a) TITLE

Liquid biopsies based minimal residual disease monitoring in Diffuse Large B-Cell Lymphoma patients

b) INTRODUCTION

Diffuse Large B-Cell Lymphoma (DLBCL) is the most common type of Non-Hodgkin Lymphoma (NHL). The median age of DLBCL at diagnosis is 60-70 years old [1-2] and its incidence is estimated as 25,380 new cases in the United States in 2016 [3].
DLBCL is considered a more aggressive malignancy than other indolent lymphomas, such as follicular lymphoma, with a median survival <1 year in untreated patients [4].
In recent years, the addition of the anti-CD20 monoclonal antibody (rituximab, R) to the cyclophosphamide, doxorubicin, vincristine, and prednisone combination (R-CHOP) has improved the efficacy of chemotherapy and it is considered the gold standard in the treatment of DLBCL. However, in 30-40% of patients relapse occurs and they die within two years [5-6].
Therefore, a better understanding of DLBCL biology might improve the therapeutic outcome. Recently, gene expression profiling (GEP) studies have defined three prognostic subgroups of patients based on the cell of origin (COO), classifying patients in germinal centre-like B cells (GCB), activated B cells (ABC), and unclassified[7].
Current evidence suggested that ABC patients treated with conventional R-CHOP therapy shown significantly poorer outcomes compared with GCB cases[8]. Moreover, in the last years, the employing of next generation sequencing technologies (NGS) notably improved the minimal residual disease monitoring (MRD) in the context of different hematological malignancies, introducing liquid biopsies (e.g. plasma) and cell-free DNA (cfDNA), fragmented extracellular DNA released from apoptotic and necrotic cells[9], as tissues to investigate for MRD purposes. Notably, in cancer patients, a fraction of the total cfDNA consists of circulating tumor DNA (ctDNA) which originates from neoplastic lesions cells and it is usually present at very low amount in plasma, as well as in other biological fluids, including urine or cerebrospinal fluid[10-11]. In this context, the immunoglobulin gene rearrangements (IG), detected on bone marrow and peripheral blood, were assessed as powerful molecular markers for MRD monitoring and outcome predictors in different B-cell lymphomas as mantle cell lymphoma, acute lymphoblastic leukemia and multiple myeloma[12]. Moreover, regarding DLBCL, NGS-based MRD studies on IG-ctDNA, from retrospective series of DLBCL patients, were able to identify patients at risk of recurrence before clinical or radiological evidence of disease[13-14]. Nevertheless, the methods proposed in those papers were strictly dependent from a biotechnology company, resulting not reproducible in academic translational research.

c) AIMS OF THE PROJECT

Based on this evidence, the aims of the project will be:

1- To employ an academical and reproducible NGS based approach, previously developed to MRD monitoring in acute lymphoblastic leukemia patients[15], within DLBCL context.
2- To assess the feasibility of a less invasive diagnostics by exploring the role of MRD on ctDNA at different time points as an early predictor of clinical outcome and relapse.

d) METHODS

Formaline-fixed paraffine-embedded (FFPE) lymph node and PB samples are prospectively collected from 2019 at baseline and during follow-up (FU) from a multicentric series of patients affected by DLBCL. As October 2023, 40 FFPE and 55 baseline plasma samples are yet available for the molecular marker assessment. Furthermore, at least 43/63 patients had collected almost 3 plasma FU samples to MRD monitoring. FU samples centralization will be expected through 2024 for 20 patients. All patients provided written informed consent for the research use of their biological samples, in accordance with Institutional Review Boards requirements, and the Helsinki's declaration.
For each patient, PB samples were collected and subsequently processed by centrifugation to separate plasma from cellular counterpart. Plasma samples were stored at -80°C until cfDNA extraction.

More in detail, cfDNA will be extracted from 3 ml of plasma using Maxwell RSC ccfDNA LV Plasma kit and Maxwell semiautomated extraction instrument (Promega, San Francisco). Differently, baseline FFPE tissues will be extracted using QIAsymphony platform (Qiagen, Germany). Subsequently, control genes quantitative analysis (ALBUMIN and TERT) by ddPCR will be performed to quantify the extracted material and then to check cfDNA integrity and amplifiability. One step PCR NGS based approach targeting IG rearrangements will be employed both to assess molecular marker on matched diagnostic tissue (FFPE) and cfDNA samples at baseline timepoint and then to monitor MRD in plasma samples collected during clinical FU. Briefly, sequencing libraries will be prepared using barcoded primers specifically designed for IGH-VJcfFR3/VJFR3, IGH-DJ and IGK loci amplification. Moreover, each sample will be added by a spike-in DNA control, a mixture of 9 cell lines according to manufacturer's instructions. PCR conditions thermal cycler will be: an initial denaturation of 94 °C for 1 minute, 94 °C for 10 minutes as denaturation step followed by annealing with 35 cycles at 50 °C for VJcfFR3/VJFR3, 63 °C for IGH-DJ and IGK for 1 minute and extension at 72°C for 30 seconds. The PCR reaction will end with a final extension step at 72°C for 30 seconds; PCR products will be stored at 12°C and then checked on 2% agarose gel electrophoresis to verify the correct target genes amplification. Then PCR products will be excised from the agarose gel and then purified using MinElute Gel Extraction Kit (Qiagen, Germany). In detail, gel slides will be resuspended in a buffer containing a pH indicator, incubated in a heat/shake block at 50°C for 10 minutes and then centrifuged several times at RT. Ethanol based buffers will be used to perform PCR products washes twice. Finally, the purified library patient will be eluted in 10 µL of elution buffer twice. Then the purified target libraries will be quantified using Quantus fluorometer (Promega, San Francisco) and the fragment length quality check will be performed by Agilent High Sensitivity D1000 ScreenTape Assay (Agilent Technologies, Santa Clara, CA, USA). The final library pool will be created by an equimolar combination of all patients target libraries. The final pool will be diluited to 4nM or 2nM, spiked with 10% of PhIX control (as an internal genome reference) and then will be sequenced employing MiSeq sequence platform and reagent Kit V2 or V3 based on the expected read depth, according to manufacturer's instructions. For each run, a dedicated samplesheet will be designed using Illumina Experiment Manager (Illumina) to allow FASTQ files demultiplex at the end of the sequencing. Finally, MiSeq raw data will be firstly visualized by Sequencing Analysis Viewer program (Illumina) to assess run quality parameters as cluster density, passing filter and number of aligned reads; then ARResT/Interrogate (at http://arrest.tools/interrogate/), a standardized bioinformatic tool developed for high-throughput sequencing data, by will used to assess and precisely define the clonal IG molecular marker at baseline. For each diagnostic FFPE and plasma samples, IG locus and aminoacidic sequences rearranged will be annotated in terms of reads number. Finally, a FU plasma sample will be defined as MRD positive if almost one IG aminoacidic sequence read will be found by ARResT/Interrogate. MRD quantification will be performed based on spike-in cellular line sequencing output, thus correcting the raw date also considering the DNA quantity input as limit of quantification.
Based on FFPE and plasma samples yet collected from 2019 to 2023, the project will be conducted during a period of 1 year, suitable to assess molecular marker and to generate solid preliminary results.

e) REFERENCES

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