNA double-strand break repair by homologous recombination (HR). Despite of BRCA1 and BRCA2 initially appearing to be genes with similar functions, it is now clear that these two genes are different in terms of their molecular biology, protein interactions and the cancer risks they confer[123]. BRCA1 associates with multiple repair proteins and cell cycle regulators, being capable of forming multiple protein complexes

which contribute to its role in maintaining chromosome stability and tumour suppression[124]. BRCA1 is a substrate of the central DNA damage response kinases ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related protein) that control the DNA damage response. BRCA1 is required for homology directed repair, a pathway that facilitates error free repair of double-strand breaks (DSBs) and resolution of stalled DNA replication forks through HR, as well as post-replicative repair in response to UV damage. BRCA1 also regulates the transcription of several genes in cancer including ATM[125], and homeostasis of itself so that levels remain capable of maintaining genome integrity in response to genotoxic insult[126]. BRCA2 primary function is in HR and it is based upon its ability to bind to the strand invasion recombinase RAD51. BRCA2 contains eight BRCT repeats, each of which can bind and recruit RAD51 to sites of DNA damage. BRCA2 also interacts with PALB2, through which it localizes to DSBs together with BRCA1[127].

BRCA1 and BRCA2 are involved in maintaining genome integrity, at least in part, by engaging in DNA repair, cell cycle checkpoint control and even the regulation of key mitotic or cell division steps. Thus, the complete loss of function of either protein leads to a dramatic increase in genomic instability.

The estimated BRCA1 and BRCA2 mutation carrier frequencies in the general population are between 1 in 300 and 1 in 800[128].

TP53 was first identified in 1979 and it is now the most common altered gene in solid tumours. P53 is essential in cell-cycle control, resulting in either a delay in cell-cycle progression or apoptosis. Inherited germline mutations are rare. However, they are known to result in Li-Fraumeni syndrome (LFS). LFS causes childhood tumours: soft tissue, osteosarcomas, gliomas, adrenocortical carcinoma, and very early onset BC. Over 70% of classical LFS families inherited TP53 mutations. LFS only accounts for less than 0.1% of BC, but mutations in TP53 confer an 18- to 60-fold increased risk of BC under the age of 45 years old compared to the general population[129].

Moderate breast cancer risk alleles

CHEK2

Germline CHEK2 (checkpoint kinase 2) sequence variants have been reported in families with LFS that do not carry TP53 mutations[130]. CHEK2 encodes a cell cycle checkpoint kinase implicated in DNA repair. CHEK2 emerged from the sequencing of plausible candidate genes in families with multiple cases of BC families without BRCA1/2 mutations[131]. Its association with increased BC risk has been explored in many studies since 2002, and nowadays, its significance has been demonstrated with a high statistical degree.

A particular germline mutation, CHEK2 1100delC, has been associated with a two-fold to three-fold increase in BC risk. Many large studies have reported this finding. In 2008, a meta-analysis of studies assessing CHEK2 risk in populations of northern and eastern European descendent, calculated odds ratios for BC in unselected populations, early-onset BC, and familial BC. For early-onset BC, the study estimated an OR of 2.6 (95% CI 1.3–5.5) and also found that for patients selected from a population with familial BC, CHEK2 1100delC heterozygotes had a much higher OR 4.8 (95% CI 3.3–7.2). CHEK2 mutation carriers either with a strong family history of BC or a history of bilateral disease was found to be at comparable risk to BRCA carriers with an estimated lifetime risk of 37% and 59% respectively.

These results suggest that CHEK2 1100delC screening should be considered in patients with a strong family history of BC.

ATM

Biallelic mutations in Ataxia telangiectasia mutated (ATM) gene cause the autosomal recessive disease Ataxia-telangiectasia (AT). This is a neurodegenerative disorder that is characterized by cerebellar ataxia, telangiectases, immunodeficiency, hypersensitivity to ionizing radiation and approximately 100 times increased risk of cancer[132]. ATM is a protein kinase involved in the response

to DSBs in a pathway that includes TP53, BRCA1 and CHEK2. DSBs activate ATM, which in turns, activates the full DNA damage response[133]. Heterozygous ATM mutation carriers, found in approximately 0.5–1.4% of the general population, do not display the symptoms observed in patients. However, several studies have shown an increased risk of cancer in them: tumours of breast, pancreas, stomach, bladder, ovary, and chronic lymphocytic leukaemia[134].

Extensive research has been carried out into the association of ATM mutations and BC, showing that up to 13% of BC may be due to heterozygous ATM mutations[135]. The relative risk of BC in heterozygous ATM female carriers has been estimated in 2.37% (95% CI, 1.51–3.78) of the general population[136]. Moreover, it has been described that ATM mutations are more frequent in BC patients selected on the basis of a family history of BC than from those compared to unselected patients[137].

MRN (MRE11–RAD50–NBS1) COMPLEX

The MRN complex is composed of three proteins, MRE11, RAD50 and NBS1. It binds to damaged DNA, undergoes a series of conformational changes to activate and increase ATM affinity for its substrates, and retains active ATM at sites of DSBs. MRN complex plays a key role in DNA damage detection and activation of the ATM kinase[138].

Mutations in all three members of the MRN complex have been noted in human cancers. Mutations of MRE11, NBS1 and RAD50 manifest as ataxia telangiectasia-like disorder (ATL), Nijmegen breakage syndrome (NBS) and NBS-like disorder, respectively. Unsurprisingly, carriers of MRE11, NBS1 and RAD50 mutations have been implicated in BC.

Screening for mutations in all the three MRN genes in Finnish population discovered a founder mutation in RAD50 associated with a 4.3-fold increase in BC predisposition (OR 4.3, 95% CI 1.5–12.5)[139]. However, this mutation has not yet been found in any other populations, including other Nordic states, making difficult the confirmation of this association.

FANC FAMILY

Fanconi anaemia (FA) is a genetic disease characterized by progressive aplastic anemia, multiple congenital defects, and susceptibility to both hematologic and solid malignancies. A defect in any of the proteins along the FA pathway prevents cells from repairing interstrand crosslinks and predisposes them to chromosomal breakage and cell death. The relationship between FA and BC susceptibility did not become fully apparent until it was discovered that BRCA2 and FANCD1 were two different names for the same gene. Constitutional inactivating mutations in genes responsible for FA have been clearly associated with an increased susceptibility to both BC and OC[140] and include the genes BRCA2 (FANCD1), FANCN (PALB2) and FANCJ (BRIP1). One third of patients homozygous for a FA gene mutation will develop cancer by the age of 40 years old. These included squamous cancer of the head and neck, meduloblastomas, oesophageal and skin cancers, gynecological cancers, as well as liver and kidney tumours[141]. Strong associations exist between heterozygous mutations of BRCA2/FANCD1 and breast and/or ovarian cancer development, as described in BRCA1/BRCA2 families. However, heterozygous mutations in other FA genes have also been shown to be associated with an increased risk of BC. Evidence that other FA pathway related proteins were also BC susceptibility genes did not unfold until BRIP1 was identified in FANC-J patients[142]. FANCJ, also known as BACH1 or BRIP1, is a BRCA1-associated DEAH helicase involved in translesion synthesis helping the polymerase bypass the interstrand crosslink, placing its role distal to the monoubiquitinated FANCD2 of the FA pathway. BRCA1–FANCJ interaction is essential for promoting error-free repair, checkpoint control and for limiting DNA damage tolerance[143]. The most common germline FANCJ/BRIP1 mutant allele is found both in BC and FA patients. In 2006, truncating mutations in BRIP1 were identified in BC families. Segregation analysis assessed a Relative Risk (RR) of BC of 2.0 (95% CI 1.2–3.2), that increased to 3.5 (95% CI 1.9–5.7) for carriers younger than 50 years old[144]. The discovery of another BC predisposition gene in the FA pathway PALB2 or FANCN[145], suggests that proteins acting downstream of monoubiquitinated FANCD2 and FANCI increase the risk of BC, while those acting upstream do not[146]. The role of PALB2 in homologous repair is to behave as a protein that functionally bridges BRCA1 and BRCA2 and also cooperates with RAD51 to stimulate recombination[147]. Biallelic PALB2 mutations have been described as responsible for FA subtype FANCN. Research on BC

families without BRCA1/2 mutations resulted in identifying PALB2 mutations. However the PALB2 mutations rarity makes accurate estimation of its penetrance more difficult. A familial-based case-control association study of PALB2 showed that a monoallelic mutation confers an OR of 2.3 (95% CI 1.4–3.9) for BC[148]. As with BRCA2 heterozygotes, PALB2 mutations have been associated with an increased risk of pancreatic cancer[149].

RAD51 FAMILY

The RAD51 family consists of several proteins, which preferentially bind to single-stranded DNA, and form complexes with each other. RAD51 unwinds duplex DNA and forms helical nucleoprotein filaments at the site of a DNA break[150]. BRCA2 stimulates RAD51-dependent strand exchange[151]. RAD51C was discovered to be the cause of a Fanconi-like disorder[152] and is a new cancer susceptibility gene[153]. Six clearly pathogenic mutations were found among 1,100 breast/ovarian cancer families. The mutations were found exclusively within 480 pedigrees with the occurrence of both breast and ovarian tumors and not in 620 pedigrees with breast cancer only or in 2,912 healthy controls. This is a distinctive behaviour from the situation observed with monoallelic mutations in the FA-related genes PALB2 and BRIP1, which are rarely present in OC. What is even more striking is that apparently there was complete segregation of the mutations in affected individuals, suggesting a penetrance level similar to BRCA1/2. Recently, a mutational screening of the RAD51C gene in a large series of 785 Spanish breast and/or ovarian cancer families identified 1.3% of mutations, and suggested the inclusion of the genetic testing of RAD51C into the clinical setting[154]. Investigators have recently sequenced RAD51D in 911 wild-type BRCA1/2 breast-ovarian cancer families as well as 1,060 controls[155]. Inactivating mutations were identified in 8 out of 911 breast and ovarian cancer families (0.9%), 0 in 737 BC families, and 1 in 1060 controls (0.09%). There was a higher prevalence of mutations in families with more cases of OC. The RR of OC for carriers of deleterious RAD51D mutations was estimated at 6.3 (95% CI 2.86–13.85) whereas RR of BC was non-significantly increased. New data support the previous observation that loss-of-function mutations in RAD51D predispose to OC but do not to BC. The XRCC2 and XRCC3, members of RAD51 family, maintain

chromosomal stability during HR[156]. A homozygous frameshift mutation in XRCC2 being associated with a previously unrecognized form of FA was recently reported[157]. XRCC2 binds directly to the C-terminal portion of the product of the BC susceptibility pathway gene RAD51, which is central to HR. XRCC2 also complexes in vivo with RAD51B (RAD51L1), the product of the breast and ovarian cancer susceptibility gene RAD51C9 and with the product of the OC risk gene RAD51D, and localizes together on sites of DNA damage[158]. An exome-sequencing study of families with multiple BC individuals identified two families with XRCC2 mutations. One of them with a protein-truncating mutation and the other one with a probably deleterious missense mutation[159]. From other XRCC genes investigated, XRCC1 399Gln allele acts as a recessive allele in association with BC risk[160].

Breast cancer low-risk alleles

Part of the unexplained fraction of familial relative risk is likely to be explained by a polygenic model involving a combination of many individual variants with weak associations to risk, the so called low-penetrance polymorphisms. The frequency of these alleles may range from 5% to 50% and could possibly be higher in families with BC. Individually they only have a small effect on BC risk (relative risk ≥1.01 and <1.5). Nevertheless, they may collectively account for a large component of BC heritability.

Other identified loci associated with BC in large studies involving thousands of subjects are, MAP3K1 (mitogen-activated kinase 1), LSP1 (lymphocytespecific protein), and TNRC9 (trinucleotide repeatcontaining 9), along with a 110 kb region of chromosome 8q24[161]. Associations with other chromosomal regions, 2q35, 5p12, 6q22, and 16q12, also have been reported[162]. Further analysis showed that allelic variation at FGFR2, TNRC9, 8q24, 2q35, and 5p12 are associated with physiological characteristics of breast tumours, such as ER status[163]. Moreover, it has been shown that specific FGFR2, MAP3K1, and TNRC9 variants may interact with BRCA1 and BRCA2 mutations to increase BC risk[164].

Circulating cell-free DNA (cfDNA)

The first description of the presence of DNA freely circulating into the bloodstream dates back to 1948, when Mandel and Métais identified extracellular nucleic acids in blood of both healthy and diseased people[165].

History

During the 60s several studies conduced in patients affected by Lupus Erythematosus (LE) discovered important features of DNA in blood. Ceppellini and colleagues identified a DNA-reacting factor in serum obtained from a patient affected by LE Diffusus. Starting from the hypothesis that DNA can elicit specific antibodies (Ab), they purified DNA from different samples of both human and animal origin and verified if they observed a reaction after incubation with serum. The choice of a LED affected patient was due to two main reasons: a) nuclear material released by nucleolysis, that is typical of this disease, can get in contact with immunological components; b) subjects affected by LE produce a huge amount of auto- and iso-Ab, thus increasing the probability to have anti-DNA Ab[166]. They effectively observed a factor able to interact with DNA from different sources and species that behave like an antibody, but the fact that other sera obtained from eight other patients did not showed reactivity drive the explanation of the phenomenon toward a patient-specific autoimmune hyperreactivity[166].

Following experiments demonstrated not only an association between the presence of anti-DNA Abs in the serum and the acute phase of SLE, but also the interaction between these Abs and autologous DNA in addition to eterologous ones, suggesting an active role of the DNA-anti-DNA complex in disease progression, in particular in driving renal disease and vasculitis[167].

Two years after, Tan and colleagues started from these discoveries to set up a technique to detect soluble tissue antigens, among which we find DNA, in SLE patient’s blood and that can react with Abs to produce renal injury. They demonstrated the possibility to use sera obtained from SLE patient to detect the presence of DNA in blood. Starting from calf thymus DNA, they performed experiments

on DNA-anti-DNA recognition in three different settings: native DNA (i.e. dsDNA), sonicated DNA (i.e. small dsDNA fragments) and heat denatured DNA (i.e. ssDNA). They did not observed differences in reaction between native and sonicated DNA, suggesting an identical antigenicity of the two DNAs. The difference was observed in ssDNA and, more interestingly, serum that had reacted with dsDNAs was able to react also with ssDNA, suggesting the presence of Abs recognising the two forms independently[168]. The complete abolishment of the reaction after DNase treatment of the sera pointed definitely toward DNA as the antigen in sera[168].

Even if the presence of DNA in plasma of patients affected by cancer was demonstrated by Tan and colleagues in 1966, it took around a decade to specifically study how circulating DNA behave in cancer-affected patients. Leon and colleagues developed a radioimmunoassay to quantify ng quantities of DNA, based on [I^{125}] iododeoxyuridine-labeled DNA working as antigen and LE patient's serum as source of Abs. They evaluated the amount of cfDNA in serum of 173 cancer patients and 55 healthy people with this assay, identifying a median concentration of DNA of 13 ± 3 ng/ml plasma and 180 ± 38 ng/ml plasma in healthy and cancer patients, respectively. They also detected higher amount of DNA in patients with metastatic cancer respect to nonmetastatic ones[169].

The application of the assay after radiation therapy of the patients revealed a decrease amount of cfDNA in those patients in which clinical conditions improved, i.e. decrease of tumour size or reduction of pain, whereas a lack of response to the therapy was associated to increase or no changes in DNA levels[169].

One of the critics that can be moved to these results concerns the 50% of patients affected by cancer that showed DNA levels comparable to healthy people. This scenario can be explained by the fact that the only inclusion criteria of this research was to be selected for radiation therapy without taking into account previous surgery or chemotherapy that could have affected the amount of free DNA[169].

These data taken together suggested the hypothesis that also tumours are capable to release circulating DNA, as previously observed by this group in cultured human normal lymphocytes[170] and confirmed by the analysis of DNA released in the medium by leukemic cells obtained from a patient, even if

conditions that drive this phenomenon were not known. Researchers finally hypothesized that cancerous DNA can play a transfectant role and contribute to the diffusion of the tumour[171].

All these information were reinforced in the first half of the 90s, when researchers started analysing cfDNA at molecular level. In particular Vasioukhin and colleagues looked at K-RAS mutation in circulating, tumour and blood-isolated cell DNA of patients affected by colorectal adenocarcinoma[172] and N-RAS mutation in DNA obtained from plasma, circulating cells and bone marrow of patients with myeloid leukemia or myelodisplastic syndrome[173]. Interestingly, N-RAS mutations identified in the plasma DNA were not always detected analysing the other sources of DNA. This observation had strong medical implication: absence of the mutation in blood cells can be linked to the apparent remission state of patients or to the fact that cancer cells can remain into bone marrow; no identification of mutations into bone marrow DNA can be explained by the fact that needle biopsy collect just a small fraction of bone marrow, thus it is likely that not all the malignant clones are included into the analysis.

This last observation can be considered a precursor of the ‘liquid biopsy’ concept: the possibility to identify a mutation in plasma DNA, avoiding biopsy invasiveness and its associated risks and reducing the probability to miss the malignant clone, can furnish fundamental clinical information, considering that presence of RAS mutations in myeloid disorders is a poor prognostic factor at diagnosis[174] and reflects efficacy of chemotherapy in achieving clinical remission[175].

Nearly in the same years, another clinical setting become involved in cfDNA study. In 1997 Lo and colleagues firstly described the presence of fetal DNA in both plasma and serum of pregnant women, by amplifying Y sequences in male fetus bearing women[176]. This discovery paved the way toward non-invasive prenatal diagnosis of fetal trisomies and genetic anomalies, such as Down syndrome[177], but also important biological characteristics such as sex[178] and Rhesus factor or blood group[179]. Even more interestingly, the possibility to analyse extracellular DNA released by embryonic cells in culture can prevent the aspiration of one or two cells from embryos after in vitro fertilization (IVF) with the conceivable complications and risks associated to the procedure, thus maintaining the levels of information obtained by sequence and structure analysis[180,181].

Biological features of cfDNA

Fragment size

We have already described the work done by Tan and colleagues in 1966, that demonstrated the presence of DNA-anti-DNA complexes in blood of SLE patients. The isolation of the DNA-anti-DNA complex allowed a better characterization of circulating DNA. It was demonstrated that DNA in these complexes is double stranded and fragments were of 30-40 bp in length[182].

The first evidence of tumour-released cfDNA and its characterization came up in 1987 with Stroun and Anker work. They analysed circulating DNA from patients with different malignancies. They firstly confirmed that DNA in cancer patient is mainly double stranded and observed a heterogeneous fragment composition, with size of the fragments ranging between 21 kb to less than 500 bp, thus smaller than genomic DNA[183].

The application of an in vitro DNA synthesis test, consisting in a DNA synthesis reaction performed in presence of carcinogens, allowed to determine if DNA was originated from cancer, if the reaction takes place, or normal cells, i.e. no synthesis. Results of this test confirmed the presence of cancer-derived circulating DNA in 5 out of 7 samples analysed, while the other 2 presented only non-tumoral DNA. The hyperchromicity test performed on these samples revealed an increase in absorbance in all the samples in which DNA synthesis occurred, suggesting that amplified DNA was characterized by double strand instability, a typical characteristic of tumoral DNA[184].

Fragmentation profiles revealed the coexistence of at least three sets of fragments, characterized by different length:

- 180-200 bp fragments and their multiples
- more than 10000 bp fragments
- fragments smaller than 150 bp

Signature of the release mechanism

The description of cfDNA fragment composition can furnish several information on processes generating these fragments.

The analysis of cfDNA by PAGE in cancer patients performed by Jahr and colleagues revealed two main sets of fragments well distinguishable on the base of their size. Despite a strong variation between samples, they observed two main sets of fragments well distinguishable on the base of their size. They observed a mono and oligonucleosomal DNA ladder, i.e. DNA of ~180 bp and multiple thereof in lengths, and a high molecular weight fraction of ~ 10000 bp.

Model studies on both cell lines and animals confirmed apoptosis and necrosis as the two mechanisms generating the oligonucleosomal ladder and the high molecular weight fraction, respectively[185].

A third set of fragments, that is probably the most clinically relevant among cfDNA, was discovered nearly a decade after these findings.

Mouliere and colleagues, in fact, observed a great variation in cfDNA quantification on the base of the length of the amplicon amplified by PCR. In particular, they determined that nearly 80% of tumoral derived cfDNA was missed when 150-300 bp amplicons are used for the analysis and suggested amplicons of <100 bp as the best (Fig. 2).

The fact that most of the cfDNA fragments originated by tumour are smaller than 145 bp, i.e. are characterized by a reduced DNA integrity, can be explained with an increased apoptotic rate. This data strongly correlates with the high proliferation and apoptosis rates observed in tumours and supports apoptosis as an important source of tumoral circulating DNA[186,187].

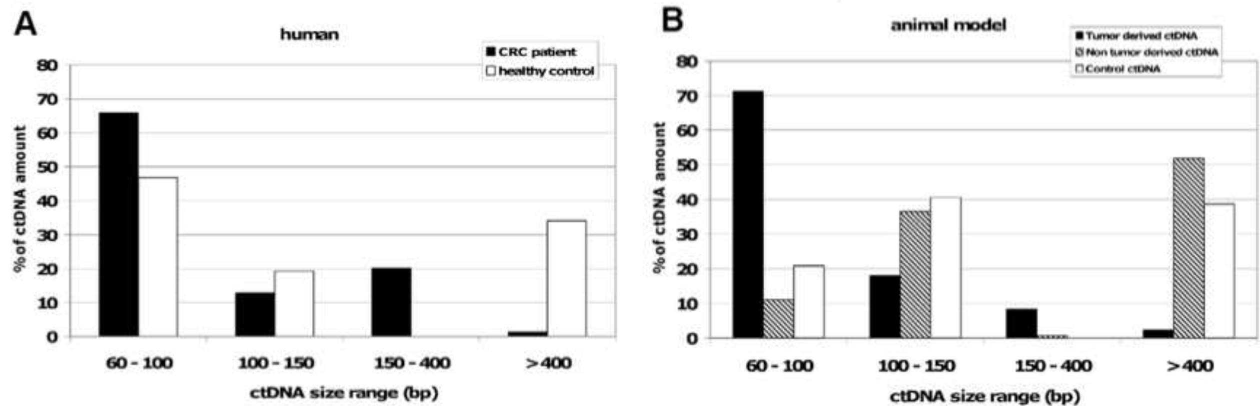


Fig.2 : cfDNA fragment size distribution of mutant and wild type cfDNA. A) plasma of CRC affected patients B) xenografted animal model. From Mouliere et al, 2011.

The identification of tumour causative mutations[172-173] or the lower length of fragments[186,187] clearly indicates that tumours produce and release DNA fragments into the bloodstream.

The identification of cfDNA in healthy people raised the question on which tissues contributes to this population. The lacking of a genetic biomarker, as mutations, that clearly indicates the origin of the fragment has been a limitation in this field and in those pathological conditions associated with an increased cfDNA, such as myocardial infarction[188], stroke[189] or autoimmune disorders[190], that do not present a genetic discriminant between healthy and pathologic cellular status. In this scenario, Snyder and his colleagues pointed out nucleosomal occupancy as a powerful marker of contributing tissues. Starting from the established association between cfDNA fragments and nucleosomal DNA, they hypothesized that cfDNA sequences must reflect at least in part the epigenetic landscape of the cell, and thus tissue, of origin. By deep sequencing of cfDNA fragments and maps of nucleosomal occupancy, they were able to indicate lymphoid and myeloid cell lines as the source of physiological cfDNA, confirming previous works[191], and revealing the contribution of different tissues in samples of different cancer patients[192].

An alternative “genotype-independent” method to analyse cfDNA focused on methylation. It is widely established that cancer harbours specific DNA methylation patterns and epigenomic profiling can furnish important clinical issues in diagnosis, prognosis and disease monitoring and can be considered a marker of tumour DNA[193].

Cancer-associated methylation modifications, as nucleosomal occupancy described earlier, are more widely applicable approaches compared to tumour-specific mutations that require knowledge of the patient specific mutational profile or the application of libraries of assays covering common mutation.

Lehmann-Werman and colleagues analysed patients affected by various diseases to identify, through cfDNA methylation analysis, cell death in different tissues. They firstly identified tissue-specific DNA methylation patterns and then looked at those markers into cfDNA. They were this way able to identify pancreatic β cells as the source of cfDNA in insulin-dependent diabetic samples, oligodendrocytes in patients affected by multiple sclerosis, neuronal/glial origin of cfDNA after both cerebrovascular accident or heat attack and exocrine pancreatic cell in subjects with pancreatitis or pancreatic cancer[194].

cfDNA bioavailability

A critical step in cfDNA comprehension concern mechanisms by which it is released and circulates into the bloodstream and how it interacts with other cells (Fig. 3).

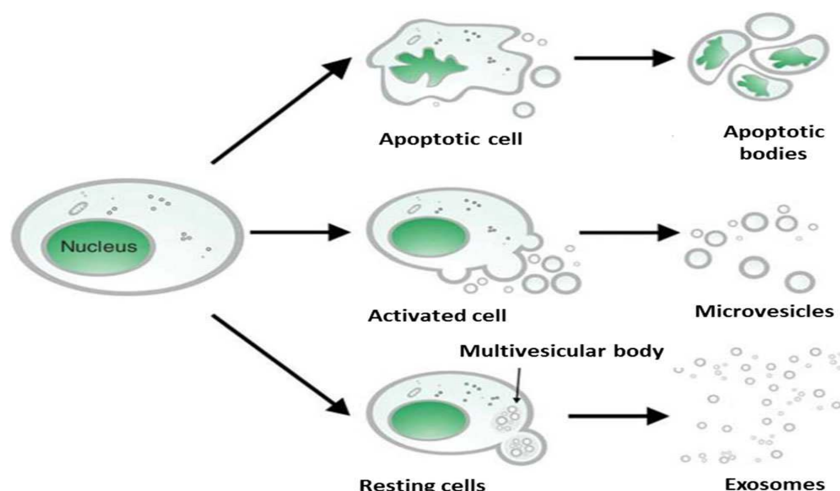


Fig. 3: potential vesicles that transport cfDNA. From Thierry *et al.* (2016).

Apoptotic bodies

Apoptotic bodies have been addressed as a source of cfDNA, even if nowadays there are still open questions on this hypothesis. These vesicles measure 1-5 μm, are produced in the late stage of apoptosis by membrane blebbing and contain part of the cytoplasm and DNA degraded by caspase-activated DNase during apoptosis[195, 196].

Surely the characteristic ladder pattern observed in cfDNA[185] indicates apoptosis as a source of cfDNA and, as a result, apoptotic bodies as the way in which it is released. Several studies associated the amount of cfDNA to apoptosis of neoplastic cells due to fragment composition and comparison with other markers of apoptosis.

On the other hand, apoptotic bodies should be cleared *in situ* by epithelial cells and macrophages, thus contribution to circulating DNA should be limited[195].

Microvesicles

Microvesicles are membrane-surrounded particles containing an aqueous compartment.

It has been observed that cancer patients produce an abnormal amount of microvesicles that are released into the bloodstream[186, 197]. These structures are strictly associated with cfDNA and are a way of genetic information transfer and cell-to-cell communication[198, 199].

Depending on the process generating them, microvesicles are divided into exosomes: 30-100 nm in diameter, these structures are secreted by most cells and can transfer material laterally, i.e. horizontally between different cells. They contain proteins, particular lipids, messenger RNA (mRNA), micro RNA (miRNA) and variable amount of DNA[200]. Interestingly, it has been demonstrated that this DNA component is characterized by two sets of fragments: a membrane-bound, large size dsDNA and a small fragment DNA inside the exosome[200]. More recently, Rohan-Fernando and colleagues observed that a large proportion of plasma cfDNA is localized into exosomes[201].

Macromolecular complexes and the Virtosome

The possibility that DNA circulates in macromolecular complexes was confirmed in several SLE studies, describing the association between DNA and Abs. A new macromolecular complex was discovered in 2010 by the Stroun group, who firstly described a DNA/RNA-lipoprotein complex that is released in a regulated fashion[202]. This discovery confirmed studies on active release of DNA in the medium of cultured lymphocytes[203,170], frog heart auricles[204], rat spleen[205] or leukemia cell line HL-60[206] and the hypothesis of an active DNA release mechanism[195].

Further studies on this complex revealed that all the components are newly synthesized only by living cells[170,195,205], the complex is released in a energy-dependent process[205] and in a controlled manner[170,195]. Analysis in chick embryo fibroblasts demonstrated that this complex could be uptaken by cells[207].

Extracellular DNA Traps

It is well known that both neutrophils and eosinophil are involved in tumour-associated inflammatory infiltrate, and both are able to generate extracellular DNA traps, i.e. DNA associated with other cellular structures.

Neutrophils in tumour microenvironment can acts as both pro- and anti-tumorigenic, depending on their activation status. In particular, NETosis is a cell death program following neutrophil activation consisting in chromatin decondensation, lysis of membranes and neutrophil extracellular DNA traps (NETs) release[208]. This complex of DNA fibers and anti-microbial granules is involved in pathogen trapping and killing[209]. There is also a so-called “vital NETosis”, a process in which NETs are released without cell lysis[210].

Eosinophil extracellular DNA traps (EETs) are released under eosinophil activation and are composed of only mitochondrial DNA, actively secreted by a catapult-like manner[211]. An important feature of EETs is that eosinophils remain viable during this process and no “EETosis” is evidenced[211,212].

So far NET release has been described in several pathological conditions, such as infections, autoimmune pathologies, trombotic illnesses and inflammatory response[213,214], metastases promotion and progression[215,216] or after exercise[217], that are characterized by high cfDNA levels, suggesting a contribution of this structure to circulating DNA population.

Serum Proteins

Since the 60s it is well known that both single and double strand DNA can interact with Ab, forming the so-called DNA-anti-DNA complex, that is involved in SLE progression[167,168].

It has been described that DNA can link with proteins, considering its electrostatic nature, in particular with albumin, fibronectin and the C1q complement component[218].

Thus, pathological conditions altering serum protein availability can affect the amount of cfDNA in blood[181].

Cell Surface-Bound cfDNA

Both DNA and RNA can be found on leukocytes and erythrocytes membranes[218]. *In vitro* studies revealed 20 kbp DNA fragments on cell surfaces[219], either naked or associated with macromolecules.

cfDNA half life and clearance

Starting from the 60s, several works were focused on deciphering free circulating DNA half life and kinetics. Tsumita and Iwanaga firstly studied externally introduced DNA fate by detecting radioactivity at different times after tritiated calf thymus DNA injection into mice. They observed two phases of radioactivity decrease: the first was quite rapid, 30 mins, and corresponded to >99% reduction in radioactivity; the second presented a slow decrease in radioactivity loss. Analysis of organs revealed that kidneys represented the main way of excretion, even if a fraction of radioactivity was trapped in liver tissues even 3h after injection[220]. Chused et al implemented previous data by

analysing clearance of dsDNA and ssDNA in different mice strains. They observed that ≥50% of injected nucleic acids, whether ss or ds, was cleared in 1 min, rising to 90% in 20 mins, with denaturated DNA removed slighty faster than dsDNA. Incubation with endogenous nucleases revealed that most of the DNA is uptaken as macromolecule or complexed with antibodies, more than digested, and analysis of organs suggested a pivotal role of liver and spleen, via reticulo-endothelial uptake, in DNA clearance[221].

Dorsch and colleagues compared clearance rate of ssDNA in rabbits non-immunized and immunized with ssDNA, obtained by heat denaturation.

They injected both rabbits with I^{125}-calf thymus ssDNA and they observed an inversely correlated relation between clearance rate and relative amount of anti-DNA antibodies in immunized rabbit, i.e. the presence of anti-ssDNA Abs delayed ssDNA clearance and persistence of DNA-anti-DNA complexes can affect pathogenesis of immune complexes disease.

These observations seemed in contrast with the high levels of circulating DNA observed in SLE patients. Emlen and Mannik thus hypothesized that in some conditions either DNA clearance is altered, or SLE DNA is somehow different from experimental DNA or larger amounts of DNA are released.

To better understand this phenomenon, they administered several doses of I^{125}-ssDNA, ranging from 2 μg to 500 μg, and collected blood at different times (30 s - 8h). As expected, ssDNA at doses comparable to previous experiments was cleared in an analogous time and the liver was the main organ involved, suggesting phagocytosis by Kupffer cells as the main physiological process at the base of clearance. As phagocytosis is a process subjected to saturation, an amount of ssDNA higher than the threshold can explain why DNA can accumulate into the bloodstream. The kinetics observed for high dose ssDNAs effectively were saturable for both blood clearance and liver uptake processes. Kidneys showed an uptake independent on DNA dose administered, in the amount of 2-5% of total removal, and spleen started uptaking DNA once liver got saturated but played a minor role in the process. Data obtained supported a circulating endonuclease that rapidly cleaves large molecular weight ssDNA into smaller fragments that, as well as big ssDNA fragments, are bound to the liver

where a membrane exonuclease release nucleotides into bloodstream. The latter step is the one that gave the saturable feature at the system, supporting the fact that SLE patients show high levels of circulating DNA[222].

Pancer and colleagues observed that splenic lymphoid cells maintained in culture spontaneously released into the media a unique species of DNA of 145-185 bp in length, predominantly double stranded and able to induce anti-DNA Ab, against both ssDNA and dsDNA, if injected into mice[223].

In 1984 Emlen and Mannik went deeper in understanding DNA behaviour studying clearance and organ uptake of both ssDNA and dsDNA of different sizes in normal mice. Clearance presented two exponential components: organ uptake, that was more rapid for ssDNA than dsDNA, and excretion of waste products, that did not showed differences between different preparations. They concluded that ssDNA is cleared in around 20 mins mainly by the liver, while dsDNA remains in the circulation longer, till 40 mins, and is mainly metabolized by circulating nucleases. This latter result can also explain why SLE patients, who are characterized by lower levels of nucleases into the bloodstream, can accumulate circulating dsDNA that forms DNA-anti-DNA complexes[224].

A direct analysis of cfDNA was performed by Lo and colleagues in 1999. They looked at fetal circulating DNA clearance in 8 pregnant women about to give birth and observed a two phases process that pointed out a median 16.3 mins half-life for cfDNA[225]. Several years after, a massively parallel sequencing approach indicated a 1h half life for the first phase and a 13 h half life time for the second phase[226].

The analysis of post-surgery cfDNA clearance in a patient affected by CRC revealed a half life of 114 mins[227].

Discrepancies observed in half-life determination can be explained accounting for different clinical and experimental settings, i.e. externally injected DNA vs effective cfDNA, model organisms vs patients, different pathological conditions, and a still not clear characterization of release rate, but determination of fate and turnover of cfDNA is necessary to comprehensively understand the biological role of cfDNA and to develop tools for biomedical and clinical applications[181].

cfDNA functions

Several functions have been attributed to cfDNA, on the base of its structure and way of circulation.

Intracellular messenger

We have already described Stroun, Anker and Gahan works that described virtosome[202]. Studies on intracellular messengers date back to the 60s and involved the concept of non-sexual transmission of characters observed mainly in plant species and graft experiments. Experiments on plant grafts revealed that characteristics of the graft are transmitted to the progeny, originated by the stock[228]. An analogous situation was observed in feather colour modification in white chickens after blood transfusion from Guinea hens[228].

The discovery of virtosome and its active and regulated secretion in the medium of cultured cells suggested virtosome could play an intracellular messenger role.

Actually, the virtosome ability to penetrate into cells was demonstrated much earlier than its description. Several works described the intercommunication between different types of cells already in the 80s. it was already known that lymphocytes in culture release actively and spontaneously newly synthesized DNA into the culture medium[170]. Experiments on T and B cells in culture revealed that T cell released DNA after HSV exposure was able, once added to B cell culture medium, to induce anti-HSV antibody synthesis by unexposed B cells. More interestingly, those Ab presented peculiar anti-allotypes of T cells[229]. Analogous experiments were performed on mice that were injected with DNA released by T cell in the medium after HSV or polio virus exposure. Parallel to the previous experiment, mice started producing human-like anti-polio and anti-HSV Abs[230].

Genometastases

Strictly associated to the ability to act as an intracellular messenger is the concept of genometastases, i.e. the capacity of tumoral cfDNA to transfect healthy cells far from the primary tumour and this way

direct the formation of metastases. The possible involvement of cfDNA in oncogenesis was hypothesized back in 1965, when it was observed that the injection of tumour DNA in mouse bloodstream results in tumour development[231]. In 1994, Anker and colleagues observed that NIH/3T3 cells grown into medium of cultured SW480 cell, that carry a K-RAS mutation, acquired the mutation identified in SW480 and became tumorous[173]. An analogous situation was observed with the addition of plasma obtained from colorectal cancer patient carrying a mutated *KRAS*[232]. Furthermore, NIH/3T3 cells treated this way were able to induce tumour formation once injected in nude mice[232] (Fig. 4).

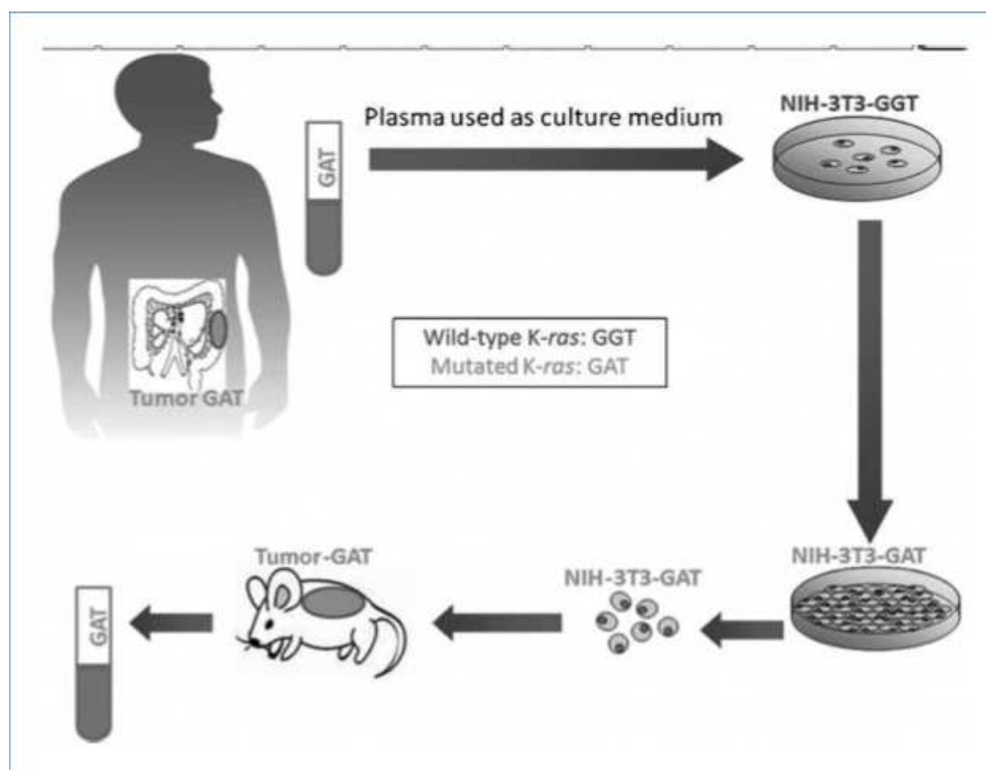


Fig. 4: schematic representation of genometastases proving experiment. Plasma from colon cancer patients, containing DNA carryng the mutated K-RAS (GAT trinucleotide), added to the medium of NIH/3T3 (wild type K-ras) resulted in the acquisition of the mutant K-RAS from the cells. Mice injected with the transformed cells develop K-RAS mutated tumors (from Garcia Olmo et al, 2010).

Mechanisms sustaining this phenomenon can be multiple and involve several cfDNA carriers. All the cfDNA-containing vesicles, i.e. apoptotic bodies, microvesicles and exosomes, can be responsible for

genetic material transfer[233]. Nucleosomes are equally able to cross plasma membrane, thus penetrating into the cell[234]. Macromolecular complexes involving proteins into the bloodstream also favours DNA internalization. The virtosome, finally, has a clear ability to penetrate into cells and alter their genetic landscape, as shown by experiments on NIH/3T3 cells described earlier.

A series of experiments shed light on events that follow cfDNA uptake. Mittra and colleagues discovered that cfDNA, once uptaken, is integrated by host cells into their genome[235] and that this event induces apoptosis and DNA damage responses[235] (Fig. 5).

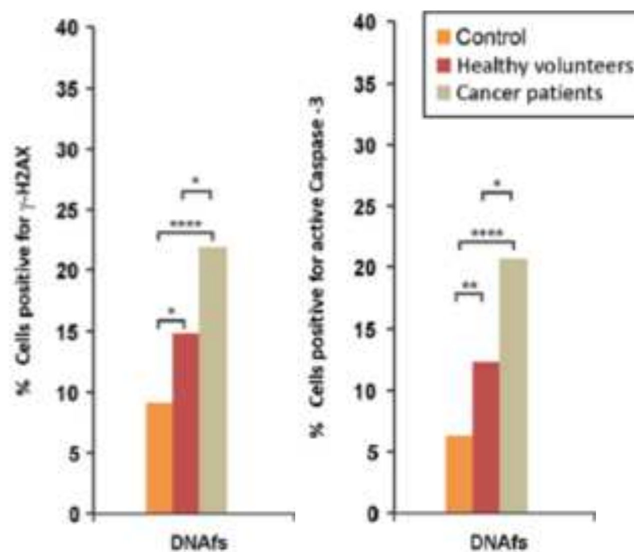


Fig. 5: induction of γ-H2AX, an indicator of dsDNA breaks, and active Caspase-3, an indicator of apoptosis, in NIH/3T3 cells treated with fragmented circulating DNA (DNAfs). DNAfs from cancer patients (grey bars) induces a higher response compared to those coming from healthy donors (adapted from Mittra et al, 2015).

Pro-inflammation mediator

It is well known that DNA is an immunostimulating molecule, considering its double-helix structure, the ability to interact with other molecules and sequence motifs. This data is supported by the observation of activation of interferon (INF) and pro-inflammatory molecules secretion in cells of the innate immune system after DNA exposure[236]. The abundance of nucleic acid specific receptors further supports an immunological function of DNA[237]. We have already described the role of circulating DNases in maintaining scarce levels of DNA, thus blocking its ability to stimulate cells, and the case of SLE patients, presenting low levels of DNase activity, characterized by autoimmunity and accumulation of cfDNA.

Several studies indicate that cfDNA acts like a damage-associated motif pattern (DAMP), i.e. endogenous ligands that can be recognized by Toll Like Receptors (TLRs) and activate the TLR-MyD88 (Myeloid differentiation primary response gene 88) pathway, leading to NF- κB and AP1 production[238] (Fig. 6). These transcription factors, then, regulate the expression of inflammatory cytokines, as tumour necrosis factor or interleukines (IL) 1 and 6, and costimulatory molecules such as CD80 and CD86[239].

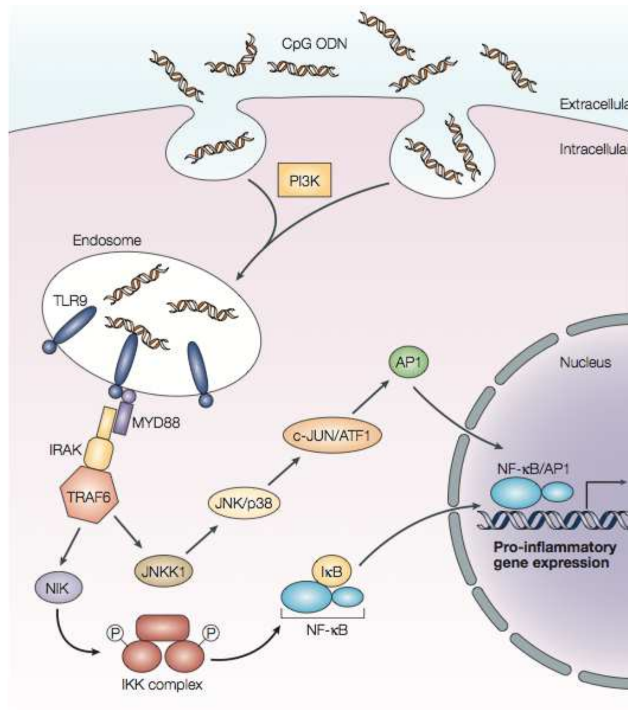


Fig. 6: schematic representation of the interaction between DNA and TLR9 receptor. DNA internalization is facilitated by class III Phosphatidylinositol 3-kinase. Once in the endosome DNA binds to TLR9 and TLR-MyD88 pathway is activated. Several downstream factors are activated, leading to NF-κB and AP1 activation and gene transcription. CpG ODN: CpG OligoDeoxyNucleotides (from Klinman, 2004).

TLR9 is a DNA receptor that recognizes oligonucleotides of 20-30 nt containing demethylated CpG motifs. For this reason, it is physiologically involved in sensing microbial DNA, either viral or bacterial, and it is usually located into intracellular vesicle membranes in dendritic cells and macrophages. The expression of TLR9 has been evidenced also in cancer cells and its high levels are associated with poor survival in patients affected by several cancers, among which glioma, prostate and esophageal adenocarcinoma. Even if a direct correlation between cfDNA and TLR9 in cancer has

not been determined, the hypomethylated DNA profile in several cancers can be a condition favouring cfDNA and TLR9 interaction and subsequent effects on cancer cells[240].

A study on CRC derived cell lines revealed that cfDNA of tumour origin is able to induce proliferation, promote cell migration and invasion and stimulate secretion of IL8, which has a pivotal role in malignancy, due to interaction with TLR9[240].

Tumour progression

Discovery of NETS and EETS opened a new field of investigation. NETS, in particular, have already been associated to cancer progression[241].

Polynuclear cells produce NETS and EETS after stimulation by pathogens and/or particular pathophysiological conditions, as inflammation[242]. Polynuclear neutrophil levels in the bloodstream correlate negatively with overall survival rate, due to their involvement in forming "bridges" between circulating tumour cells and epithelia of both lung and liver. It has also been demonstrated that NETS can sequester circulating tumour cells promoting metastasis formation by increasing adhesion to epithelial cells[243].

Therapeutic target

Considering all these physiological and pathological conditions in which cfDNA is involved, it can be reasonably considered a potential target for therapies. In particular, tumour progression via genometastases and DAMP-induced activation of the immune system are the main field in which therapy can be hypothesized.

Experiments on cancer cell lines xenografted onto mice were used to verify the efficacy of DNase and RNase treatments. Lung tumour-bearing mice treated daily with low doses of DNase and RNase presented reduced metastatic progression[244] (Fig. 7). Reduced tumour progression was observed also in mice injected with DNA obtained from the medium of SW480 cell cultures, after DNase treatment[245].

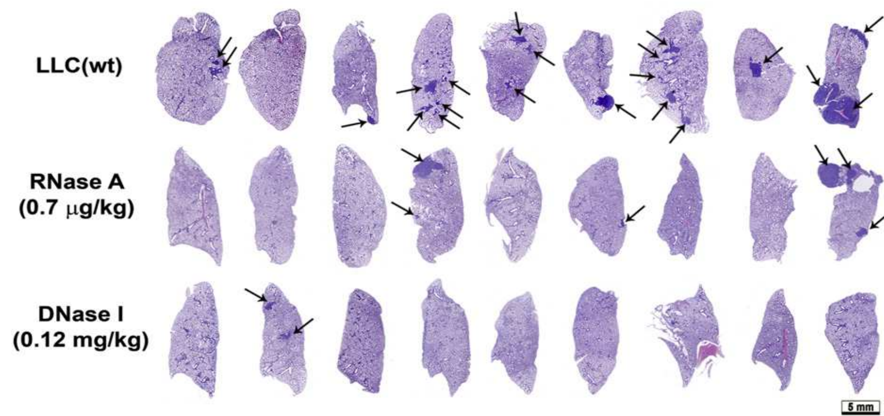


Fig. 7: Effects of RNase A and DNase I treatment on metastasis development in a Lewis lung carcinoma (LLC) metastatic mouse model. As we can see, lung lobes in control animals are characterized by several large metastasis (arrow). Mice treated with either RNase A (second line) or DNase I (third line) showed a great reduction in metastasis number and dimension (from Patutina et al, 2011).

NETS destruction can be achieved with either DNase or neuro-elastase inhibitors[243].

A third therapeutic option involves cationic polymers that electrostatically interact with cfDNA inhibiting the inflammatory response without affecting immune system[246].

MATERIALS AND METHODS

Project structure and inclusion criteria

This research project was submitted to the Ethics Commitee of the Circolo Varese Hospital and, after receiving the approval, we started enrolling patients with Breast Cancer who underwent surgery at the Senology Research Center - ASST Sette Laghi Varese.

This is a monocentric, explorative and translational study.

Inclusion criteria are: a) only female patients; b) cytologically or histologically confirmed breast cancer; c) candidate for surgery; d) no exclusion on the base of histological subtype or lymph node involvement; e) diameter size of the neoplasia: ≥ 20 mm; f) no previous detection of gene mutation (i.e. sporadic cancers, no BRCA1-2 mutated patients).

Exclusion criteria consist of : a) previous breast cancer (i.e. recurrence); b) the patient has undergone treatments, either chemotherapy or radiotherapy.

Validation of the extraction protocol

Samples collection

We firstly collect samples to validate the extraction protocol. Considering that patients presenting neoplasia bigger than 2 cm in diameter are not so common, we decided to perform this step including also samples with smaller tumours.

Blood was collected from 13 patients with an average age of 62 yo (range 43-88 yo), neoplasia diameter of 15 mm on average (range 2-28 mm) and no BRCA 1-2 mutations. Data are summarized in Table 4 (see results).

Blood collection and plasma isolation

We collected 10 ml of peripheral blood in K_3EDTA tubes commonly used for molecular applications. Plasma was isolated within 30 mins from the collection of blood in order to minimize the risk of white blood cells (WBCs) haemolysis.

Plasma isolation was performed as follow:

- Blood is firstly centrifuged at 1600 *xg* for 10 minutes to separate blood cells.
- Supernatant is collected and transferred to 1.5 ml microcentrifuge tubes, being careful not to take away cells or proteins.
- Supernatant is centrifuged again at 12000-16000 *xg* for 10 mins, in order to remove cellular debris.
- Plasma is collected and transferred to new microcentrifuge tubes, being careful not to collect pellet formed at the bottom of the tube.
- The obtained plasma is processed immediately or stored at -80° C.

We decided to exclude from the analysis all those samples that present any sign of haemolysis (i.e. those plasma whose colour show any shade of red).

cfDNA extraction

We decided to firstly compare two different extraction procedures, both automatized, one working on the MaxWell® RSC (Promega) platform with the MaxWell® RSC ccfDNA Plasma Kit (Promega), and the other working on the Abbott m2000sp (Abbott).

The extraction with MaxWell® RSC was done on 1 mL of plasma obtained as previously described. The quantification step was performed using both the fluorometer associated to the MaxWell@ RSC instrument and the Qubit™ dsDNA HS Kit (Thermo Fisher). The second extraction was performed on a volume of 300 µL of plasma and the quantification was performed with the Qubit™ dsDNA HS Kit (Thermo Fisher).

Due to the limited amount of cfDNA obtained and to avoid the risk to consume all the material for just one analysis, we decided to evaluate extraction protocols that allow us to scale up the starting volume of plasma.

We had the opportunity to test the NextPrep-Mag cfDNA isolation kit (Bioo Scientific) and the QIAamp MinElute ccfDNA Midi Kit (Qiagen), whose protocols allow to scale up the extraction till 3 mL and from 3 to 10 mL of plasma, respectively.

Both kits are based on magnetic beads purification of cfDNA. The Qiagen one uses beads to collect and concentrate cfDNA from plasma and then the purification is based on columns, while the Bioo Scientific kits requires the use of magnetic beads in all the steps of the protocol. We performed both protocols following manufacturer instructions.

We validate the extraction protocols with samples from a different project and we decided to proceed with the QIAamp MinElute ccfDNA Midi Kit extracting from a starting volume of plasma of 4 ml.

Preservative-containing tubes

We then test the utilization of the Blood STASIS 21-ccfDNA tubes (MagBio Genomics Inc.). These tubes are specific for cfDNA isolation due to the presence of an additive that stabilize cells and prevent coagulation, thus reducing the risk of genomic contamination by blood cell haemolysis and stabilizing cfDNA levels when stored for up to 21 days at room temperature.

16 mL of blood were collected into 2 Blood STASIS tubes and centrifuged as described above.

We then extract in parallel cfDNA from plasma obtained from Blood STASIS 21-ccfDNA and common K_3EDTA tubes with the QIAamp MinElute ccfDNA Midi Kit.

Amount of cfDNA obtained from Blood STASIS 21-ccfDNA tubes was higher than K_3EDTA ones, being equal the haemolysis of the plasma.

We thus decided to proceed collecting samples in Blood STASIS 21-ccfDNA tubes.

Experimental project

Sample collection and plasma separation

We collected blood samples in Blood STASIS 21-cffDNA tubes and fresh tissue in culture medium from 19 patients that match including criteria indicated.

Plasma has been separated as described in “blood collection and plasma isolation” paragraph.

Fresh tissue has been placed in a culture media after surgery and then stored dry at -80°Cwithin 3 h.

Genomic DNA extraction

Genomic DNA has been extracted with the DNeasy Blood & Tissue Kit, starting from 25 mg of fresh tissue, following manufacturer instructions.

The protocol consists of a first mechanical homogenization of the tissue followed by a Proteinase K digestion step and silica-based membrane purification with several centrifugations.

DNA has been quantified with the Qubit dsDNA BR kit (Thermo Fisher) following manufacturer protocol.

cfDNA extraction

Circulating DNA has been extracted as described in the paragraph above “cfDNA extraction”.

Target sequencing procedure

Genomic DNA

The preparation of genomic DNA, extracted from fresh tissue, was performed with the Homologous Recombination Solution by Sophia Genetics Kit (Sophia Genetics) and the Kapa™ Hyperplus Library preparation kit (Roche). These kits are usually used for formalin-fixed paraffin-embedded (FFPE) extracted DNA, characterized by DNA fragmentation and reduced molecule integrity. The usage of genomic DNA extracted from fresh tissue allows us to avoid these problems and have high-quality starting material.

As suggested by the protocol, we used the maximum amount of DNA, i.e. 200 ng, to generate high-quality sequencing data.

200 ng of DNA in a 25 µl volume are firstly enzymatically fragmented at 37°C for 20 mins, then the End Repair and A-tailing (ER&AT) two step procedure is performed, with an incubation at 20°C for 30 mins anda second at 65°C for 30 minutes. Fragments are then ligated to Dual Index Adapters and the reaction is cleaned up using AMPure XP magnetic beads to remove all the reagents of the previous reactions.

Libraries this way prepared are then amplified with a PCR reaction whose conditions are summarized in the Table 2 (below). Step 1st to 3rd are repeated 8 times.

	Temperature (°C)	Time (s)
Lid	99	
Initial step	98	45
1st step	98	15
2nd step	60	30
3rd step	72	30
Final step	72	60
Holding	10	

Amplified libraries are purified again with AMPure XP magnetic beads and individual libraries are quantified and their quality is evaluated.

2 μl of the purified library is diluted with 6 μl of nuclease-free water, quantification is performed on 2 μl of the diluted solution with a fluorometric method while quality control (QC) is performed by capillary electrophoresis and library profile should present fragments ranging between 200 bp and 800 bp.

Libraries passing QC analysis are pooled mixing 300 ng of each library in a DNA low-binding tube; Blocking oligos and human Cot DNA are added to the mixture to mask adaptors and block nonspecific hybridization, respectively, and the mixture is lyophilized and stored at -20°C if not immediately utilized.

Lyophilized libraries are resuspended in the Hybridation mix and hybridization step is performed as indicated by manufacturer (65°C for 4 to 16 hours). Streptavidin beads are used to bind and enrich hybridized targets by incubation at 65°C for 45 minutes.

After a wash step to remove unbound DNA, the post-capture libraries are amplified. PCR conditions are listed in the Table 3 (below).

	Temperature (°C)	Time (s)
Lid	99	
Initial step	98	45
1st step	98	15
2nd step	60	30
3rd step	72	30
Final step	72	60
Holding	10	

Steps 1st to 3rd are repeated for 15 cycles.

Post-capture amplified libraries are cleaned up with AMPure XP magnetic beads and purified libraries are checked for concentration and quality, as described in the previous step of control.

The last step of the protocol consists in library preparation for sequencing. Molarity of each pool is determined by the ratio between library concentration (ng/µl) and the product of the average size (bp) and 649.5 (Fig. 8) multiplied for 10^6.

$$\text{Library molarity (nM)} = \frac{\text{Library concentration (ng/µl)}}{\text{Average size in base pairs} \times 649.5} \times 10^6$$

Pools are sequenced on MiSeq platform.

cfDNA

The preparation of circulating DNA was performed following the Solid Tumour Solution by Sophia Genetics protocol for circulating cell-free DNA (Sophia Genetics) and the Kapa™ Hyperplus Library preparation kit (Roche).

Compared to the protocol described for the genomic DNA, this one does not include the enzymatic fragmentation, due to the fragmented nature of cfDNA.

We decided to use as much cfDNA as we have, in order to reduce the risk of having bad results in library preparation and sequencing. Considering the low amount of cfDNA, the first PCR was set to 12 cycles.

The protocol starts from the End Repair & A-tailing step (ER&AT) and follows the one previously described for genomic DNA.

Data analysis

Data analysis was performed on the Sophia DDM® platform. A three-step procedure allows to rapidly and efficiently obtain information from row sequencing data.

Step 1 consists in row data uploading and processing, resulting in a list of information.

In step 2 results of step 1 are interpreted, thanks to the classification from highly pathogenic to benign made from the Sophia AI.

Last step consists in a variant report definition.

Variants detected were evaluated with the ClinVar (NCBI) database, that allows a direct association between the variant and its role in health.

RESULTS

Validation of the extraction protocols

Samples collected to validate the extraction procedure have been analysed as described in Matherial and Methods. Quantifications of the samples are summarized in Table 4 rappresented below (reference from materials and methods).

	MaxWell™ RSC (Promega)		Abbott m2000 (Abbot)	
SAMPLE	ng/µL	ng tot	ng/µL	ng tot
E.coli 0.5 MF	0.096	5.664	<0.010	-
87173141	0.100	5.90	<0.010	-
87180260	0.067	3.95	<0.010	-
87182036	0.110	6.49	<0.010	-
87187622	0.060	3.54	<0.010	-
87189259 (1)	0.138	5.38	<0.010	-
87189259 (2)	0.248	9.67	<0.010	-
87191680	0.185	10.92	<0.010	-
117002299	0.0156	0.9204	<0.010	-
117002296	0.0132	0.7788	<0.010	-
117002837	0.0139	0.8201	<0.010	-
117003112	0.0160	0.944	<0.010	-

117003115	0.0128	0.7552	<0.010	-
117003410	0.0127	0.7493	<0.010	-

Table 4: summary of quantification of cfDNA from samples coming from breast cancer affected patients used to validate the extraction procedure.

The Table 5 (below) sum up characteristics of patients used to validate the extraction method. Thirteen patients were included, median age 62 years (range 43 – 88), the average diameter of the neoplasia were 15 mm (range 2 – 28).

Ten patients underwent breast conservative surgery and only 3 had mastectomy. All had sentinel lymph node biopsy and in only two cases axillary lymph node dissection were performed. The average lymph nodes removed were 10 (range 4 – 18) and the average of metastatic lymph nodes were 1,7 (range 1 – 3).

The majority of the tumors (11 patients) were invasive ductal carcinoma with a moderate grade of differentiation (G2), only two patients were G3. Twelve had a strong positivity for the ER and PGR receptor status, 10 patients were HER2 negative and the average Ki67 were 15 (range 4 – 30).

Ten patient underwent to complete adjuvant radiotherapy after breast conservative surgery. Almost all patients received adjuvant hormonal treatment, only 3 received also adjuvant chemotherapy.

Table 5

Clinical Features	
Number of patients	13
Average Age	62 y.o. (range 43 - 88)
Average diameter of the neoplasm	15 mm (range 2 - 28)
BRCA 1-2 mutations	all negative

Surgery

Sugical Procedure	
Mastectomy	3
Lumpectomy	10
BLNS	13
Lymph node dissection	0
BLNS ➔ Lymph node dissection	2

LNS	
negative	10
positive	3 (1 micro MTS)
n° LN MTS average	average 1,7 (range 1 - 3)
n° LN removed	average 10 (range 4 - 18)

Histology

Histological Type	
ductal	11
lobular	2

Grading	
G 1	3
G 2	8
G 3	2

ER, PGR status	
positive	12
negative	1

HER-2 status	
positive	3
negative	10

Ki67
average 15 (range 4 - 30)

Therapy

Adiuvant Therapy	
Radiotherapy	10
Ormonal therapy	12
Chemotherapy	3

Experimental project

DNA extractions

DNA obtained from patients enrolled in the project was extracted and quantified as previously described, starting from 4 mL of plasma.

Genomic DNA was extracted from 25 mg of fresh tissue and quantified as described in Materials and Methods.

Quantification of these samples are summarized in Table 6.

	cfDNA		Fresh tissue	
SAMPLE	ng/μL	ng tot	ng/μL	ng tot
Patient 1	0,35	8,75	49,4	7410
Patient 2	0,39	9,75	35,1	5265
Patient 3	0,56	14	42	6300
Patient 4	0,26	6,50	40	6000
Patient 5	0,65	17	71,1	10755
Patient 6	1,07	20	85,9	12885
Patient 7	0,26	1,3	47,1	7065
Patient 8	0,44	2,2	47	7050
Patient 9	0,14	3,5	46	6900
Patient 10	1,42	35,5	163,3	24495
Patient 11	1,16	29	144,6	21690
Patient 12	0,07	1,75	42,1	2105
Patient 13	0,93	23,25	33,8	5070
Patient 14	3,17	79,25	229	34350
Patient 15	8,7	174	150,5	22575
Patient 16	17,4	348	207,2	31050
Patient 17	28,3	566	63,8	9570

Target enrichment analysis

Three runs of target enrichment and sequencing were by now performed.

The first one comprised four samples of genomic DNA of patients 1 (ID 278399), 2 (ID 278400), 3 (ID 278401) and 4 (ID 278402); the second one cfDNA samples of patients 1 (ID 293372), 2 (ID 293373), 3 (ID 293374), and 4 (ID 293375); and the third genomic and circulating DNA of patients 5 (IDs 295272 and 295274, respectively) and 6 (IDs 295273 and 295275, respectively).

Figure 9 summarizes data regarding the total number of reads, those mapping and those with problemating matching of the three runs.

Sample ID	Number of reads	Number of Mapped reads	Number of reads with problematic mate	Percentage of Mapped reads
278399-88-S1	10,509,348	10,077,063	57,505	95.89%
278400-89-S2	7,510,838	7,098,394	48,924	94.51%
278401-90-S3	8,438,848	8,076,739	40,511	95.71%
278402-91-S4	10,218,280	9,840,043	65,485	96.30%
Total	**36,677,314**	**35,092,239**	**212,425**	**95.68%**

I run

Sample ID	Number of reads	Number of Mapped reads	Number of reads with problematic mate	Percentage of Mapped reads
293372-119-S1	7,681,986	7,540,466	61,288	98.16%
293373-120-S2	10,575,478	10,294,352	663,780	97.34%
293374-121-S3	10,234,072	10,033,745	565,641	98.04%
293375-122-S4	12,717,022	12,488,636	1,032,418	98.20%
Total	**41,208,558**	**40,357,199**	**2,323,127**	**97.93%**

II run

Sample ID	Number of reads	Number of Mapped reads	Number of reads with problematic mate	Percentage of Mapped reads
295272-135-S1	5,831,670	5,703,480	36,776	97.80%
295273-136-S2	4,809,330	4,698,258	35,644	97.69%
295274-137-S3	5,444,018	5,359,192	761,842	98.44%
295275-138-S4	11,183,516	11,040,379	564,787	98.72%
Total	**27,268,534**	**26,801,309**	**1,399,049**	**98.29%**

III run

Fig. 9: total, mapped and problematic read of the samples of the three runs.

Quality of the sequencing was determined as mean phred quality score, distribution of raw reads quality and raw reads length. An example of data obtained is described in Figure 10.

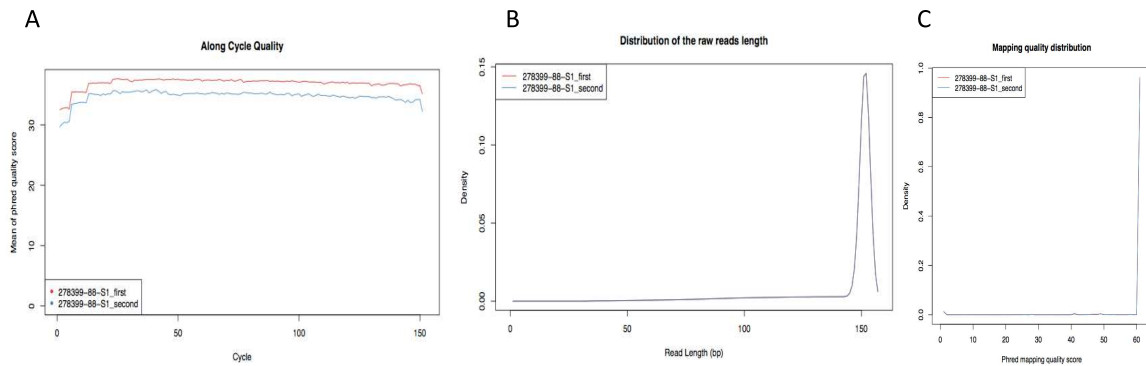


Fig. 10: example of reads quality evaluation in genomic DNA of patient 1. A) Along Cycle Quality measure the phred score, i.e. an evaluation of the correct base call, along the dimension of the fragment. A phred score value of 30 corresponds to a 99,9% accuracy of the base called during the sequencing. B) Distribution of the raw reads length: the peak at the end of the scale indicates thatmost reads have a high length, i.e. are of good quality. C) Mapping quality distribution: the location of the peak indicates the quality of the mapping. Optimally, most of the mapping are of high quality with a peak around 40, generally the higher the peak the higher the quality is.

Soft-clipping is a value associated to incomplete matching between the full length read and the reference genome. This data is essential to understand if a read can be considered in the analysis or must be discarded. In this protocol, percentage of no soft clip above 75% can be considered reliable.

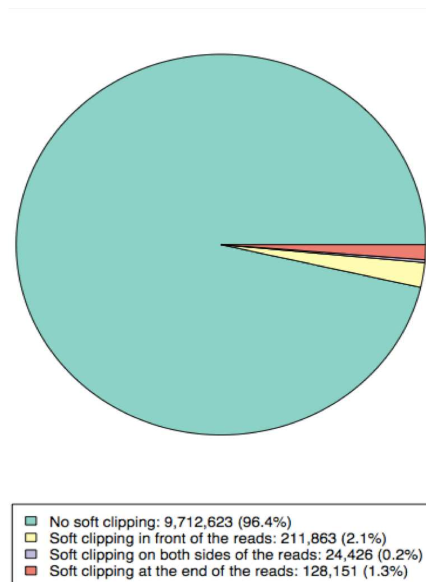


Fig. 11: pie chart illustrating the proportion of no soft clipping and soft clipping reads in genomic DNA of patient 1. As we can see in the box below the pie chart, >96% of the reads are no soft clipping, thus can be considered reliable.

Mapping statistics give information on where the sequences mapped on the reference genome. This data indicated the accuracy of the target enrichment capture. If the sum of “onTarget” and “flankTarget” overcome 80%, target enrichment has captured the correct fragments, otherwise fragments in not-of-interest regions have been captured and analysed during the process (Fig. 11).

The duplicate fraction percentage indicates if reads analysed are the result of an unbalanced PCR amplification or they belong to different amplicons. In genomic DNA analysis, this value should be between 20% and 40% to consider the analysis reliable, while values <20% can be considered excellent (Fig. 12).

	onTarget	flankTarget	offTarget_HighCov	offTarget_LowCov
295272-135-S1	72.47%	13.86%	4.29%	9.38%
295273-136-S2	72.78%	13.44%	4.44%	9.34%
295274-137-S3	76.88%	8.50%	3.99%	10.62%
295275-138-S4	76.46%	9.38%	4.40%	9.76%

Fig. 12: Mapping statistics of genomic and circulating DNA from samples 5 (IDs 295272 and 295274, respectively) and 6 (IDs 295273 and 295275, respectively). As we can see, the sum of "onTarget" and "flankTarget" percentages is more than the 80% considered as a threshold for good capture results.

	duplicate fraction	2	3	4	5	6-10	11-100	>100
295272-135-S1	18.42%	343704	62431	12098	2373	695	1	0
295273-136-S2	17.08%	272207	45537	7913	1591	433	0	0
295274-137-S3	57.51%	235861	138322	83006	48831	65618	10608	1
295275-138-S4	66.06%	347,616	227,288	157,430	109,191	188,566	45,404	24

Fig. 13: Analysis of the duplicate fraction of genomic (IDs 295272 and 295273) and circulating DNA (ID 295274 and 295275) from patients 5 and 6, respectively. Percentages observed in genomic DNA are below 20%, suggesting a low levels of PCR amplification unbalance. cfDNA samples, instead, have an higher percentage of sequences amplified by PCR. Columns named 2, 3, 4, 5, 6-10, 11-100 and >100 indicates total number of reads having an equivalent number of copies (2 means two copies, 3 three copies etc).

Identification of mutation

The Sophia DDM software analysis allows to identify among the sequences the presence of mutation/s and simultaneously to classify it on the base of their effect: 1 - benign, 2 - likely benign, 3 - uncertain, 4 - pathogenetic and 5 - definitely pathogenetic. Those mutation that have not been described yet in the ClinVar database are classified on the base of their predicted role in: A - definitively pathogenetic, B - potentially pathogenetic, C - unknown significance.

The analysis also allows to distinguish between sequences presenting a low coverage respect to the threshold (x1000), i.e. are located in the "onTarget" region but data cannot be considered reliable,those retained, i.e. have an above threshold coverage and thus data can be analysed, and those called "low confidence" SNVs/INDELS, grouping modifications associated to intronic or untranslated (UTRs) regions predicted to be of unknown significance (class C).

Patient 1

The analysis of the genomic DNA highlighted only a pathogenic mutation in the RAD51B gene with a variant fraction (VF) of 5.2% and classified as definitely pathogenic.

The mutation consists of an insertion of a 100 bp fragment between nucleotides 80 and 81that causes the formation of a stop codon, resulting in the truncation of the protein at Cys27 (c.80_81ins100 p.Cys27*) (Fig. 14).

Gene Transcript	Exon	c.DNA Protein alteration	Variant Fraction Coverage (ref / alt)	Coding consequence	Pathogenicity	ClinVar
RAD51B NM_002877	2	c.80_81ins100 p.(Cys27*)	5.2 % (3295 / 235)	nonsense	Flagged Pathogenicity 5 I Definitely Pathogenic	

The analysis of the cfDNA, however, did not detect any mutation of interest.

Patient 2

The analysis of the genomic DNA in this patient highlighted two pathogenic mutations. The first one is an insertion of a T between nucleotide 390 and 391, resulting in a stop codon and a truncated protein at Arg131 (c.390_391insT p.Arg131*). It has a VF of 7.2% and is classified as definitely pathogenic (5).

The second one is a missense mutation (T>A) at nucleotide 526 of the TP53 gene, resulting in the substitution of a cysteine with a serine (aminoacid 176), with a VF of 2.5% and a classification of 4 (Fig. 15).

Gene Transcript	Exon	c.DNA Protein alteration	Variant Fraction Coverage (ref / alt)	Coding consequence	Pathogenicity	ClinVar
PALB2 NM_024675	4	c.390_391insT p.(Arg131*)	7.2 % (3117 / 219)	nonsense	Flagged Pathogenicity 5 I Definitely Pathogenic	
TP53 NM_000546	5	c.526T>A p.(Cys176Ser)	2.5 % (4924 / 122)	missense	Flagged Pathogenicity 4 I Likely Pathogenic	Conflicting interpretations of pathogenicity rs967461896

The analysis of the cfDNA detected the mutation in the TP53 genes described also in genomic DNA but, interestingly, with a VF of 4.8% (Fig. 16).

Gene Transcript	Exon	c.DNA Protein alteration	Variant Fraction Coverage (ref / alt)	Coding consequence	Pathogenicity	ClinVar
TP53 NM_000546	5	c.526T>A p.(Cys176Ser)	4.8 % (15132 / 722)	missense	Flagged Pathogenicity 4 I Likely Pathogenic	Conflicting interpretations of pathogenicity rs967461896

Patients 3, 4 and 5

The analysis on both genomic and circulating DNA in these patients did not revealed the presence of somatic mutation in genes of the panel.

Patient 6

The analysis of the genomic DNA in this patient highlighted two pathogenic mutations. The first one involved the BRCA2 gene and consist in the formation of a splice acceptor site due to the substitution of two A with one T at nucleotide 7436 (c.7436-2A>T). Its VF is of 13.7% and is classified as 5.

The second one is a likely pathogenic (4),splice donor site in the TP53 gene, involving a deletion of nucleotides from 372 to 375 and an insertion of 2 G (c.372_375+2delinsG). The observed VF of this mutation is 14.1% (Fig. 17).

Gene Transcript	Exon	c.DNA Protein alteration	Variant Fraction Coverage (ref / alt)	Coding consequence	Pathogenicity	ClinVar
BRCA2 NM_000059	15	c.7436-2A>T p.(?)	13.7 % (3046 / 489)	splice_acceptor_	Flagged Pathogenicity 5 I Definitely Pathogenic	Pathogenic rs397507917
TP53 NM_000546	4	c.372_375 + 2delinsG p.(?)	14.1 % (4939 / 798)	splice_donor_cd	Flagged Pathogenicity 4 I Likely Pathogenic	

As for patient 1, the analysis of the cfDNA does not detect any mutation.

DISCUSSION AND CONCLUSIONS

The analysis of genetic alterations in tumours is becoming a routine in clinical practice due to the possibility to predict response to targeted-therapies or as prognostic markers, affecting progression-free survival (PFS) and overall survival (OS) of many cancers.

Nowadays, the golden standard in somatic alteration assessment is represented by tumour biopsy. This approach, however, have some limitations. First of all, it is an invasive approach that carries a high complications rate [247,248]. Then, biopsies allow to analyse only a small fraction of the tumour lesion, with a high risk of considering just a snapshot of the highly heterogeneous cancer [249]. It is often necessary to repeat sampling of the tumour tissue and this procedure, besides intrinsic risks described above, is often considered unethical or impossible in cases of irradiated tumours[250].

The discovery of DNA circulating in blood opened the way to a surrogate marker for tumour tissue biopsy, called ‘liquid biopsy’. Blood drawing is a minimally invasive procedure that can be repeated several times without causing deep complication for the patient, but at the same time can furnish information on early cancer detection[251], analyse the evolution of genetic abnormalities and monitoring tumour dynamics[252].

Limitations on clinical application of cfDNA analysis are mainly related to the necessary development of novel, highly sensitive assays able to detect very low frequency mutations in small amounts of material with high sensitivity and specificity, to the lack of homogeneity on pre-analytical procedure between different studies and to procedure that validate cfDNA based assay in comparison with actual gold standard approaches.

The first biomarker we can consider is the total amount of cfDNA. Several studies have pointed out that cancer-affected patients have higher levels of circulating DNA, compared with healthy subjects.

In non-small cells lung cancer (NSCLC) patients median concentration is eight time higher compared to heavy smokers controls, achieving a 75% sensitivity and 86% specificity in detecting NSCLC [253].

cfDNA integrity has been evaluated as a diagnostic marker in colorectal cancers (CRC), periampullary cancers and BC.

These two markers proved not to be widely applicable due to the inability to clearly discriminate between malignant and non malignant diseases such as chronic inflammatory disorders, thus having a high risk of false positive detection[254].

Genetic alterations can be highly specific biomarkers that allow discriminating between malignant and non-malignant conditions. Cancer-specific epigenetic modification, such as hypermethylation of regulatory genes, can be a powerful marker in different cancers. Promoter methylation of *SEPT9* can help detecting CRC at early stages and a commercial test is under approval by Food and Drug Administration (FDA). Somatic mutations in oncogenes can be used as biomarkers to identify, evaluate prognosis and therapy and monitor cancer development[255].

In this work we have compared the detection of mutations in 16 genes (ATM , BARD1 , BRCA1 , BRCA2 , BRIP1 , CDK12 , CHEK1 , CHEK2 , FANCL , PALB2 , PPP2R2A , RAD51B , RAD51C , RAD51D , RAD54L , TP53) involved in homologous recombination (HR) in genomic, obtained from fresh tissue, and circulating DNA, obtained from plasma, in 6 patients affected by BC.

We firstly validated the procedure to extract cfDNA from plasma, testing four different kits, two automated and two manuals, with starting plasma volumes ranging between 300 μl and 4 ml. In a second project, we also tested the difference in terms of cfDNA quality between the commonly used K_3EDTA and the Blood STASIS 21-ccfDNA tubes, that contains an additive that prevents cells from lysis and preserve cfDNA from degradation for up to 21 days at room temperature.

We conclude that, in order to obtain good amount of high quality cfDNA, the best procedure is to collect blood in the Blood STASIS 21-ccfDNA tubes, considering that it is not always possible to separate plasma within 1 h from venepuncture.

The extraction has to be performed starting from at least 3 ml of plasma, considering that the more plasma can be processed the more cfDNA can be collected and analysed, increasing dramatically the representativeness of the sample in particular in the presence of mutations with very low VF.

We choose the QIAamp MinElute ccfDNA Midi Kit (Qiagen) because it allows us to scale the input plasma volume from 3 ml to 10 ml and it gives better yield in comparison with the other manual kit.

We observe a great variability in the levels of cfDNA in different samples, according with the literature.

We performed the target enrichment protocol following manufacturer instructions and all the samples passed the QC.

No mutations have been detected in 3 out of 6 patients analysed (numbers 3, 4 and 5), either in genomic or circulating DNA.

Mutations in at least one of genomic and circulating DNA have been identified in the other 3 patients.

In two genomic samples we observe the presence of mutations. Surprisingly, no mutations have been detected in cfDNA obtained from these two patients. Looking at QC reports of the target enrichment procedure, we can exclude that this result is caused by unsuccessful preparation and sequencing of the libraries. One possible explanation of what we have observed can be associated to the proliferation rate of the tumour. Both patients 1 and 6 have, indeed, a Ki67 value of 20 at the diagnosis. The minimum Ki67 value indicating high proliferation rate is 25, suggesting these two patients have slow proliferating tumours. This fact can affect deeply the amount of cfDNA released in the circulation, resulting in a higher possibility to miss tumour-derived fragment among the physiological background cfDNA. To obtain a more representative view of the whole cfDNA composition that allows us to identify also mutation with low frequency, parallel analysis of different cfDNA aliquots or increased starting amount of sample must be considered.

In addition to what already described, patient 6 harbours a splice acceptor mutation in the BCRA2 gene. Patients enrolled in this project have been tested for mutation in BRCA 1/2 genes. This analysis is performed on whole blood, i.e. white blood cell DNA, and allows identifying germline mutations.

We enrolled only those patients with sporadic cancers, i.e. both BRCA 1 and BRCA 2 are wild type. We can thus conclude that the identified mutation is somatic, i.e. it is present only in the tumour tissue.

In the patient 2, a mutation in TP53 is observed in both genomic and circulating DNA, while the second mutation affecting PALB2 is present only in the DNA extracted from the fresh tissue.

Interestingly, TP53 mutation VF is higher in cfDNA compared to DNA obtained from the fresh tissue. A similar scenario has already been described in the literature and confirms that tissue biopsies are only representative of the fraction of material collected and do not detect tumour heterogeneity, while cfDNA can furnish information on the whole clones composing the tumour.

Regarding the mutation in the PALB2 gene, the absence of the mutation in cfDNA can have different explanations. It is possible that the two mutations identified are associated with different clones, the one carrying the PALB2 having low proliferation rates, thus being undetectable as described in patients 1 and 6. By now, analytical procedures on cfDNA are not able to discriminate if mutations are harboured by the same clone or belong to different ones. Patient 2 has a Ki67 of 21, thus a low proliferation rate, but it is hard to determine if this value represent the contribution of just a clone or reflect the entire tumour proliferation, considering the limited sample analysed in the biopsy.

Future perspectives

In this study we identify mutations in a panel of 16 genes, associated with homologous recombination deficiency, in both circulating and fresh-tissue-extracted DNA.

One limitation of this study is the reduced amount of patients analysed. We have by now 11 samples ready to be analysed and we are proceeding collecting samples of patients matching our inclusion criteria in order to increase the amount of data and this way have a better understanding of sensitivity and specificity of this method.

Patients analysed will be monitored with further blood collection in order to verify the eventual occurrence of new mutations and data obtained will be crossed with the clinical data on disease status and development.

Analysis of DNA extracted from patient's white blood cells (WBC) will give us a deeper understanding of the germline mutational landscape in genes of the panel, allowing us to better assign the role of mutations observed during the analysis.

The further identification of mutations clearly associated with approved therapies (PARPi and platinum salts) can direct the clinical management of the patient toward a more efficient and specific treatment of cancer.

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