UNIVERSITY OF INSUBRIA

PhD program in EXPERIMENTAL AND TRANSLATIONAL MEDICINE
XXXII CYCLE

Circulating cell-free DNA: a powerful biomarker for tumor management and a possible monitor tool in other pathological conditions.

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A.A. 2018/2019

Summary

The discovery of DNA circulating into bloodstream gave rise to several efforts aiming to discover its physiological and pathological roles, its clinical utility and its possible use as a powerful biomarker that can revolutionise clinical procedures and therapeutic approaches.
The possibility to obtain information on the behaviour of cancer, graft, infection status or fetus conditions through the so-called 'liquid biopsy', i.e. the analysis of cfDNA in blood or body fluids, with little-to-no invasiveness and having a live, 'total body' image representing the entire heterogeneity of the system seems promising.
The application of high throughput analytical procedures, such as Next Generation Sequencing (NGS) or digital droplet PCR (ddPCR), are necessary to obtain reliable data on such a small amount of starting material.
Actually only few applications have achieved a clinical validation, due to the great variability in pre-analytical procedures that can deeply affect the overall result of the analysis, but cfDNA analysis revealed to be useful in particular in those clinical settings in which it is difficult to obtain other tissues, such as lung cancer, or repetitive biopsies can affect dramatically the outcome of the patient, as in transplantation.

In our work we applied cfDNA analysis to three different clinical settings.

In the first, we evaluated the presence of mutation in a panel of 16 genes belonging to the Homologous Recombination (HR) pathway in cfDNA and genomic DNA extracted from nodules in patients affected by breast cancer.
This analysis revealed the presence of mutations in 3 out of 6 samples. Interestingly, in one case we observed a higher variant fraction in cfDNA compared to genomic DNA, probably due to limited ability to detect clonal heterogeneity in tissue.

In the second clinical setting we evaluated if cfDNA can be used as a monitoring tool for determining septic risk. We analysed samples taken from patients undergoing dialysis with clinical symptoms suggesting the need of an emoculture test for bacterial systemic infection.
We were able to detect with high confidence the presence of bacterial strains in two samples. Interestingly, in the first one we detected, in accordance with the emoculture, a *Staphylococcus* strain together with *Propionibacterium* and *Streptococcus* strains.
The detection of *Burkholderia multivorans* in the second sample rised the possibility to identify with our technique those bacteria that take more than the canonical 5 days of emoculture to growth,

or that completely do not grow in emoculture conditions, sustaining the hypothesis of a, even if by now far in the future, culture-independent microbiology.

The third set of samples analysed was obtained from patients undergoing kidney transplantation.
We wanted to verify the applicability of the AmpF*l*STR® Identifiler® Plus PCR Amplification kit to cfDNA in order to monitor graft status.
We started analysing 3 patients having kidney biopsy due to clinical symptoms of graft rejection. We were not able to detect any donor-derived polymorphism in these samples.
We thus decided to analyse samples in which we were sure to have donor-derived cfDNA (dd-cfDNA). We collect blood samples from patients undergoing transplantation just after reperfusion of the graft and 1, 4 and 8 days after surgery.
We were able to identify more than 50% of donor derived polymorphisms just after reperfusion, thus falling down to 10% one day after surgery and disappearing from day 4 on.
This data are in accordance with the literature, describing a huge amount of dd-cfDNA just after transplantation released by reperfusion injury.

Data by now obtained seems promising, even if preliminary.
The possibility to cross our data with clinical parameters will help us to better describe the pertinence of our results to the effective status of patients.
In all the three settings, we are collecting other samples to have a broader amount of data that will allow us to perform statistical analysis to effectively validate our procedures.

1 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 (Mandel and Métais, 1948).

1.1 History

During the 60s several studies conduced in patients affected by Lupus Erythematosus (LE) discovered important features of DNA in blood.

Ceppellini and colleagues, starting from the hypothesis that DNA can elicit specific antibodies (Ab), identified a DNA-reacting factor in serum obtained from a patient affected by LE Diffusus. They then purified DNA from different samples of both human and animal origin and verified if they observed a reaction after incubation with LE 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 Abs (Ceppellini et al., 1957). 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 (Ceppellini et al., 1957).

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 that these Abs are able to interact with either autologous and eterologous DNA, suggesting an active role of the DNA-anti-DNA complex in disease progression, in particular in driving renal disease and vasculitis (Casals et al, 1964).

Two years after, Tan and colleagues 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 conditions: native DNA (i.e. dsDNA), sonicated DNA (i.e. small dsDNA fragments) and heat denatured DNA (i.e. ssDNA).

They observed an identical behaviour of the native and sonicated DNA, suggesting identical antigenic properties. Interestingly, they observed that serum that had reacted with dsDNAs was able to react also with ssDNA, suggesting the presence of Abs specifically and independently recognising the two forms (Tan EM, Schur PH et al, 1966). The complete abolishment of the reaction after DNase treatment pointed definitely toward DNA as the antigen in sera (Tan EM, Schur PH et al, 1966).

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. Radioimmunoassays were developed to quantify the presence of ng amounts of DNA: [I^{125}]iododeoxyuridine-labeled DNA worked as an antigen and LE patient's serum as source of Abs. Leon and colleagues were able to evaluate 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 (Leon et al, 1977).

The application of the assay after radiation therapy of patients revealed a decreased 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 (Leon et al, 1977).

50% of patients enrolled in this study showed circulating DNA levels comparable to healthy subjects, but this situation 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 (Leon et al, 1977).

These data taken together suggested the hypothesis that also tumours are capable of releasing circulating DNA, as previously observed by this group in cultured human normal lymphocytes (Anker et al, 1977) 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 (Stroun et al, 1989).

The possibility to study cfDNA at the molecular level increased information on this field. Vasioukhin and colleagues identified K-*ras* mutation in circulating, tumour and blood-isolated cell DNA of patients affected by colorectal adenocarcinoma (Vasioukhin et al, 1992; Vasioukhin et al, 1993) and N-*ras* mutation in DNA obtained from plasma, circulating cells and bone marrow of patients with myeloid leukemia or myelodisplastic syndrome (Vasioukhin et al, 1994). They

observed that N-*ras* mutations identified in plasma DNA were not always detected analysing the other sources of DNA, suggesting strong medical implication: 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, while the 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.

This last observation can be considered a precursor of the 'liquid biopsy' concept: the possibility to identify a mutation in plasma DNA, 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 (Padua et al, 1988) and reflects efficacy of chemotherapy in achieving clinical remission (Senn et al, 1988).

The detection of fetal DNA in both plasma and serum of pregnant women further expanded the field of liquid biopsies. Lo and colleagues, amplifying Y sequences in male-fetus-bearing women (Lo et al, 1997), paved the way toward non-invasive prenatal diagnosis of fetal trisomies and genetic anomalies, such as Down syndrome (Yu et al, 2014), but also important biological characteristics such as sex (Mennuti et al, 2015) and Rhesus factor or blood group (Rieneck et al, 2015). 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 (Assou et al, 2014; Thierry et al, 2016).

1.2 Biological features of cfDNA

1.2.1 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 (Sano and Morimoto, 1981).

Stroun and Anker in 1987 analysed circulating DNA from patients with different malignancies, proving the existence and characterizing tumour-released cfDNA. 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 (Stroun et al, 1987).

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, resulting in the presence of cancer-derived circulating DNA in 5 out of 7 samples analysed, while the other 2 presented only non-tumoral DNA. The increase in absorbance in those 5 samples, as the result of the hyperchromicity test, suggested double strand instability of the newly synthesized DNA, a typical characteristic of tumoral DNA (Stroun et al, 1989).

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

1.2.2 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. 1).

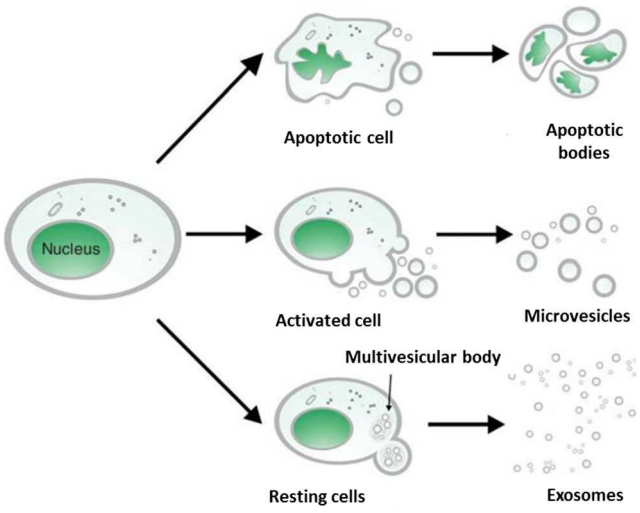

Fig. 1: potential vesicles that transport cfDNA. From Thierry *et al.* (2016).

1.2.3 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 (Stroun et al, 2006; Vlassov et al, 2007).
Surely the characteristic ladder pattern observed in cfDNA (Jahr et al, 2001) 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 (Giacona et al, 1998).
On the other hand, apoptotic bodies should be cleared *in situ* by epithelial cells and macrophages, thus contribution to circulating DNA should be limited (Stroun et al, 2006).

1.2.4 Microvesicles

Microvesicles are membrane-surrounded particles containing an aqueous compartment.
The observation of abnormal production of microvesicles in cancer patients (Mouliere et al, 2012; D'Souza-Schorey and Clancy, 2012), in addition to their role in genetic information transfer and cell-to-cell communication (Lee et al, 2012; Turturici et al, 2014), strictly connect these structures to cfDNA.
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 (De Jong et al, 2014). They contain proteins, particular lipids, messenger RNA (mRNA), micro RNA (miRNA) and variable amount of DNA (Thakur et al, 2014). 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 (Thakur et al, 2014). More recently, Rohan-Fernando and colleagues observed that a large proportion of plasma cfDNA is localized into exosomes (Rohan-Fernando et al, 2017).
- Microparticles: membrane fragments of 200 - 1000 nm in diameter, released by eukaryotic cells and they contain both DNA and RNA.
- Apoptotic bodies

1.2.5 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 DNA/RNA-lipoprotein complex was described for the first time in 2010 by the Stroun group. This macromolecular complex is actively released by cells in a regulated fashion (Gahan and Stroun, 2010). This discovery confirmed studies on active release of DNA in the medium of cultured lymphocytes (Rogers et al, 1972; Anker et al, 1975), frog heart auricles (Stroun and Anker, 1972), rat spleen (Adams and Gahan, 1983) or leukemia cell line HL-60 (Abolhassani et al, 1994) and the hypothesis of an active DNA release mechanism (Stroun et al, 2001).

Studies on this complex revealed that all the components are newly synthesized only by living cells (Anker et al, 1975; Stroun et al, 1978; Adams and Gahan, 1983), that the complex is released in a energy-dependent process (Adams and Gahan, 1983) and in a controlled manner (Anker et al, 1975; Stroun et al, 1977). Analysis in chick embryo fibroblasts demonstrated that this complex could be uptaken by cells (Adams and McIntosh, 1985).

1.2.6 Extracellular DNA Traps

It is well known that both neutrophils and eosinophil are involved in tumour-associated inflammatory infiltrate, where they can acts as both pro- and anti-tumorigenic depending on their activation status, and both are able to generate extracellular DNA traps, i.e. DNA associated with other cellular structures.

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 (Brinkman et al, 2004). This complex of DNA fibers and anti-microbial granules is involved in trapping and killing pathogens (Papayannopulos et al, 2010). A so-called “vital NETosis”, a process in which NETs are released without cell lysis, has also been described (Yipp and Kubes, 2013).

Differently from NETs, eosinophil extracellular DNA traps (EETs) are actively secreted by a catapult-like manner after eosinophil activation and they are composed of only mitochondrial DNA (Youssefi et al, 2008). An important feature of EETs is that eosinophils remain viable during this process and no “EETosis” is evidenced (Youssefi et al, 2008; Youssefi et al, 2012).

So far, NET release has been described in several pathological conditions, such as infections, autoimmune pathologies, thrombotic illnesses and inflammatory response (Kaplan and Radic, 2012; Cooper et al, 2013), metastases promotion and progression (Cools-Lartigue et al, 2013; Tohme et

al, 2016) or after exercise (Beiter et al, 2014), all conditions characterized by high cfDNA levels, suggesting a contribution of this structure to circulating DNA population.

1.2.7 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 (Casals et al, 1964; Tan et al, 1966; Rykova et al, 1994).
Due to its electrostatic nature, DNA can link with proteins such as albumin, fibronectin and the C1q complement component (Rykova et al, 2012).
Thus, pathological conditions altering serum protein availability can affect the amount of cfDNA in blood (Thierry et al, 2016).

1.2.8 Cell Surface-Bound cfDNA

Both DNA and RNA can be found on leukocytes and erythrocytes membranes (Rykova et al, 2012; Laktionov et al, 2004). *In vitro* studies revealed 20 kbp DNA fragments on cell surfaces (Morozkin et al, 2004), either naked or associated with macromolecules.

1.3 Signature of the release mechanism

Information on processes generating cfDNA fragments can be deduced by fragment themselves.
The analysis of cfDNA in cancer patients by PAGE, 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 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 two mechanisms generating the oligonucleosomal ladder and the high molecular weight fraction, respectively (Jahr et al, 2001).
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 were used for the analysis and suggested amplicons of <100 bp as the best (Fig. 2).

High proliferation and apoptosis rates observed in tumours can explain the reduced integrity observed, i.e. minor length of the fragments, and further supports apoptosis as an important source of tumoral circulating DNA (Mouliere et al, 2011; Madhavan et al, 2014).

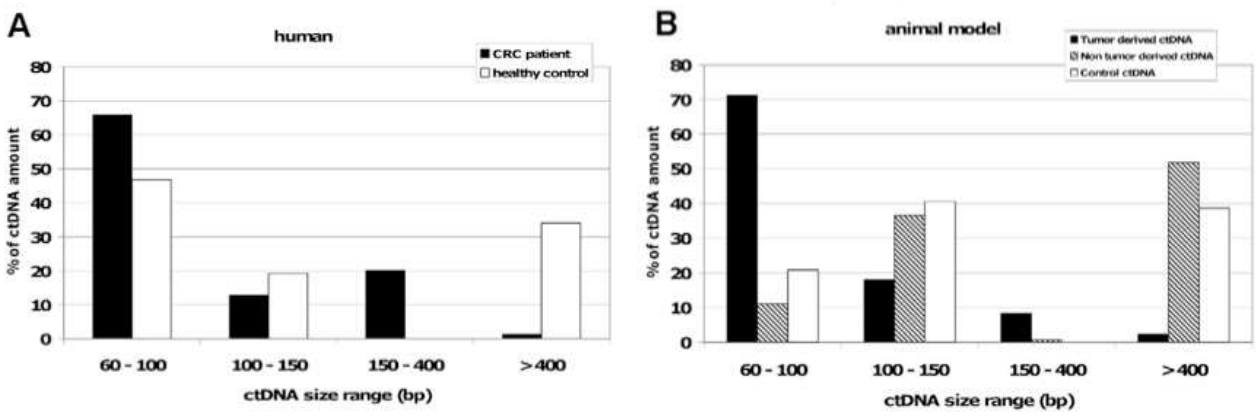

Fig 2: cfDNA fragment size distribution of mutant and wild type cfDNA. A) plasma of CRC affected patients B) xenografted animal model. From Mouliere et al, 2011.

The identification of tumour causative mutations (Vasioukhin et al, 1992-1993-1994; Sorenson 1994) or the lower length of fragments (Mouliere et al, 2011; Madhavan et al, 2014) 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 contribute to this population. Lack of a genetic biomarker as mutations that clearly indicates the origin of the fragment had been a limitation in this field and in those pathological conditions associated with an increased cfDNA, such as myocardial infarction (Chang et al, 2003), stroke (Rainer et al, 2003) or autoimmune disorders (Galeazzi et al, 2003), that do not present a genetic discriminant between healthy and pathologic cellular status. Nucleosomal occupancy can be a powerful marker of tissue contribution in the absence of a mutation, as described by Snyder and colleagues.

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 (Lui et al, 2002), and revealing the contribution of different tissues in samples of different cancer patients (Snyder et al, 2016).

An alternative “genotype-independent” method to analyse cfDNA is represented by methylation. Epigenomic profiling and cancer-specific DNA methylation patterns can furnish clinical issues in

diagnosis, prognosis and disease monitoring and thus can be considered a marker of tumour DNA (Warton et al, 2016).

One advantage of analysis on cancer-associated methylation modification or nucleosomal occupancy is that they do not require knowledge of the patient-specific mutational profile or to apply libraries of assays covering the most common mutations related to a specific type of tumour.

The first analysis on cfDNA methylation in different diseases was performed by Lehmann-Werman and colleagues. 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 heart attack and exocrine pancreatic cell in subjects with pancreatitis or pancreatic cancer (Lehman-Werman et al, 2016).

Circulating methylated DNA has also been proved to be a predictive and prognostic marker in metastatic colorectal cancer after treatment with Regorafenib. In particular, high baseline methylated DNA is associated with worst prognosis and shorter progression free survival during treatment (Amatu et al, 2019).

1.4 cfDNA halflife and clearance

Starting from the 60s, several works were focused on deciphering free circulating DNA halflife and kinetics. Tsumita and Iwanaga firstly detected radioactivity at different times after tritiated calf thymus DNA injection into mice bloodstream. 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 (Tsumita and Iwanaga, 1963). 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. Denaturated DNA was removed slighty faster than dsDNA and incubation with endogenous nucleases revealed that most of the DNA is uptaken as macromolecule or complexed with antibodies, more than digested. The analysis of organs suggested a pivotal role of liver and spleen, via reticulo-endothelial uptake, in DNA clearance (Chused et al, 1972).

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 that in the presence of anti-ssDNA Abs ssDNA clearance was delayed and persistence of DNA-anti-DNA complexes can affect pathogenesis of immune complexes disease (Dorsch et al, 1975).

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. Saturation of phagocytosis explains how an amount of ssDNA higher than the threshold resulted in DNA accumulation 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.

The mechanism of DNA clearance seemed to involve circulating nucleases that cleaves large molecular weight ssDNA into smaller fragments that are bound by liver, where a membrane exonuclease releases nucleotides into the 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 (Emlen and Mannik, 1978).

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 (Pancer et al, 1981).

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. They described the two steps clearance consists of: 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 was in agreement with lower levels of nucleases characterizing SLE patients, leading to

accumulation of circulating dsDNA that forms DNA-anti-DNA complexes (Emlen and Mannik, 1984).

A direct analysis on cfDNA, thus not involving externally injected DNA, 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 (Lo et al, 1999). 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 (Yu et al, 2013).

The analysis of post-surgery cfDNA clearance in a patient affected by CRC revealed a half-life of 114 mins (Diehl et al, 2008).

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 (Thierry et al, 2016).

1.5 cfDNA functions

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

1.5.1 Intracellular messenger

We have already described Stroun, Anker and Gahan works that described virtosome (Gahan and Stroun, 2010). Studies on intracellular messengers dated 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 were transmitted to the progeny, originated by the stock (Stroun, 1962). An analogous situation was observed in feather colour modification in white chickens after blood transfusion from Guinea hens (Stroun et al, 1962).

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 (Anker et al, 1975). 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 (Anker et al, 1980). Analogous experiments were performed on mice that were injected with DNA released in the medium by T cell after HSV or poliovirus exposure. Parallel to the previous experiment, mice started producing human-like anti-polio and anti-HSV Abs (Anker et al, 1984).

1.5.2 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 (Bendich et al, 1965). 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 (Anker et al, 1994). An analogous situation was observed with the addition of plasma obtained from colorectal cancer patient carrying a mutated *KRAS* (Garcia Olmo et al, 2010). Furthermore, NIH/3T3 cells treated this way were able to induce tumour formation once injected into nude mice (Fig. 3) (Garcia Olmo et al, 2010).

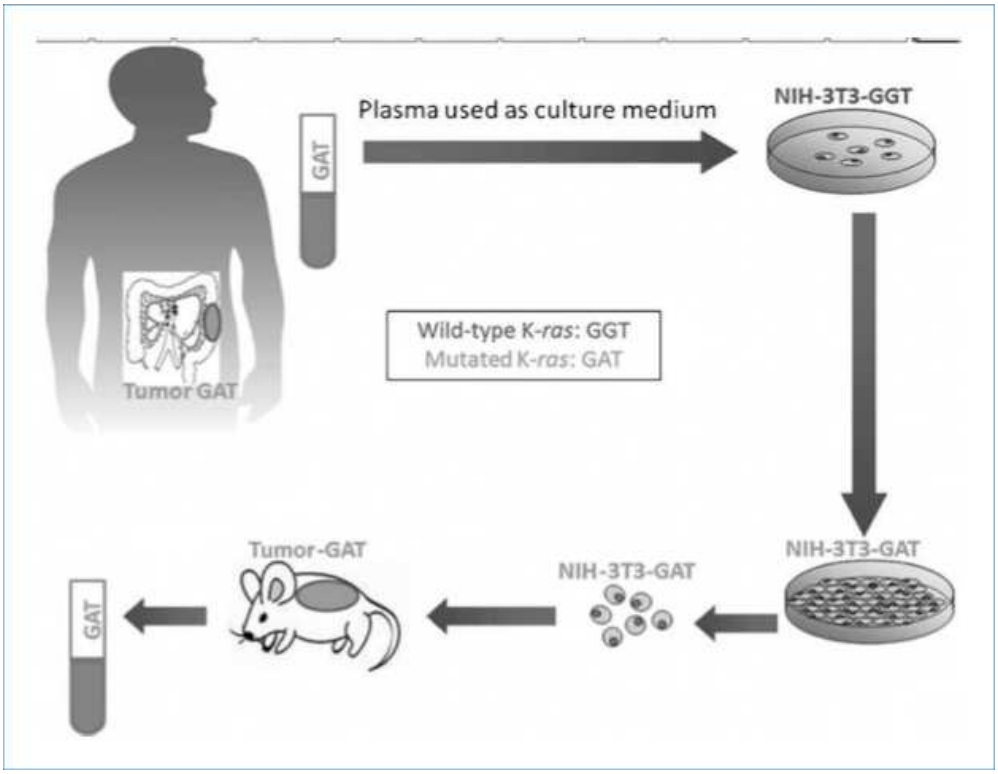

Fig. 3: schematic representation of genometastases proving experiment. Plasma from colon cancer patients, containing DNA carryng the mutated K-ras (GAT trinucleotide), added to the medium of NIH/3T3 (wild type K-ras) resulted in the acquisition of the mutant K-ras from the cells. Mice injected with the 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 (Peters and Pretorius, 2012). Nucleosomes are equally able to cross plasma membrane, thus penetrating into the cell (Hariton-Gazal et al, 2003). 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 (Mittra et al, 2015) and that this event induces apoptosis and DNA damage responses (Fig. 4) (Mittra, 2015).

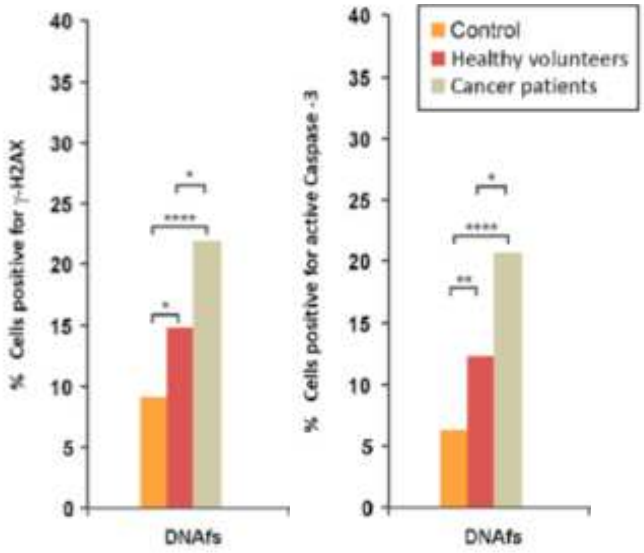

Fig. 4: induction of γ-H2AX, an indicator of dsDNA breaks, and active Caspase-3, an indicator of apoptosis, in NIH/3T3 cells treated with fragmented circulating DNA (DNAfs). DNAfs from cancer patients (grey bars) induces a higher response compared to those coming from healthy donors (adapted from Mittra et al, 2015).

1.5.3 Pro-inflammation mediator

It is well known that DNA is an immunostimulating molecule, considering its double-helix structure and 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 (Pisetsky, 2012). The abundance of nucleic acid specific receptors further supports an immunological function of DNA (Kawasaki et al, 2011). 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 indicated 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 (Fig. 5) (Klinman, 2004). 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 (Kawai and Akira, 2010).

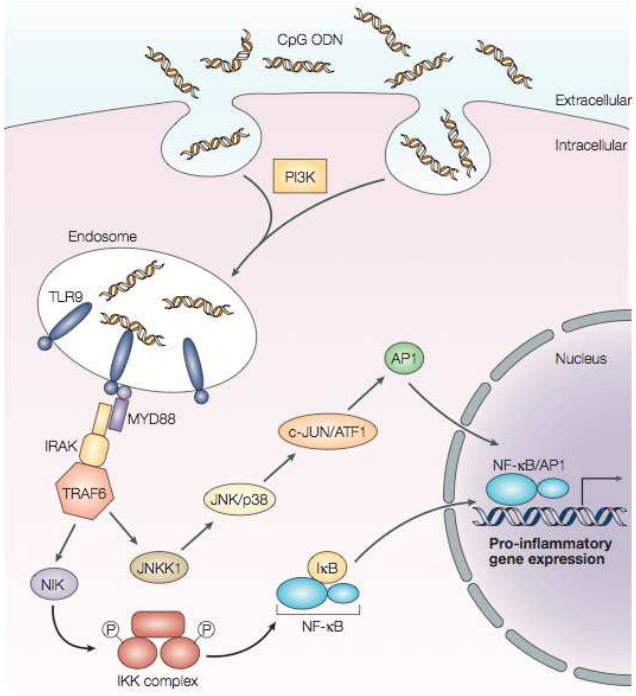

Fig. 5: schematic representation of the interaction between DNA and TLR9 receptor. DNA internalization is facilitated by class III Phosphatidylinositol 3-kinase. Once in the endosome DNA binds to TLR9 and TLR-MyD88 pathway is activated. Several downstream factors are activated, leading to NF-κB and AP1 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 (Yasuda et al, 2009). 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 (Fehri et al, 2016; Nakagawa and Gallo, 2015). 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 (Vaisanen et al, 2013; Zhang et al, 2014). 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 (Niu et al, 2018).

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 (Niu et al, 2018).

1.5.4 Tumour progression

Discovery of NETS and EETS opened a new field of investigation. NETS, in particular, have already been associated to cancer progression (Demers and Wagner, 2013).

Polynuclear cells produce NETS and EETS after stimulation by pathogens and/or particular pathophysiological conditions, as inflammation (Wartha et al, 2007). 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 (McDonald et al, 2009). It has also been demonstrated that NETS can sequester circulating tumour cells promoting metastasis formation by increasing adhesion to epithelial cells (Cools-Lartigue et al, 2013).

1.5.6 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 (Fig. 6) (Patutina et al, 2011). Reduced tumour progression was observed also in mice injected with DNA obtained from the medium of SW480 cell cultures, after DNase treatment (Trejo-Becerril et al, 2012).

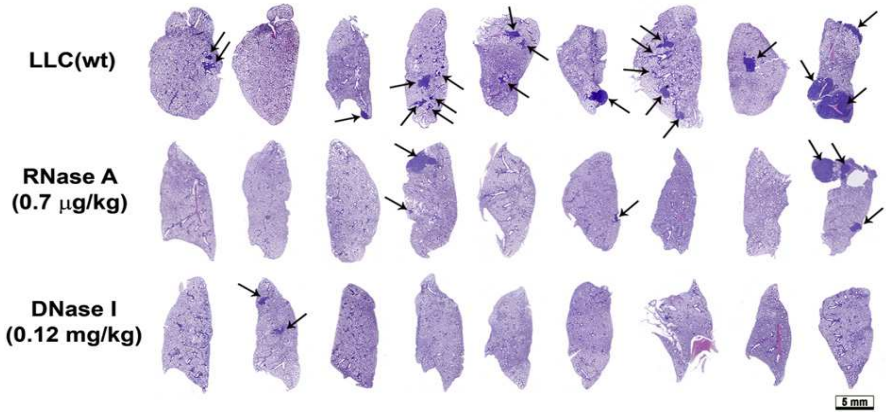

Fig. 6: effects of RNase A and DNase I treatment on metastasis development in a Lewis lung carcinoma (LLC) metastatic mouse model. As we can see, lung lobes in control animals are characterized by several large metastasis (arrow). Mice treated with either RNase A (second line) or DNase I (third line) 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 (Cools-Lartigue et al, 2013).

A third therapeutic option involves cationic polymers that electrostatically interact with cfDNA inhibiting the inflammatory response without affecting immune system (Holl et al, 2013).

2 Breast Cancer

2.1 Epidemiology

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 (DeSantis et al, 2013). In Italy, in 2018, about 52,800 new cases were diagnosed (AIOM, 2018).

It is also the leading cause of death, representing the 29% of the causes of oncological death before the age of 50, 21% between 50 and 69 years and 14% after 70 years (AIOM, 2018). In Italy the relative survival, is 87% at 5 years and 80% at 10 years.

2.1.1 Risk Factors

Considering that most breast cancers are sporadic, it is difficult to clearly determine specific, and therefore predictable, risk factors.

Those factors that mostly determine the risk of developing breast cancer are:

SEX: The vast majority (99-99.5%) of breast cancer affects women. Male breast cancer is rare (Ruddy et al, 2013).

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 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.

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 (Rhodes 2002). Only in 7-8% of cases the tumor is defined as hereditary (Rovera et al, 2018). The mainly involved genes are BRCA1 and BRCA2 (genes with autosomal dominant transmission), which are normally involved in DNA repair processes (Karami and Mehdipour, 2013; Chen and Parmigiani, 2007). 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) (Michailidou et al, 2013).

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 (Kelsey et al, 1993; Colditz and Rosner, 2000). Nulliparity, a first full-term pregnancy in late age and failure to breastfeed are associated with an increased risk (Colditz and Rosner, 2000). 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 estrogens and the longer the glandular epithelium of the breast is exposed to them, the greater the risk of developing breast cancer is (Rovera et al, 2018). The literature of the last 20 years on the association between hormone replacement therapy (HRT) and breast cancer is very extensive and articulated (ESHRE Capri Workshop Group, 2004; Moskowitz et al, 2014). Users of hormonal contraceptives have a modest increase in the likelihood of illness compared to non-users and this risk returns to non-user population about 10 years after the suspension (Collaborative Group on Hormonal Factors in Breast Cancer, 1996).

DIETARY AND METABOLIC FACTORS: A high consumption of animal fats and alcohol seems to be associated with the development of breast cancer (Rovera et al, 2018). Obesity and the metabolic syndrome are recognized risk factors, probably linked to the excessive presence of adipose tissue, the main source of estrogen in menopause (Rosato et al, 2011). Protective role is instead played by physical education (Gonçalves et al, 2014).

RADIATION EXPOSURE: Women exposed to radiation therapy in the thoracic district, especially if at a young age, have a higher frequency of breast cancer (Clemons et al, 2000). The risk of disease increases proportionally to the amount of radiation received and to the earliness of the exposure (Moskowitz et al, 2014).

PRECANCEROUS LESIONS: A condition of epithelial hyperplasia without atypia is associated to a 1.5 to 2 times greater risk of developing a carcinoma. In atypical ductal or 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.

2.2 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 of 2013, breast cancers can be divided into five molecular subtypes according to their receptor status and gene expression profile. Each subtype is characterized by a specific prognosis and response to therapy:

Luminal A (40-55%): it is usually associated with a better prognosis. These tumors express hormonal receptors but not HER2. These tumors express ER, PgR> 20% and Ki67 <20% (Ahn et al, 2013); they are 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%; they are less differentiated and may benefit from chemotherapy as well as hormone therapy.

Luminal B HER2-positive (15-20%): they are positive to hormonal receptors, overexpress HER2 regardless of the proliferation index and they are of medium-high grade; they also benefit from monoclonal antibody therapy.

HER2-positive (non-luminal, 7-12%): overexpression of HER2 but no hormonal receptors, generally poorly differentiated, highly proliferating and associated to high risk of metastasis.

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

2.3 Therapy

The association between surgery and radiotherapy is the gold standard in conservative treatment of invasive breast cancer, in order to eliminate any foci of residual tumor cells after surgery. Indeed, radiotherapy after conservative surgery strongly impacts the risk of local recurrence (Truong et al, 2006; EBCTCG et al, 2014).

Radiation therapy can lead to acute (asthenia, skin erythema, epidemiolysis) or late toxicity (hardening and retraction of the breast, cutaneous dystrophy, telangiectasia, lymphedema of the arm) (Hickey et al, 2016; Coen et al, 2003).

Pharmacological approaches to breast cancer vary based on molecular characterization of the tumour. It can be divided into hormonal therapy, chemotherapy and targeted therapy, depending on the target affected by drugs.

Hormonal therapy may be used for patients with hormone-responsive breast cancer (i.e. with ER expression ≥ 1% or PgR ≥ 1%) (EBCTCG 2005). These tumors (Luminal A and B) are the most frequent in all age groups and particularly in older women (Clarke et al, 2012). The side effects of these drugs include sudden hot flushes, fluid retention and mood changes; less frequently it can be observed thromboembolic phenomena or an increased incidence of endometrial tumour (Zervoudis et al, 2010).

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) and its primary objectives are: obtain a downstaging of the neoplasia and control distant micrometastases through an early "sterilization" of the whole organism. This treatment aims to achieve a complete response 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 (EBCTCG 2005). The most widely used drugs are anthracyclines and taxanes.

In case of metastatic disease, it 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 metastatic 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) (Zervoudis et al, 2010; Sonmezer et al, 2006).

More than 20% of breast carcinomas express the HER2 protein (Santa-Maria et al, 2016), thus can be treated with targeted therapy. Although this characteristic is generally associated with unfavorable immunohistochemical characteristics, such as the lack of hormonal receptor expression, it has a further specific therapeutical perspective. This is represented by trastuzumab: monoclonal antibody drug able to selectively act on HER2 receptors (Moasser et al, 2015; Ahmed et al, 2015; Figueroa-Magalhães et al, 2014).

2.4 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 (Helleday, 2011; Ray Chaudhuri et al, 2016). 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 (Krejci et al, 2012), 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) (vonMinckwitz et al, 2014; Sikov et al, 2015; Tutt et al, 2015).

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 (Wendie et al, 2017). 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% (Akashi-Tanaka et al, 2015) 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 path- ways 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 7).

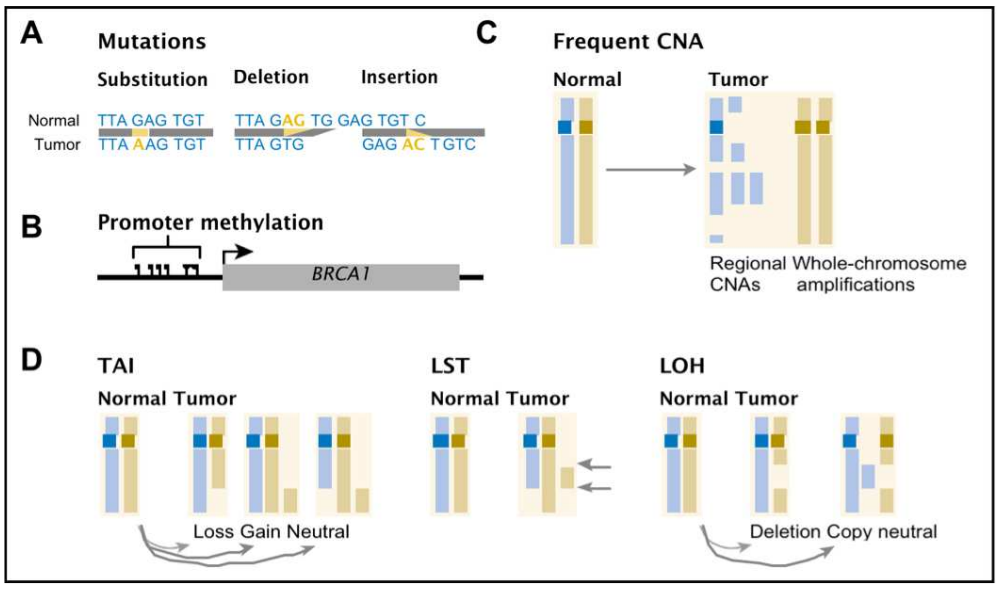

Fig. 7: possible driver modifications responsible for HR defects.

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.

2.5 TESTS OF DRIVER MUTATIONS

Sequence variants (or mutations) include substitutions, deletions, or insertions of nucleotides (Fig 7A). 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.

2.5.1 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 (Couch et al, 2016).

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 (Lord and Ashworth, 2016).

2.5.2 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 (Nik-Zainal et al, 2016). 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 (Nik-Zainal et al, 2016; Hennessy et al, 2010; Winter et al, 2016; Shah et al, 2012) 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 stand to 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.

2.5.3 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 (Fig 7C). 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 (Bergamaschi et al, 2006).

2.5.4 Structural Rearrangements

Inversions, translocations, and recombination change the location or orientation of a DNA sequence (Watkins et al, 2014). 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 7D). 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 (Birkbak et al, 2012) 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 (Abkevich et al, 2012). 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 (Timms et al, 2014).

While Triple Negative Breast Cancer (TNBC) is heterogeneous, a significant portion of TNBC is characterized by defective homologous recombination (HR) (Lord and Ashworth, 2016; Telli et al, 2018). 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. 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 (Lord and Ashworth, 2016; Telli et al, 2015).

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 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.

2.6 Breast Cancer Genes

Family linkage studies have identified high-penetrance genes, BRCA1, BRCA2, PTEN and TP53, which 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.

2.6.1 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. This was closely followed by discovery of BRCA2 on chromosome 13q12–13 in 1995. 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. 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. 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, and homeostasis of itself so that levels remain capable of maintaining genome integrity in response to genotoxic insult. 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.

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.

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.

2.6.2 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. 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. 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 telangiectasiamutated (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. 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. 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.

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. The relative risk of BC in heterozygous ATM female carriers has been estimated in 2.37 (95% CI, 1.51–3.78) that of the general population. 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.

MRN (MRE11–RAD50–NBS1) COMPLEX

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

Mutations in all three members of the MRN complex have been noted in human cancers. Mutations of MRE11, NBS1 and RAD50 manifest as ataxiatelangiectasia-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). 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 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. 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 pathwayrelated proteins were also BC susceptibility genes did not unfold until BRIP1 was identified in FANC-J patients. 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. 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. The discovery of another BC predisposition gene in the FA pathway PALB2 or FANCN, suggests that proteins acting downstream of monoubiquitinated FANCD2 and FANCI increase the risk of BC, while those acting upstream do not. 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. 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. As with BRCA2 heterozygotes, PALB2 mutations have been associated with an increased risk of pancreatic cancer.

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. BRCA2 stimulates RAD51-dependent strand exchange. RAD51C was discovered to be the cause of a Fanconi-like disorder and is a new cancer susceptibility gene. 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 distinctive behaviour from the situation observed with monoallelic mutations in the FA-related genes PALB2 and BRIP1, which are rarely present with OC. What it is even more striking, is that apparently there was complete segregation of the mutations to 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. Investigators have recently sequenced RAD51D in 911 wild-type BRCA1/2 breast-ovarian cancer families as well as 1,060 population controls. Inactivating mutations were identified in 8 in 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 present 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.

A homozygous frameshift mutation in XRCC2 being associated with a previously unrecognized form of FA was recently reported. 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. 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. From other XRCC genes investigated, XRCC1 399Gln allele acts as a recessive allele in association with BC risk.

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. Associations with other chromosomal regions, 2q35, 5p12, 6q22, and 16q12, also have been reported. 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. Moreover, it has been shown that specific FGFR2, MAP3K1, and TNRC9 variants may interact with BRCA1 and BRCA2 mutations to increase BC risk.

3 The human blood microbiome and septic risk

3.1 The human blood microbiome (HBM)

In the last decades several studies focused on microbiome, i.e. the analysis of the entire DNA content of microorganisms living in our body.

More recently, scientists suggest the possibility of the existence of a "healthy" human blood-microbiome (HBM), in contrast with the previous concept of blood as a sterile tissue.

The first report of bacteria in blood dates back to 1674, when Antonie van Leeuwenhoek observed bacteria together with erythrocytes in salmon blood (Bessis and Delpech, 1981). In 1969, Tedeschi and colleagues firstly suggested the possible presence of mycoplasma-like or L-phase bacteria, i.e. bacteria without the cell wall, in blood of healthy individuals (Tedeschi et al, 1969).

The possibility to identify the presence of bacteria with a 16S qPCR pushed forward the analysis on HBM, and from 2000 several publications described the presence of bacteria in blood of healthy people (Nikkari et al, 2001; McLaughlin et al, 2002; Amar et al, 2013).

It has been demonstrated that in some pathological conditions, such as cirrhosis, inflammatory or cardiovascular diseases, sepsis or diabetes, bacteria probably originating from the gut can cross the gut wall and migrate toward tissues (Potgieter et al, 2015; Manzo et al, 2015; Amar et al, 2011; Amar et al, 2013; Wang et al, 2015; Bellot et al, 2010).

In 2016 Païsse and colleagues described in details blood microbiome of 30 healthy individuals, evidencing the presence of a diversified microbiome that can be implied in transfusion-transmitted bacterial infections. In this work, they performed a 16S rDNA quantification by real time qPCR and a targeted 16S metagenomic sequencing of the V3-V4 hypervariable region on whole blood and its fractions, i.e. plasma, buffy coat and red blood cells (Païsse et al, 2016).

The first approach resulted in a differential distribution of bacterial DNA between blood fractions and individuals: they observed a strong correlation between white blood cells concentration and 16S DNA copy values in the buffy coat, suggesting a constant average quantity of bacterial DNA per immune cell, while the main difference between bacterial DNA among individuals was observed in the red blood cell compartment (Païsse et al, 2016).

Bacterial profiles obtained with the metagenomic analysis described that the Proteobacteria phylum was the most represented, followed by the Actinobacteria one, while Firmicutes and Bacteroidetes phyla are less represented, differently from what is observed in gut microbiota (Païsse et al, 2016).

Taken together, these data represent the first comprehensive description of the HBM in healthy donors.

Further analysis revealed how HBM diversity is affected in some pathological conditions: Traykova

and colleagues, targeting the 16S gene by qPCR, evidenced an increased number of bacterial species and DNA in patients affected by cirrhosis (Traykova et al, 2017); higher number of 16S DNA gene copies was observed also in patients with severe acute pancreatitis, with an increase in *Bacteroides* and *Actinobacteria* compared to healty controls (Li et al, 2018); an interesting study conducted in type-2 diabetes affected patients associated an increased risk in developing diabetes in people 'carrying' the *Sediminibacterium* genus, while those presenting the *Bacteroides* genus showed a lower risk (Qui et al, 2019).

Bacterial DNA in blood can be used as a powerful marker in several clinical settings. Bossola and colleagues associated the presence of bacterial-derived DNA fragments in blood of patients undergoing chronic haemodialysis with increased levels of C-reactive protein and interleukin 6 (IL-6), two well known markers of inflammation, a typical condition associated with maintenance haemodyalisys (Bossola et al, 2008). Simimlarly, in 2013 the abundance of bacterial-derived DNA in blood of patients subjected to peritoneal dialysis correlates to the systemic inflammatory status, only hypothesizing the possible use of bacterial-derived DNA as a prognostic marker of renal failure (Kwan et al, 2013).

All these works analysed DNA extracted from whole blood or its fractions, thus considering live, dormant and/or dead bacteria, either free circulating and associated with blood cells. A couple of works focused on circulating cell-free DNA as the unique source of information.

A first study on lung transplanted patients aimed to set up a diagnostic assay able to monitor simultaneously rejection and infection status by performing shotgun sequencing of cfDNA followed by identification of donor-derived single nucleotide polymorphisms (SNPs) and nonhuman cfDNA sequences (De Vlaminck et al, 2015). They observed a good correlation between donor-derived cfDNA and clinical indicators of graft dysfunction, such as pulmonary function tests or tests for cytomegalovirus, that is strictly associated to allograft injury (De Vlaminck et al, 2015).

The analysis of nonhuman sequences revealed cases of undiagnosed infection among patient tested associated to pathogens, such as human herpesviruses and adenoviruses, which are not always tested. These data taken together suggest the utility to utilize cfDNA as a monitoring marker for multiple parallel analysis, such as detection of broad spectrum of infections and graft rejection (De Vlaminck et al, 2015).

A further analysis of patients with different transplants (bone marrow, lung, heart) or pregnant women by shotgun sequencing of cell-free DNA identifyed novel contigs of DNA sequences with no to little homology in databases, suggesting the presence of uncharacterized and divergent microbes colonizing the human body. Interestingly, these contigs were observed in almost every sample analysed (Kowarsky et al, 2017).

Recently, a microbial cell-free DNA sequencing test, the Karius test, was validated clinically and analytically to identify and quantify 1250 bacteria, DNA viruses, fungi and eukaryotic parasites of clinical interest in a clinically relevant time (Blauwkamp et al, 2019).

All these information lead us to the introduction of the concept of culture independent microbiology, i.e. the possibility microbial cfDNA furnishes to obtain information on microbes and drive clinician toward therapy without the need of cultures (Peaper and Durant, 2019). Several open questions need to be answered, such as which cutoff must be chosen to differentiate pathogens from nonpathogens, RNA-based assays to obtain information on clinically relevant RNA viruses, how to interpret multiple microbe identification or the lack of information on the localization of the infection (Peaper and Durant, 2019).

3.2 Septic risk

In this perspective, we asked if cfDNA analysis and 'liquid biopsy' can be a monitoring tool in conditions in which no time course monitoring is possible.

Patients with renal disease and that require dialysis are frequently affected by systemic infections that often lead to death (Sarnak and Jaber, 2000). Overwhelming uremia is surely one leading cause of the high number of infection because it is associated to alteration in host defence mechanisms (Naqvi and Collins, 2006). Sepsis and pneumonia are the most frequent infection in patient affected by chronic kidney disease (CKD). Haemodialysis patients are exposed to several infection risks, due to vascular access, such as central venous catheter, that plays an important role in bloodstream infection development (Naqvi and Collins, 2006). The main problem associated with sepsis in haemodialysis patients is that diagnosis is not so simple to be formulated. By now, possible septic patients are identified by the simultaneous presence of an infection and two or more systemic inflammatory response syndrome (SIRS) criteria (Abou Dagher et al. 2015). SIRS criteria are: a) temperature higher than 38°C or lower than 36°C; b) heart rate higher than 90 beats/min; c) respiratory rate higher than 20 breaths/min or $PaCO_2$ lower than 32 torr; d) white blood cell counts higher than 12000 cells/mm^3 or lower than 4000 cells/mm^3 or the presence of more than 10% immature neutrophils ("band cells") (Jaimes et al 2003).

4 Transplant monitoring

The possibility to monitor graft status by cfDNA analysis dates back to 1998, when Lo and colleagues performed a Y-specific PCR in women who had received liver or kidney transplant from a male donor (Lo et al, 1998), resembling the analysis performed on pregnant women with male foetuses one year before. Even if working, this approach was limited by the necessity to have a 'Y-free' background and a 'Y-positive' graft.

The discovery of Single Nucleotide Polymorphisms (SNPs) expanded monitoring possibility toward every transplant in which the donor and the recipient present variation in one or several SNPs.

Several works take advantage of this characteristic to identify donor-derived cfDNA (dd-cfDNA) among recipient cfDNA in heart (Snyder et al, 2011; Beck et al, 2013; De Vlamink et al, 2014), liver (Beck et al, 2013) kidney (Beck et al, 2013; Bloom et al, 2016) or lung (De Vlaminck et al, 2015).

Both Snyder and Bloom observed how a 1% dd-cfDNA/recipient cfDNA corresponds to a normal graft status while increases in this ratio are significantly associated to active Ab mediated rejection (ABMR; Bloom et al, 2016) or an occurring organ rejection (Snyder et al, 2011).

Beck and colleagues had the possibility to monitor dd-cfDNA dynamics from liver transplantation onwards, detecting a ~90% graft-derived cfDNA just after transplantation, falling down to <15% at day 10 in complication free patients or rising back to >60% in two patients with biopsy-proven rejection (Beck et al, 2013).

Interestingly, the analysis on SNPs in cfDNA of heart transplanted patients performed by NGS sequencing revealed elevated dd-cfDNA even 5 months before moderate-to-severe rejection events, suggesting a powerful predicting ability not observed in concomitant biopsies (De Vlamink et al, 2014).

Analysis of dd-cfDNA can also be combined with other clinical parameters that indicate graft rejection, such as creatinine (Bloom et al, 2016) or Donor Specific Antibodies (DSA) to HLA antigens (Stanley et al, 2018) in kidney, in order to achieve a better evaluation of transplant status.

We thus decided to verify the applicability of the AmpFlSTR® Identifiler® Plus PCR Amplification kit on the analysis of cfDNA in kidney transplanted patients, in order to monitor graft rejection events with a certified kit commonly used to genotype donor and recipient and determine their compatibility.

1 Validation of the cfDNA extraction protocol

1.1 Preanalytical considerations

Preanalytical procedures have a significant impact on results, accounting for 46-68% of the error rate (Carraro et al, 2012; Plebani, 2012). This data suggest the necessity of standard operating procedures that ensure reproducible results among different source area and clinical settings (Basso et al, 2017).

We followed indications by El Massaoudi and colleagues to reduce effects on cfDNA concentration and fragmentation: plasma as a source, first centrifugation within 4h, a second high-speed centrifugation and only one plasma freeze-thaw cycle, if necessary (El Massaoudi et al, 2015).

1.2 Samples collection

We firstly collect samples of patients with breast cancer, due to higher availability, to validate the cfDNA 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 Tab (see results).

1.3 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).

1.4 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.

1.5 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.

2 Breast cancer

2.1 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:≥20mm; 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.

2.2 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.

2.3 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.

2.4 cfDNA extraction

Circulating DNA has been extracted as described in the paragraph 1.3.

2.5 Target sequencing procedure

2.5.1 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 and a 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 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 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 below) 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.

2.5.2 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.

2.5.3 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.

3 Septic risk

3.1 Project structure and inclusion criteria

We enrolled patients undergoing dialysis and presenting clinical symptoms suggesting an emoculture analysis to verify the presence of bacterial systemic infection.
Blood collection, plasma separation and cfDNA extraction have been performed as described in 1.2 and 1.3.

3.2 Library preparation and sequencing

Library preparation was performed following manufacturer indications and libraries passed quality control (QC) analysis. Shotgun sequencing was performed on a MiSeq v3 platform with these conditions: 2x150 bp, 10 million paired-end reads per sample.

3.3 Metagenomic analysis

QC analysis on reads was evaluated in terms of *phred* score. Sequencing data with *phred* score above 20, indicating a good reliability of the base read, were sent to CosmosID® to be analysed.
This company has curated and proprietary databases of 31064 bacterial, microbial and animal genomes. The ownership bioinformatics algorithms, summed to their database, allow them to finely determine strain level of microorganisms and to identify the presence of genes conferring resistance to antibiotics or coding for virulence factors.

4 Transplant monitoring

4.1 Project structure and inclusion criteria

We started collecting blood samples from kidney-transplanted patients with clinical symptoms indicating a possible rejection event, thus directed to renal biopsy to evaluate graft status.
Blood collection, plasma separation and cfDNA extraction have been performed as described in 1.2 and 1.3.

4.2 Detection of polymorphisms

The analysis on microsatellite polymorphisms was performed with the AmpF*l*STR® Identifiler® Plus PCR Amplification kit, that simultaneously amplify 15 microsatellite loci characterized by polymorphisms (Fig. 9). Each patient is analysed in parallel to DNA extracted from blood of the donor and of the recipient.

Locus designation	Chromosome location	Alleles included in Identifiler® Plus Allelic Ladder	Dye label	Control DNA 9947A
D8S1179	8	8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	6-FAM™	13‡
D21S11	21q11.2-q21	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38		30§
D7S820	7q11.21-22	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 11
CSF1PO	5q33.3-34	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 12
D3S1358	3p	12, 13, 14, 15, 16, 17, 18, 19	VIC®	14, 15
TH01	11p15.5	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3		8, 9.3
D13S317	13q22-31	8, 9, 10, 11, 12, 13, 14, 15		11#
D16S539	16q24-qter	5, 8, 9, 10, 11, 12,13, 14, 15		11, 12
D2S1338	2q35-37.1	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28		19, 23
D19S433	19q12-13.1	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2	NED™	14, 15
vWA	12p12-pter	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24		17, 18
TPOX	2p23-2per	6, 7, 8, 9, 10, 11, 12, 13		8‡‡
D18S51	18q21.3	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27		15, 19
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y	PET®	X
D5S818	5q21-31	7, 8, 9, 10, 11, 12, 13, 14, 15, 16		11§§
FGA	4q28	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2		23, 24

Fig. 9: list of microsatellites and relatives polymorphisms analysed by the AmpF*l*STR® Identifiler® Plus PCR Amplification kit.

4.2.1 PCR amplification

PCR reaction was performed on 1ng cfDNA in 10 µL of TE buffer. The reaction consists of 10 µL cfDNA, 10 µL AmpFlSTR® Identifiler® Plus Master Mix and 5 µL AmpFlSTR® Identifiler® Plus Primer Set, that allows the amplification of all the 15 polymorphic loci.

4.2.2 Electrophoresis

Each PCR products is analysed, together with an allelic ladder, by capillary electrophoresis in order to identify the length of the amplified polymorphisms.
Data are represented by an electropherogram (Fig 10) in which the presence of a specific polymorphism is revealed by a peak at a specific position on the x axis. The area under the peak is proportional to the abundance of the polymorphism.

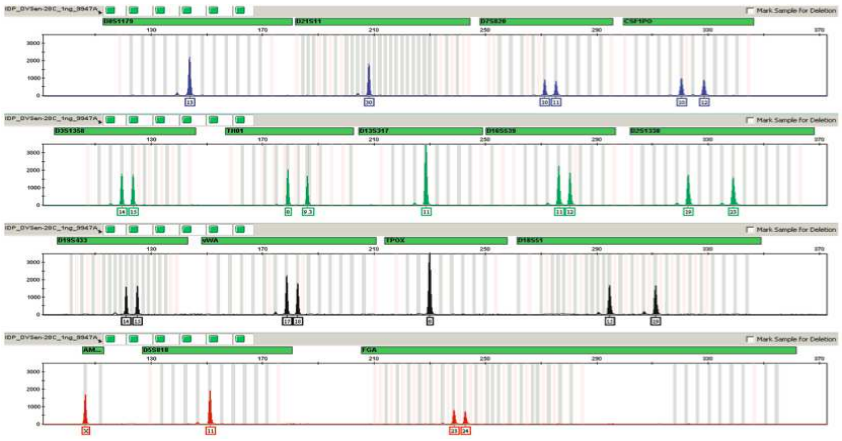

Fig. 10: electropherogram of a control DNA sample. Each green bar represent a microsatellite; each peak correspond to the amplification of a specific polymorphism. The area of the peak corresponds to the relative amount of the polymorphism.

4.2.3 Data analysis

Each peak in the electropherogram has two main informations: the size of the polymorphism and the area under the curve. The size of each polymorphism is used to determine if the polymorphism belongs to the donor or the recipient, while the area under the curve is proportional to the relative amount of the associated polymorphism.

4.3 Second sample collection

We then decided to apply this procedure to monitor dd-cfDNA dynamics from transplantation onward, to verify the possibility to use this analysis to monitor graft status and detect early rejection events, following the same procedure for plasma separation, cfDNA extraction and analysis described previously.

1 Validation of the extraction protocol

Samples collected to validate the extraction procedure have been analysed as described in Matherial and Methods (2.3). Quantifications of the samples are summarized in Table 1 below.

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

2 Breast cancer

2.1 DNA extraction

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 (3.2).

Quantification of these samples are summarized in Table 2 below.

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

2.2 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).

Fig 11 summarizes data regarding the total number of reads, those mapping and those with problematic 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%	I run
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%**	
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%	II run
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%**	
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%	III run
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%**	

Fig. 11: 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 Fig. 12.

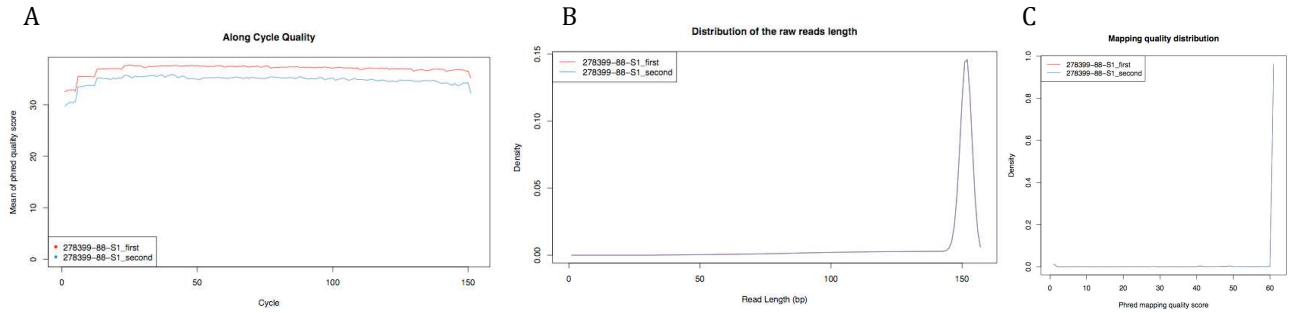

Fig. 12: example of reads quality evaluation in genomic DNA of patient 1. A) Along Cycle Quality measure the *phred* score, i.e. an evaluation of the correct base call, along the dimension of the fragment. A *phred* score value of 30 corresponds to a 99,9% accuracy of the base called during the sequencing. B) Distribution of the raw reads length: the peak at the end of the scale indicates that most 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 mappings 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. 13).

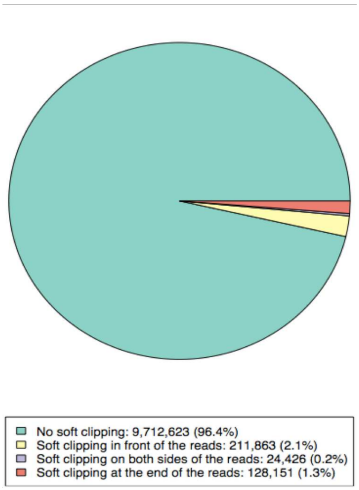

Fig. 13: pie chart illustrating the proportion of no soft clipping and soft clipping reads in genomic DNA of patient 1. As we can see in the box below the pie chart, >96% of the reads are no soft clipping, thus can be considered reliable.

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. 14).
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. 15).

	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. 14: 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. 15: 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).

2.3 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 can not 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).

2.3.1 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 81 that causes the formation of a stop codon, resulting in the truncation of the protein at Cys27 (c.80_81ins100 p.Cys27*) (box below).

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 | Definitely Pathogenic	

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

2.3.2 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 (box below).

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 | Definitely Pathogenic	
TP53 NM_000546	5	c.526T>A p.(Cys176Ser)	2.5 % (4924 / 122)	missense	Flagged Pathogenicity 4 | 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% (figure below).

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 | Likely Pathogenic	Conflicting interpretations of pathogenicity rs967461896

2.3.3 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.

2.3.4 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% (box below).

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 | Definitely Pathogenic	Pathogenic rs397507917
TP53 NM_000546	4	c.372_375 + 2delinsG p.(?)	14.1 % (4939 / 798)	splice_donor_cd	Flagged Pathogenicity 4 | Likely Pathogenic	

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

3 Septic risk

3.1 Validation of the analysis

We have firstly performed the analysis on 5 samples, to verify feasibility of the study.

Circulating DNA was extracted as previously described.

We performed a shotgun sequencing on whole cfDNA on a Illumina MiSeq v3 platform, following these conditions: 1x150 bp, single reads and 5 million reads/sample.

All the samples have high quality (*phred* score higher than 20, fig. 16) so sequencing results have been sent to CosmosID to perform a metagenomic analysis on bacterial sequences.

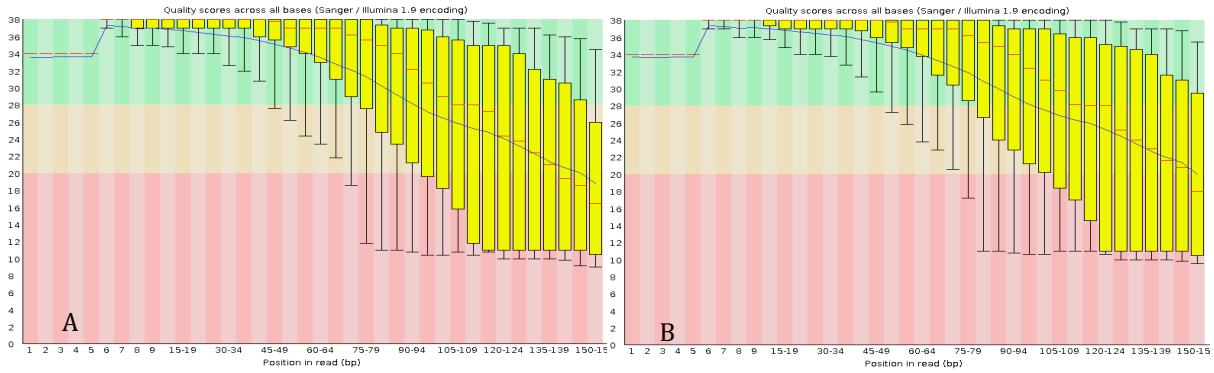

Fig. 16: diagrams repreesentig the *phred score* correlated to bp position in the sequence. Quality drop observed in last bases is due to Illumina Chemistry during sequencing. A) Sample: 92014656. As we can see *phred score* indicates a good sequencing. B) Sample: B.A. CVP. As for the previous sample, *phred score* is enough high to consider sequences obtained reliable

The metagenomic analysis revealed the presence of variable percentage of bacterial sequences in each sample (Tab. 2). Only sequences belonging to the *Propionibacterium* branch overcome the threshold required to consider the detection reliable (Fig. 17).

SAMPLE	% OF HUMAN SEQUENCES
92014656	17.21
U.AG. CVP	40.67
B.A. CVP	54.21
A.F. CVP	10.02
91778569	19.74

Tab. 2: percentage of human reads in cfDNA samples analyzed.

Database	Organism	Relative Abundance	Above_Threshold
Bacteria	Propionibacterium_20962 Branch	66,83	Y
Bacteria	Pseudomonas_sp_FH4	13,41	N
Bacteria	Rubrobacter_xylanophilus_DSM_9941	2,887	N
Bacteria	Staphylococcus_hominis_6333 Branch	2,785	N
Bacteria	Streptomyces_sp_NRRL_B_2375	2,201	N
Bacteria	Acinetobacter_johnsonii_SH046	1,941	N
Bacteria	Lactococcus_lactis_subsp_cremoris_4115 Branch	1,913	N
Bacteria	Streptomyces_flaveus_strain_NRRL_B_1652	1,697	N
Bacteria	Acinetobacter_lwoffii_SH145	1,665	N
Bacteria	Rothia_mucilaginosa_20589 Branch	1,067	N
Bacteria	Brevundimonas_diminuta_ATCC_11568	1,023	N
Bacteria	Paracoccus_yeei_ATCC_BAA_599	1,015	N
Bacteria	Acinetobacter_sp_MII	0,821	N
Bacteria	Enhydrobacter_aerosaccus_SK60	0,7406	N
Antibiotic Resistance	Aminoglycoside_aph6_Id		N
Antibiotic Resistance	Aminoglycoside_aac6'_aph2''		N
Virulence Factor	Staphylococcus_aureus_GENE_AACA-APHD		N

Database	Organism	Relative Abundance	Above_Threshold
Bacteria	Propionibacterium_20956 Branch	83,2	Y
Bacteria	Pseudomonas_sp_FH4	7,696	N
Bacteria	Rubrobacter_xylanophilus_DSM_9941	1,515	N
Bacteria	Propionibacterium_acnes_HL201PA1	1,226	N
Bacteria	Acinetobacter_johnsonii_SH046	1,164	N
Bacteria	Streptomyces_flaveus_strain_NRRL_B_1652	1,152	N
Bacteria	Pseudomonas_syringae_pv_syringae_1212	1,126	N
Bacteria	Streptomyces_sp_NRRL_B_2375	0,9366	N
Bacteria	Acinetobacter_lwoffii_NIPH_478	0,833	N
Bacteria	Stenotrophomonas_maltophilia_K279a	0,6043	N
Bacteria	Acinetobacter_sp_CIP_51_11	0,5502	N
Virulence Factor	Staphylococcus_aureus_GENE_blaR1		N

Fig. 17: metagenomic analysis of cfDNA. As shown by the 'Above_Threshold' data, most of sequences analyzed are not enough confident to be considered sure. This analysis also allows us to detect the presence of antibiotic resistance genes and virulens factors. Box on the left represents results of the analysis performed on sample 92014656. Box on the right represent results of the anaysis performed on B.A. CVP.

This data can be explained by different conditions: bacterial circulating DNA is a fraction of total cfDNA, thus 5 million reads/samples may not be enough to detect with an high coverage and confidence bacterial sequences, resulting in below threshold results; the identification of a branch is less specific than bacterial strain, in this sense the metagenomic analysis sum sequences belonging to different *Propionibacterium* strains, resulting in a higher confidence.
To overcome these problems, we decided to improve the sequencing step by performing at least 10-20000 reads/sample.

We thus collect six more samples of blood from as much patients. We sequenced samples with these conditions: 1x150 bp, single reads and 10000 reads/sample. In four cases, plasma presented faint signs of haemolysis. We decided to perform analysis on these samples to verify the sensitivity of our technique in a sub-optimal condition.

Percentage of human reads in four slightly haemolysed samples was high, ranging from 94% to 97.6%, revealing a huge genomic contamination. As expected, no bacterial sequences were detected in 3 out of 4 samples. In sample PL, only *E.coli* was detected, but the low amount of reads did not allow us to consider it as a confident value (Fig. 18).

Bacteria

44072_ID1431_3-PL

Fasta/q details

Total results

Metric	Value
Reads Hits	966
Reads Total	10625051

Table

Name	Frequency	Unique Matches %	Total Matches %	Relative Adundance %	Tax ID
Escherichia coli	2	0.23	0.23	100.00	562

Fig. 18: identification of *Escherichia coli* in sample PL. As we can see, less than 1000 reads covers bacterial sequences, thus we can not consider this call statistically significant.

The analysis of the two other samples revealed the presence of three and two bacterial strains, respectively (Fig. 19).

Emoculture from patient summarized in fig. A revealed the presence of *Staphylococcus epidermidis*, while *Propionibacterium* or *Streptococcus* colonies were not detected.

The presence of sequences belonging to *Burkholderia multivorans* in patient B is particularly of interest. This bacterium has a culture time of 7-14 days, much more than the common 5 days of emocultures, thus limiting the possibility of detection by this procedure.

A

Database	Organism	Taxonomy	Above_Threshold
Bacteria	**Propionibacterium_species**		**Y**
Bacteria	**Staphylococcus_sp_E463**	**Bacteria > Firmicutes > Baci**	**Y**
Bacteria	Streptococcus_species Branch		Y
Bacteria	Clostridiales_Order_26997 Branch		N
Bacteria	Rothia_mucilaginosa_20589 Branch	Bacteria > Actinobacteria > A	N
Bacteria	Streptococcus_mitis_4053 Branch	Bacteria > Firmicutes > Bacil	N
Bacteria	Staphylococcus_haemolyticus_R1P1	Bacteria > Firmicutes > Bacil	N
Bacteria	Propionibacterium_acnes_SK182B_JCVI	Bacteria > Actinobacteria > A	N

B

Database	Organism	Taxonomy	Above_Threshold
Bacteria	**Burkholderia_multivorans_25203 Branch**	**Bacteria > Proteobacteria > Bet**	**Y**
Bacteria	Staphylococcus_sp_URHA0057	Bacteria > Firmicutes > Bacilli >	Y
Bacteria	StaphEpidermidis_d101_6055 Branch		N
Bacteria	Escherichia_coli_MS_153_1	Bacteria > Proteobacteria > Gar	N
Bacteria	Micrococcus_luteus_20595 Branch	Bacteria > Actinobacteria > Acti	N

Fig. 19: metagenomic analysis of two cfDNA samples. In green: data whose confidence is above threshold, i.e. the presence is sure. A) Patient A. B) Patient B.

4 Transplant monitoring

The analysis of the three samples showing clinical symptoms suggesting an occurring graft rejection and, for this reason, undergoing renal biopsy did not presented dd-cfDNA, suggesting that rejection was not occurring. These data were confirmed by biopsy analysis.

Fourth sample was analysed at four times: day 0 corresponding to 20 minutes after surgery; day 1, 4 and 8 are days after surgery.

As we can see from the figure 20 below, at the time of surgery we can detect nearly the same amount of polymorphisms belonging to the donor (e.g. sz148 and sz161 in panel E, sz166 or sz188 in panel F) and the recipier (e.g. sz153 and sz157 in panel E and sz172 and sz184 in panel F), while 1 day after surgery the areas of peaks corresponding to donor polymorphisms are highly reduced (panels G and H).

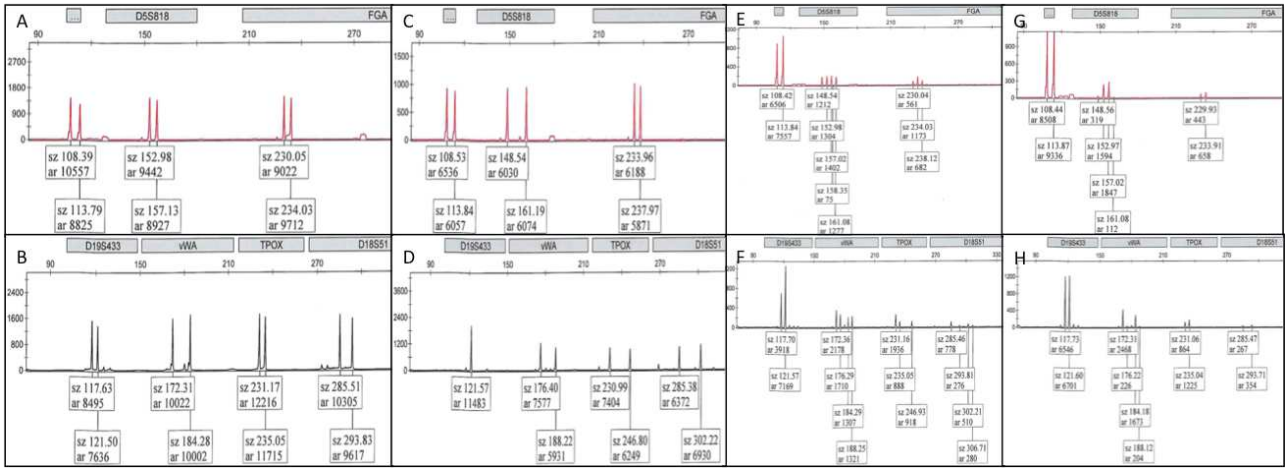

Fig. 20: electropherograms of polymorphisms belonging to recipier (panels A and B), donor (panels C and D), transplanted recipier at surgery (panels E and F) and 1 day after surgery (panels G and H). We can see in panels E and F the relative amount of polymorphisms detected, described by the ar value, with a nearly equal amount of donor and recipient polymorphisms just after surgery. Panels G and H showed reduced amount of donor derived cfDNA compared to recipient one.

Table 3 below summarizes percentage of recipient or donor cfDNA detected at different timepoints. The sudden reduction of percentage of dd-cfDNA at day 1 indicates that reperfusion injury is solving and graft damage is reducing. The complete abolishment of detection of dd-cfDNA at day 4 and 8 is an indicator of good transplant outcome.

		I.D.	TIME	RESULTS
4	DONOR	V.G.	DAY 0	≈ 53% DONOR
			DAY 1	≈ 10% DONOR
	RECIPIENT	T.G.	DAY 4	100% RECIPIER
			DAY 8	100% RECIPIER

Tab. 3: summary of detection of donor derived cfDNA in blood sample of recipient at day 0, 1, 4 and 8.

1 Breast cancer

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 gold 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 (Overman et al, 2013; Raaijmakers, Kirkels and Roobol, 2002). 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 (Shah et al, 2009). 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 (Schwarzenbach, Hoon and Pantel, 2011).

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 (Church et al, 2014), analyse the evolution of genetic abnormalities and monitoring tumour dynamics (Dawson et al, 2013; Ollson et al, 2013).

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 (Sozzi et al, 2003).

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

These two marker 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 (Bettegowda et al, 2014).

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 (Franczak et al, 2018).

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 samples analysed, either in genomic or circulating DNA.

In two genomic samples we observe the presence of mutations. Surprisingly, no mutations have been detected in cfDNA obtained from these two patients. One of the reason underlying this result is that, despite QC of the target enrichment protocol did not revealed problems during the protocol, starting cfDNA was not enough to detect mutations at that frequencies. To better verify this

hypothesis and avoid such an outcome, future experiments will be performed on a greater amount of starting material, in order to reduce the risk of analyse a sample that results unrepresentative of the circulating DNA pool.

In the last patient, a mutation in TP53 is observed in both genomic and circulating DNA, while the second mutation identified is present only in the DNA extracted from the fresh tissue. This latter situation can be explained as the two mentioned above.

Interestingly, the VF of the mutation in TP53 is higher in cfDNA compared to genomic DNA. This data can be explained by the fact that it is probable that the tissue analysed contained only a fraction of the clone harbouring this mutation, while cfDNA, reflecting the whole tumour heterogeneity, is characterized by a higher frequency of mutated sequences.

2 Septic risk

The analysis of our 11 patients allowed us to set up the workflow and to verify the feasibility of our project.

With the first analysis we observed a significant detection of just a *Propionibacterium* branch in two out of five samples. If from one side this data is compatible with the low amount of reads per sample, on the other the presence of *Propionibacterium* can be due to sample contamination. It is well reported in the literature that *Propionibacterium* bacteria can colonize foreign-body (Braun et al, 2013), especially prosthesis and venous accesses, giving rise to severe infections (Brook and Frazier, 1991). By now there is no way to determine, based only on sequence type and amount, a threshold that distinguishes a pathogenic from a non-pathogenic bacterium (Peaper and Durant, 2019).

Second set of samples allows us to further confirm the necessity to perform high numbers of reads per sample, as observed in patients A and B where we detected three and two bacterial strain, respectively. Parallelly, the analysis of slightly haemolysed samples results in no statistical relevant information on bacterial DNA fragments, suggesting the necessity to have high quality plasma and the possibility to introduce quality control analysis of plasma haemolysis more sensitive than visual inspection.

Data obtained for samples A and B are quite promising. In the first case we have the simultaneous detection of *Staphylococcus* in emoculture and metagenomic analysis, while this latter indicates the presence of *Propionibacterium* and *Streptococcus* too.

Metagenomic analysis on patient B revealed the presence of *Burkholderia multivorans*, a slowly growing bacterium that is hardly identified in emoculture due to a 7-14 days growing time. This data is probably the most interesting: a culture independent microbiology approach (Peaper and Durant, 2019), i.e. the possibility to identify the presence of bacteria independently of emoculture growing time and of bacterial ability to grow in standard emoculture conditions, by analysing circulating bacterial DNA can revolutionise microbiology the way we have seen it till now.

3 Transplant monitoring

The application of cfDNA analysis on graft monitoring has been widely described in the literature as a promising tool to avoid invasive and risky biopsies.

In our project we want to verify the possibility to apply a well-established kit, commonly used to genotype and evaluate compatibility between donor and recipient, to monitor graft status and to early detect rejection events.

The application of the AmpF*l*STR® Identifiler® Plus PCR Amplification kit to plasma samples allows us to identify the presence of donor derived polymorphisms in cfDNA collected just after and 1 day after surgery. Since 4 days after surgery we do not observe dd-cfDNA, suggesting a complete recovery from reperfusion damage.

There is limited information in the literatures regarding dd-cfDNA dynamics during early days after transplant. Beck and colleagues observed a 90% graft-derived cfDNA just after liver transplantation, rapidly decreasing to >15% at day 10 (Beck et al, 2013). Shen and colleagues observed a 20,69% dd-cfDNA levels after transplant, that decrease to 5.22% on the first day and then remain stable (Shen and Zhou et al, 2018).

Our data are in accordance with the literature in observing a decreasing level of dd-cfDNA in the first days post surgery but a huge amount of data must be collected to perform statistical analysis.

FUTURE PERSPECTIVES

1 Breast cancer

One limitation of this study is the reduced amount of samples analysed. 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 status and development of the disease.
Analysis of DNA extracted from peripheral white blood cells (WBC) can give us a deeper understanding of the somatic and germline mutational landscape in our patients, 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.

2 Septic risk

We will increase sample collected and analysed, in order to verify the accuracy of our technique. The collection of control samples, possibly from healthcare professionals that are in contact with patients analysed, will allow us to determine if the detection of some bacteria can be ascribed to environmental/procedural contamination or it is reasonable to consider it as an infection.
The possibility to have long-term emocultures will help us in verifying sensitivity of our technique in detecting low growing bacteria such as *Burkholderia.*
Further increase in read per sample will be considered in order to increase information obtained by the metagenomic analysis, in particular regarding the presence of viral factors and, even more, genes conferring resistance to antibiotics, to better direct therapeutic approaches.

3 Transplant monitoring

Our preliminary data suggest the possibility to effectively monitor graft status with this technique, as evidenced by data obtained on the fourth patient.

To further strengthen these data we are collecting more samples with time points described but also at several months after surgery or until the occurrence of clinical markers suggesting a rejection event.

The correlation with clinical data will allow us to better understand the appropriateness of our conclusion based on cfDNA data.

Finally, the availability of samples to retrospectively analyse patient having a rejection event will be extremely important to validate the sensitivity of our procedure in detecting early rejection events, thus considering it as a real monitor tool that can furnish information earlier compared with standard clinical procedures.

Bibliography

Abkevich V, Timms KM, Hennessy BT, et al: Patterns of genomic loss of heterozygosity predict homologous re- combination repair defects in epithelial ovarian cancer. Br J Cancer 107:1776-1782, 2012

Abolhassani M, Tillotson J and Chiao J. (1994). Characterization of the release of DNA by a human leukemia-cell line HL-60. International Journal of Oncology. 4(2):417–421.

Adams DH and Gahan PB. (1983). The DNA extruded by rat spleen cells in culture. The International Journal of Biochemistry. 15(4): 547–552.

Adams DH and McIntosh AA. (1985). Studies on the cytosolic DNA of chick embryo fibroblasts and its uptake by recipient cultured cells. The International Journal of Biochemistry. 17(10):1041– 1051.

Ahmed S, Sami A, Xiang J. HER2-directed therapy: current treatment options for HER2-positive breast cancer. Breast Cancer. 2015;22(2):101-116.

Ahn HJ, Jung SJ, Kim TH, Oh MK, Yoon H-K. Differences in Clinical Outcomes between Luminal A and B Type Breast Cancers according to the St. Gallen Consensus 2013. J Breast Cancer. 2015;18(2):149-159.

AIOM. AIRTUM. Fondazione AIOM. I numeri del cancro in Italia 2018. 2018.

Akashi-Tanaka S, Watanabe C, Takamaru T, et al: BRCAness predicts resistance to taxane-containing regimens in triple negative breast cancer during neoadjuvant chemotherapy. Clin Breast Cancer 15:80-85, 2015

Amatu A, Schirripa M, Tosi F, et al (2019) High Circulating Methylated DNA Is a Negative Predictive and Prognostic Marker in Metastatic Colorectal Cancer Patients Treated With Regorafenib. Front. Oncol. 9:622.

Anker P and Stroun M. (1972). Bacterial ribonucleic acid synthesis in frog organs after intraperitoneal injection of bacteria. Biochemical Journal. 128(3):101P.

Anker P, Jachertz D, Maurice PA and Stroun M (1984) Nude mice injected with DNA released by antigen stimulated human T lymphocytes produce specific antibodies expressing human characteristics. Cell Biochemistry and Function 2(1):33–37.

Anker P, Stroun M and Maurice P (1977) Characteristics of nucleic acids excreted by non-stimulated normal human lymphocytes. Schweizerische Medizinische Wochenschrift 107(41):1457.

Anker P, Stroun M and Maurice PA. (1975). Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. Cancer Research. 35(9):2375–2382.

Assou S, Aït-Ahmed O, El Messaoudi S, et al. (2014). Non-invasive pre-implantation genetic diagnosis of X- linked disorders. Medical Hypotheses. 83(4):506–508.

Basso D, Padoan A, Laufer T, et al. Relevance of pre-analytical blood management on the emerging cardiovascular protein biomarkers TWEAK and HMGB1 and on miRNA serum and plasma profiling. Clin Biochem. 2017 Mar;50(4-5):186-193.

Basso D, Padoan A, Laufer T, et al., Relevance of pre-analytical blood management on the emerging cardiovascular protein biomarkers TWEAK and HMGB1 and on miRNA serum and plasma profiling. Clin Biochem (2016).

Beiter T, Fragasso A, Hudemann J, et al (2014) Neutrophils release extracellular DNA traps in response to exercise. Journal of Applied Physiology (Bethesda, Md: 1985) 117(3):325–333.

Bendich A, Wilczok T and Borenfreund E (1965) Circulating DNA as a possible factor in oncogenesis. Science (New York, NY) 148(3668):374–376.

Bergamaschi A, Kim YH, Wang P, et al: Distinct patterns of DNA copy number alteration are associated with different clinicopathological features and gene-expression subtypes of breast cancer. Genes Chromosomes Cancer 45:1033-1040, 2006

Bettegowda C, Sausen M, Leary RJ, et al Jr.Sci Transl Med. 2014 Feb 19;6(224):224ra24. doi: 10.1126/scitranslmed.3007094.

Birkbak NJ, Wang ZC, Kim JY, et al: Telomeric allelic imbalance indicates defective DNA repair and sensitivity to DNA-damaging agents. Cancer Discov 2:366-375, 2012

Brinkmann V, Reichard U, Goosmann C, et al. (2004). Neutrophil extracellular traps kill bacteria. Science (New York, NY). 303(5663):1532–1535.

Carraro P, Zago T and Plebani M Exploring the initial steps of the testing process: frequency and nature of pre-preanalytic errors, Clin. Chem. 58 (2012) 638–642.

Casals SP, Friou GJ and Myers LL. Significance of Antibody to DNA in Systemic Lupus Erythematosus. Arthritis And Rheumatism, Vol.7, No.4 (August), 1964.

Ceppellini, R., Polli, E., and Celada, F.: A DNA reacting factor in serum of a patient with lupus erythematosus diffuses. Proc. SOC.Exptl. Biol. & Med. 96:572, 1957.

Chang CPY, Chiaa RH, Wu TL, et al (2003) Elevated cell-free serum DNA detected in patients with myocardial infarction. Clinica Chimica Acta 327 (2003) 95 – 101.

Chen S, Parmigiani G. Meta-analysis of BRCA1 and BRCA2 penetrance. J Clin Oncol. 2007;25(11):1329-1333.

Church TR, Wandell M, Lofton-Day C, et al. PRESEPT Clinical Study Steering Committee, Investigators and Study Team.Gut. 2014 Feb;63(2):317-25. doi: 10.1136/gutjnl-2012-304149. Epub 2013 Feb 13.

Chused TM, Steinbergt AD and Talal N (1972) The Clearance and Localization of Nucleic Acids by New Zealand and Normal Mice. Clin. Exp. Immunol. (1972) 12, 465-476.

Clemons M, Loijens L, Goss P. Breast cancer risk following irradiation for Hodgkin's disease. Cancer Treat Rev. 2000;26(4):291-302.

Coen JJ, Taghian AG, Kachnic LA, Assaad SI, Powell SN. Risk of lymphedema after regional nodal irradiation with breast conservation therapy. Int J Radiat Oncol Biol Phys. 2003;55(5):1209-1215.

Colditz GA, Rosner B. Cumulative risk of breast cancer to age 70 years according to risk factor status: data from the Nurses' Health Study. Am J Epidemiol. 2000;152(10):950-964.

Cools-Lartigue J, Spicer J, McDonald B, et al (2013) Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. The Journal of Clinical Investigation.

Cooper PR, Palmer LJ, Chapple ILC (2013) Neutrophil extracellular traps as a new paradigm in innate immunity: friend or foe? Periodontology 2000 63(1):165–197.

Couch FJ, Hu C, Lilyquist J, et al: Breast cancer risks associated with mutations in cancer predisposition genes identified by clinical genetic testing of 60,000 breast cancer patients. San Antonio Breast Cancer Symposium, San Antonio, TX, December 5-10, 2016 SABCS abstract (abstr S2-01)

D'Souza-Schorey C and Clancy JW. Tumor-derived microvesicles: shedding light on novel microenvironment modulators and prospective cancer biomarkers. Genes Dev. (2012) Jun 15;26(12):1287-99.

De Jong OG, Van Balkom BWM, Schiffelers RM, et al. (2014). Extracellular vesicles: potential roles in regenerative medicine. Frontiers in Immunology. 5:608.

Demers M and Wagner DD (2013) Neutrophil extracellular traps: A new link to cancer-associated thrombosis and potential implica- tions for tumor progression. Oncoimmunology 2(2):e22946.

DeSantis C, Ma J, Bryan L, Jemal A. Breast cancer statistics, 2013. CA Cancer J Clin. 2014;64(1):52-62.

Diehl F, Schmidt K, Choti MA, et al (2008) Circulating mutant DNA to assess tumor dynam- ics. Nature Medicine 14(9):985–990.

Dorsch CA, Chia D, Levy L and Barnett EV. (1975). Persistence of DNA in the circulation of immunized rabbits. Rheumatology. 212:161.

EBCTCG (Early Breast Cancer Trialists' Collaborative Group), McGale P, Taylor C, et al. Effect of radiotherapy after mastectomy and axillary surgery on 10-year recurrence and 20-year breast cancer mortality: meta-analysis of individual patient data for 8135 women in 22 randomised trials. Lancet (London, England). 2014;383(9935):2127-2135.

El Messaoudi S, Rolet F, Mouliere F and Thierry AR. (2015) Circulating cell free DNA: Preanalytical considerations. Clinica Chimica Acta 424 (2013) 222–230.

Emlen W. and Mannik M (1984) Effect of DNA size and strandedness on the *invivo* clearance and organ localization of DNA. Clin. Exp. Immunol. (1984)56,185-192.

Emlen W. and Mannik M. (1978) Kinetics and mechanisms for removal of circulating single-stranded DNA in mice. J.Exp.Med.147,684.

Fehri E, Ennaifer E, Bel Haj Rhouma R, et al. The role of Toll-like receptor 9 in gynecologic cancer. Curr Res Transl Med 2016, 64: 155–159.

Fernando MR, Jiang C, Krzyzanowski GD and Ryan WL. (2017). New evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes. PLoS ONE. 12(8): e0183915.

Figueroa-Magalhães MC, Jelovac D, Connolly R, Wolff AC. Treatment of HER2-positive breast cancer. Breast. 2014;23(2):128-136.

Franczak C, Filhine-Tressarieu P, Broséus J, Gilson P, Merlin JL, Harlé A.Arch Med Res. 2018 Jul;49(5):297-305. doi: 10.1016/j.arcmed.2018.10.007. Epub 2018 Nov 7.

Gahan PB and Stroun M (2010) The virtosome-a novel cytosolic informative entity and intercellular messenger. Cell Biochemistry and Function. 28(7):529–538.

Galeazzi M, Morozzi G, Piccini M, et al (2003) Dosage and characterization of circulating DNA: present usage and possible applications in systemic autoimmune disorders. Autoimmunity Reviews 2(1) 50-55.

García-Olmo DC, Domínguez C, García-Arranz M, et al (2010) Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. Cancer Research 70(2):560–567.

Giacona MB, Ruben GC, Iczkowski KA, et al. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. Pancreas. 1998 Jul;17(1):89-97.

Gonçalves AK, Florêncio GLD, de Atayde Silva MJM, Cobucci RN, Giraldo PC, Cote NM. Effects of Physical Activity on Breast Cancer Prevention: A Systematic Review. J Phys Act Heal. 2014;11(2):445-454.

Hariton-Gazal E, Rosenbluh J, Graessmann A, et al (2003) Direct translocation of histone molecules across cell membranes. Journal of Cell Science 116(Pt 22):4577–4586.

Helleday T: The underlying mechanism for the PARP and BRCA synthetic lethality: Clearing up the mis- understandings. Mol Oncol 5:387-393, 2011

Hennessy BTJ, Timms KM, Carey MS, et al: Somatic mutations in BRCA1 and BRCA2 could expand the number of patients that benefit from poly (ADP ribose) polymerase inhibitors in ovarian cancer. J Clin Oncol 28:3570-3576, 2010

Hickey BE, Lehman M, Francis DP, See AM. Partial breast irradiation for early breast cancer. Cochrane database Syst Rev. 2016;7:CD007077.

Holl EK, Shumansky KL, Pitoc G, et al (2013) Nucleic acid scavenging polymers inhibit extracellular DNA-mediated innate immune activation without inhibiting anti-viral responses. PloS One 8(7):e69413.

Jahr S, Hentze H, Englisch S, et al. (2001). DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Research. 61(4):1659–1665.

Kaplan MJ, Radic M. (2012). Neutrophil extracellular traps (NETs): Double-edged swords of innate immunity. Journal of Immunology (Baltimore, Md : 1950). 189(6):2689–2695.

Karami F, Mehdipour P. A comprehensive focus on global spectrum of BRCA1 and BRCA2 mutations in breast cancer. Biomed Res Int. 2013;2013:928562.

Kawai T and Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature Immunology 11(5):373–384.

Kawasaki T, Kawai T and Akira S (2011) Recognition of nucleic acids by pattern-recognition receptors and its relevance in autoimmuni- ty. Immunological Reviews 243(1):61–73.

Kelsey JL, Gammon MD, John EM. Reproductive factors and breast cancer. Epidemiol Rev. 1993;15(1):36-47.

Klinman DM (2004) Immunotherapeutic uses of CpG oligodeoxynucleotides. Nature Reviews Immunology 4(4):249– 259.

Krejci L, Altmannova V, Spirek M, et al: Homologous recombination and its regulation. Nucleic Acids Res 40: 5795-5818, 2012

Laktionov PP, Tamkovich SN, Rykova EY, et al (2004) Cell-surface-bound nucleic acids: Free and cell-surface-bound nucleic acids in blood of healthy donors and breast cancer patients. Annals of the New York Academy of Sciences 1022:221–227.

Lee Y, El Andaloussi S and Wood MJA. (2012). Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. Human Molecular Genetics. 21(R1):R125– 134.

Lehmann-Werman R, Neiman D, Zemmour H, et al (2016) Identification of tissue-specific cell death using methylation patterns of circulating DNA. Proc Natl Acad Sci U S A. 2016 Mar 29;113(13):E1826-34.

Leon SA, Shapiro B, Sklaroff DM and Yaros MJ. (1977). Free DNA in the serum of cancer patients and the effect of therapy. Cancer Research 37(3):646–650.

Lo YM, Corbetta N, Chamberlain PF, et al. (1997). Presence of fetal DNA in maternal plasma and serum. Lancet. 350(9076):485–487.

Lo YM, Zhang J, Leung TN, et al (1999) Rapid clearance of fetal DNA from maternal plasma. American Journal of Human Genetics 64(1):218–224.

Lord CJ, Ashworth A: BRCAness revisited. Nat Rev Cancer 16:110-120, 2016

Lui YYN, Chik KW, Chiu RWK, et al. (2002). Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin. Chem. 48, 421–427.

Madhavan D, Wallwiener M, Bent K, et al (2014) Plasma DNA integrity as a biomarker for primary and metastatic breast cancer and potential marker for early diagnosis. Breast Cancer Research and Treatment, 146(1), 163–174.

Mandel P, Métais P (1948) Les acides nucléiques du plasma sanguin chez l'Homme. Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales 142:241–243.

McDonald B, Spicer J, Giannais B, et al (2009) Systemic inflammation increases cancer cell adhesion to hepatic sinusoids by neutrophil mediated mechanisms. International Journal of Cancer 125(6):1298–1305.

Mennuti MT, Chandrasekaran S, Khalek N and Dugoff L. (2015). Cell- free DNA screening and sex chromosome aneuploidies. Prenatal Diagnosis. 35(10):980–985.

Michailidou K, Hall P, Gonzalez-Neira A, et al. Large-scale genotyping identifies 41 new loci associated with breast cancer risk. Nat Genet. 2013;45(4):353-361.

Mittra I (2015) Circulating nucleic acids: a new class of physiological mobile genetic elements. F1000Research 4:924.

Mittra I, Khare NK, Raghuram GV, et al (2015) Circulating nucleic acids damage DNA of healthy cells by integrating into their genomes. Journal of Biosciences 40(1):91–111.

Moasser MM, Krop IE. The Evolving Landscape of HER2 Targeting in Breast Cancer. JAMA Oncol. 2015;1(8):1154-1161.

Morozkin ES, Laktionov PP, Rykova EY, Vlassov VV (2004) Extracellular nucleic acids in cultures of long-term cultivated eukaryotic cells. Annals of the New York Academy of Sciences 1022:244–249.

Moskowitz CS, Chou JF, Wolden SL, et al. Breast cancer after chest radiation therapy for childhood cancer. J Clin Oncol. 2014;32(21):2217-2223.

Mouliere F and Thierry AR. (2012). The importance of examining the proportion of circulating DNA originating from tumor, microenvironment and normal cells in colorectal cancer patients. Expert Opinion on Biological Therapy. 12(S1):S209–S215.

Mouliere F, Robert B, Arnau Peyrotte E, et al (2011) High fragmentation characterizes tumour-derived circulating DNA. PloS One 6(9):e23418.

Nakagawa Y and Gallo RL. Endogenous intracellular cathelicidin enhances TLR9 activation in dendritic cells and macrophages. J Immunol 2015, 194: 1274–1284.

Nik-Zainal S, Davies H, Staaf J, et al: Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature 534:47-54, 2016

Niu Z, Tang W, Liu T, et al (2018) Cell-free DNA derived from cancer cells facilitates tumor malignancy through Toll-like receptor 9 signaling-triggered interleukin-8 secretion in colorectal cancer. Acta Biochim Biophys Sin, 2018, 1–11.

Overman MJ, Modak J, Kopetz S, Murthy R, Yao JC, Hicks ME, Abbruzzese JL, Tam AL.J Clin Oncol. 2013 Jan 1;31(1):17-22. doi: 10.1200/JCO.2012.43.1718. Epub 2012 Nov 5.

Padua RA, Carter C, Hughes D, et al. (1988) *Ras* mutations in myelodysplasia detected by amplification oligonucleotide hybridization and transformation. Leukemia, 2, 503-510.

Pancer LB, Milazzo MF, Morris VL, Singhal SK and Bell DA. (1981) Immunogenicity and characterization of supernatant DNA released by murine spleen cells. J.Immunol. 127,98.

Papayannopoulos V, Metzler KD, Hakkim A and Zychlinsky A. (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. The Journal of Cell Biology. 191(3):677–691.

Patutina O, Mironova N, Ryabchikova E, et al (2011) Inhibition of metastasis development by daily administration of ultralow doses of RNase A and DNase I. Biochimie 93(4):689–696.

Peters DL and Pretorius PJ (2012) Continuous adaptation through genetic communication - a putative role for cell-free DNA. Expert Opinion on Biological Therapy 12(Suppl 1):S127–132.

Pisetsky DS (2012) The origin and properties of extracellular DNA: from PAMP to DAMP. Clinical Immunology (Orlando, Fla) 144(1):32–40.

Plebani M Quality indicators to detect pre-analytical errors in laboratory testing, Clin. Biochem. Rev. 33 (2012) 85–88.

Raaijmakers R, Kirkels WJ, Roobol MJ, Wildhagen MF, Schrder FH. Urology. 2002 Nov;60(5):826-30.

Rainer TH, Wong LKS, Lam W, et al (2003) Prognostic Use of Circulating Plasma Nucleic Acid Concentrations in Patients with Acute Stroke. Clinical Chemistry 49:4 562–569.

Ray Chaudhuri A, Callen E, Ding X, et al: Replication fork stability confers chemoresistance in BRCA-deficient cells. Nature 535:382-387, 2016

Rhodes DJ. Identifying and counseling women at increased risk for breast cancer. Mayo Clin Proc. 2002;77(4):355-60; quiz 360-1.

Rieneck K, Clausen FB and Dziegiel MH. (2015). Noninvasive Antenatal Determination of Fetal Blood Group Using Next-Generation Sequencing. Cold Spring Harbor Perspectives in Medicine. 6(1):a023093.

Rogers JC, Bold D, Kornfeld S, et al. (1972). Excretion of Deoxyribonucleic Acid by Lymphocytes Stimulated with Phytohemagglutinin or Antigen. Proc. NatI. Acad. Sci. U. S. 69: 1685-1689.

Rosato V, Bosetti C, Talamini R, Levi F, Negri E, La Vecchia C. (Metabolic syndrome and the risk of breast cancer). Recenti Prog Med. 2011;102(12):476-478.

Rovera F, Bonifacino A, Panizza P, Peccatori F. Senologia. Diagnosi, Terapia e Gestione. (Edra, ed.).; 2018.

Ruddy KJ, Winer EP. Male breast cancer: risk factors, biology, diagnosis, treatment, and survivorship. Ann Oncol Off J Eur Soc Med Oncol. 2013;24(6):1434-1443.

Rykova EY, Morozkin ES, Ponomaryova AA, et al (2012) Cell-free and cell-bound circulating nucleic acid complexes: mechanisms of generation, concentration and content. Expert Opinion on Biological Therapy 12(Suppl 1):S141–153.

Rykova EY, Pautova LV, Yakubov LA, et al (1994) Serum immunoglobulins interact with oligonucleotides. FEBS Letters 344(1):96–98.

Safia El Messaoudi, Florent Mouliere, Stanislas Du Manoir, Caroline Bascoul-Mollevi, Brigitte Gillet, Michelle Nouaille, Catherine Fiess, Evelyne Crapez, Frederic Bibeau, Charles Theillet, Thibault Mazard, Denis Pezet, Muriel Mathonnet, Marc Ychou, and Alain R. Thierry. Clin Cancer Res; 22(12) June 15, 2016.

Sano H and Morimoto C. Isolation of DNA from DNA/anti-DNA Antibody Immune Complexes in Systemic Lupus Erythematosus. J Immunol (1981). 126:538-539.

Santa-Maria CA, Nye L, Mutonga MB, Jain S, Gradishar WJ. Management of Metastatic HER2-Positive Breast Cancer: Where Are We and Where Do We Go From Here? Oncology (Williston Park). 2016;30(2):148-155.

Schwarzenbach H, Hoon DS, Pantel K.Nat Rev Cancer. 2011 Jun;11(6):426-37. doi: 10.1038/nrc3066. Epub 2011 May 12.

Senn HP, Tran-Thang Ch, Wodnar-Filipowicz A, et al. (1988). Mutation analysis of the N-*ras* proto-oncogene in active and remission phase of human acute leukemias. International Journal of Cancer. 41, 59-64.

Shah SP, Morin RD, Khattra J, et al. Nature. 2009 Oct 8;461(7265):809-13. doi: 10.1038/nature08489.

Shah SP, Roth A, Goya R, et al: The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature 486:395-399, 2012

Sikov WM, Berry DA, Perou CM, et al: Impact of the addition of carboplatin and/or bevacizumab to neoadjuvant once-per-week paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pathologic complete re- sponse rates in stage II to III triple-negative breast cancer: CALGB 40603 (Alliance). J Clin Oncol 33:13-21, 2015

Snyder MW, Kircher M, Hill AJ, et al (2016) Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. Cell 164, 57–68.

Sonmezer M, Oktay K. Fertility preservation in young women undergoing breast cancer therapy. Oncologist. 2006;11(5):422-434

Sozzi G, Conte D, Leon M, Ciricione R, Roz L, Ratcliffe C, Roz E, Cirenei N, Bellomi M, Pelosi G,

Pierotti MA, Pastorino U.J Clin Oncol. 2003 Nov 1;21(21):3902-8. Epub 2003 Sep 24.

Stroun M (1962) Modifications des caractères à la suite de greffes intervariétales chez le Solanum melongena. Comptes Rendues á l'Academie de Science Paris 255:361–363.

Stroun M and Anker P (2006) Prehistory of the notion of circulating nucleic acids in plasma/serum (CNAPS): birth of a hypothesis. Annals of the New York Academy of Sciences. 1075:10–20.

Stroun M, Anker P, Beljanski M, et al. (1978). Presence of RNA in the nucleoprotein complex spontaneously released by human lymphocytes and frog auricles in culture. Cancer Research. 38(10):3546–3554.

Stroun M, Anker P, Lyautey J, et al. (1987). Isolation and characterization of DNA from the plasma of cancer patients. European Journal of Cancer & Clinical Oncology. 23(6): 707–712.

Stroun M, Anker P, Maurice P, et al. (1989). Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology. 46(5):318–322.

Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M (1989) Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology 46(5):318–322.

Stroun M, Lyautey J, Lederrey C, et al. (2001). About the possible origin and mechanism of circulating DNA: Apoptosis and active DNA release. Clinica Chimica Acta. 313(1–2):139–142.

Stroun, M, Anker P, Gahan PB and Henri J. (1977). Spontaneous release of newly synthesized DNA from frog auricles. Archives des Sciences.

T. M. Chused, A. D. Steinberg And N. Talal. Clin. Exp. Immunol. (1972) 12, 465-476.

Tan EM, Schur PH, Carr RI and Kunkel HG.(1966). Deoxyribonucleic acid and antibodies to DNA in the serum of patients with Systemic Lupus Erythematosus. J. Clin. Invest. 45,1732.

Telli ML, Hellyer J, Audeh W, et al: Homologous recombination deficiency (HRD) status predicts response to standard neoadjuvant chemotherapy in patients with triple-negative or BRCA1/2 mutation-associated breast cancer. Breast Cancer Res Treat. 2018 Apr;168(3):625-630.

Telli ML, Jensen KC, Vinayak S, et al: Phase II study of gemcitabine, carboplatin, and iniparib as neoadjuvant therapy for triple-negative and BRCA1/2 mutation-associated breast cancer with assessment of a tumor-based measure of genomic instability: PrECOG 0105. J Clin Oncol 33:1895-1901, 2015

Thakur BK, Zhang H, Becker A, et al. (2014). Double-stranded DNA in exosomes: a novel bio-marker in cancer detection. Cell Research. 24(6):766–769.

Thierry AR, El Messaoudi S, Gahan PB, et al. (2016). Origins, structures, and functions of circulating DNA in oncology. Cancer Metastasis Rev (2016) 35:347–376.

Timms KM, Abkevich V, Hughes E, et al: Association of BRCA1/2 defects with genomic scores predictive of DNA damage repair deficiency among breast cancer subtypes. Breast Cancer Res 16:475, 2014

Tohme S, Yazdani HO, Al-Khafaji AB, et al (2016) Neutrophil Extracellular Traps Promote the Development and Progression of Liver Metastases after Surgical Stress. Cancer Research.

Trejo-Becerril C, Pérez-Cárdenas E, Taja-Chayeb L, et al (2012) Cancer progression mediated by horizontal gene transfer in an in vivo model. PloS One 7(12):e52754.

Truong PT, Bernstein V, Lesperance M, Speers CH, Olivotto IA. Radiotherapy omission after breast-conserving surgery is associated with reduced breast cancer-specific survival in elderly women with breast cancer. Am J Surg. 2006;191(6):749-755.

Tsumita, T. and Iwanaga, M. (1963) Fate of injected deoxyribonucleic acid in mice. Nature (Lond.), 198, 1088.

Turturici G, Tinnirello R, Sconzo G and Geraci F. (2014). Extracellular membrane vesicles as a mechanism of cell-to-cell communication: advantages and disadvantages. American Journal of Physiology - Cellular Physiology. 306(7):C621–633.

Tutt A, Ellis P, Kilburn L, et al: The TNT trial: A randomized phase III trial of carboplatin (C) compared with docetaxel (D) for patients with metastatic or recurrent locally advanced triple negative or BRCA1/2 breast cancer (CRUK/07/012). Cancer Res 75, 2015 (suppl; abstr S3-01)

Vaisanen MR, Jukkola-Vuorinen A, Vuopala KS, et al. Expression of Toll-like receptor-9 is associated with poor progression-free survival in prostate cancer. Oncol Lett 2013, 5: 1659–1663.

Vasioukhin V, Anker P, Maurice P, et al. (1993) Point mutations of the K-*ras* gene in the blood plasma DNA of patients with colon adenocarcinoma. In: The Challenge of Biotechnology: from laboratory diagnosis to clinical therapy(ed.by S. A. Aaronson, A. E. Shamooand R. Verna). Serono Symposia Publications.

Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M (1994) Point mutations of the N-*ras* gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute mye- logenous leukaemia. British Journal of Haematology 86(4):774– 779.

Vasioukhin V, Anker P, Stroun M, et al. (1992) Presence of a point mutation of codon 12 of the K-*ras* oncogene found in the DNA extracted from the plasma of cancer patients. Proceedings of the AACR Conference on Normal and Neoplastic Growth and Development, Chatham. Mass., October 1992.

Vlassov VV, Laktionov PP and Rykova EY (2007) Extracellular nucleic acids. BioEssays. 29(7):654–667.

Von Minckwitz G, Schneeweiss A, Loibl S, et al: Neoadjuvant carboplatin in patients with triple-negative and HER2- positive early breast cancer (GeparSixto; GBG 66): A randomised phase 2 trial. Lancet Oncol 15:747-756, 2014

W. Emlen And M. Mannik. Clin. Exp. Immunol. (1984). 56,185-192.+

W. Emlen And M. Mannik. The Journal Of Experimental Medicine. Volume 147, 1978.

Wartha F, Beiter K, Normark S and Henriques-Normark B (2007) Neutrophil extracellular traps: casting the NET over pathogenesis. Current Opinion in Microbiology 10(1):52–56.

Warton K, Mahon KL and Samimi G. (2016) Methylated circulating tumor DNA in blood: power in cancer prognosis and response. Endocr Relat Cancer. 2016 Mar;23(3):R157-71.

Watkins JA, Irshad S, Grigoriadis A, et al: Genomic scars as biomarkers of homologous recombination deficiency and drug response in breast and ovarian cancers. Breast Cancer Res 16:211, 2014.

Wendie DdB, Kasmintan AS, Sophie S et al. Homologous Recombination Deficiency in Breast Cancer: A Clinical Review. JCO Precision Oncology, -(1), 1-13 (2017)

Winter C, Nilsson MP, Olsson E, et al: Targeted sequencing of BRCA1 and BRCA2 across a large unselected breast cancer cohort suggests that one-third of mutations are somatic. Ann Oncol 27:1532-1538, 2016

Yasuda K, Richez C, Uccellini MB, et al. Requirement for DNA CpG content in TLR9- dependent dendritic cell activation induced by DNA-containing immune complexes. J Immunol 2009, 183: 3109–3117.

Yipp BG and Kubes P. (2013). NETosis: how vital is it? Blood. 122(16):2784–2794.

Yousefi S, Gold JA, Andina N, et al. (2008). Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. Nature Medicine. 14(9):949–953.

Yousefi S, Simon D and Simon H-U. (2012). Eosinophil extracellular DNA traps: molecular mechanisms and potential roles in disease. Current Opinion in Immunology. 24(6):736–739.

Yu SCY, Chan KCA, Zheng YWL, et al (2014). Size-based molecular diagnostics using plasma DNA for noninvasive prenatal testing. Proceedings of the National Academy of Sciences of the United States of America. 111(23):8583–8588.

Yu SCY, Lee SWY, Jiang P, et al (2013) High-resolution profiling of fetal DNA clearance from maternal plasma by massively parallel sequencing. Clinical Chemistry 59(8):1228–1237.

Zervoudis S, Iatrakis G, Navrozoglou I. Reproduction after breast cancer. Best Pract Res Clin Obstet Gynaecol. 2010;24(1):81-86.

Zhang Y, Wang Q, Ma A, et al. Functional expression of TLR9 in esophageal cancer. Oncol Rep 2014, 31: 2298–2304.