



Review

# Cell-Free DNA for the Management of Classical Hodgkin Lymphoma

Vincent Camus \* and Fabrice Jardin

Department of Hematology, Centre Henri Becquerel, 76038 Rouen, France; fabrice.jardin@chb.unicancer.fr

\* Correspondence: vincent.camus@chb.unicancer.fr; Tel.: +33-(0)-2-32-08-29-47; Fax: +33-(0)-2-32-08-22-83

**Abstract:** Cell-free DNA (cfDNA) testing, is an emerging “liquid biopsy” tool for noninvasive lymphoma detection, and an increased amount of data are now available to use this technique with accuracy, especially in classical Hodgkin lymphoma (cHL). The advantages of cfDNA include simplicity of repeated blood sample acquisition over time; dynamic, noninvasive, and quantitative analysis; fast turnover time; reasonable cost; and established consistency with results from tumor genomic DNA. cfDNA analysis offers an easy method for genotyping the overall molecular landscape of pediatric and adult cHL and may help in cases of diagnostic difficulties between cHL and other lymphomas. cfDNA levels are correlated with clinical, prognostic, and metabolic features, and may serve as a therapeutic response evaluation tool and as a minimal residual disease (MRD) biomarker in complement to positron emission tomography (PET). Indeed, cfDNA real-time monitoring by fast high-throughput techniques enables the prompt detection of refractory disease or may help to address PET residual hypermetabolic situations during or at the end of treatment. The major recent works presented and described here demonstrated the clinically meaningful applicability of cfDNA testing in diagnostic and theranostic settings, but also in disease risk assessment, therapeutic molecular response, and monitoring of cHL treatments.

**Keywords:** cell-free DNA; Hodgkin lymphoma; precision medicine; liquid biopsy; circulating tumor DNA



**Citation:** Camus, V.; Jardin, F. Cell-Free DNA for the Management of Classical Hodgkin Lymphoma. *Pharmaceuticals* **2021**, *14*, 207. <https://doi.org/10.3390/ph14030207>

Academic Editor: Alfredo Berzal-Herranz

Received: 13 February 2021  
Accepted: 26 February 2021  
Published: 2 March 2021

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

### 1.1. Classical Hodgkin's Lymphoma (cHL) Particularities

Classical Hodgkin's lymphoma (cHL) is a rare and curable malignancy with an annual incidence representing 10% of new lymphomas and a prevalence of 1% of all cancers in Western countries [1]. Patient outcomes are mostly excellent with multiagent chemotherapy (escalated BEACOPP, ABVD) and modern radiation techniques with a five-year progression-free survival (PFS) rate of 65% to 90% according to advanced versus localized stage disease and standard clinical risk factors [2,3]. Patients achieving an early metabolic rapid response after two cycles of frontline chemotherapy display excellent outcome and are proposed for treatment intensity de-escalation after interim positron emission tomography (PET) assessment [4]. However, approximately one-quarter of patients will display progressive disease or relapse, pinpointing the urgent need to determine the underlying biological processes involved and to select useful biomarkers. At present, no pathognomonic biomarker exists for cHL. To date, the genetic landscape of cHL has been incompletely described because Hodgkin and Reed-Sternberg (HRS) tumor cells are very scarce (0.1–3% of cells in the tissue) [5], hampering molecular biology analyses with techniques that lack sensitivity.

Standard lymph node removal or tissue biopsy is the recommended procedure for the lymphoma diagnosis and is the routine method for tumor genetic profiling, but this invasive method is associated with numerous caveats: hemorrhage, infections, anesthetic risks, or poor-quality fine-needle biopsy with artifacts or sampling issues [6]. In addition, the rarity of HRS cells augments pathological diagnosis complexity in small biopsy samples. In line

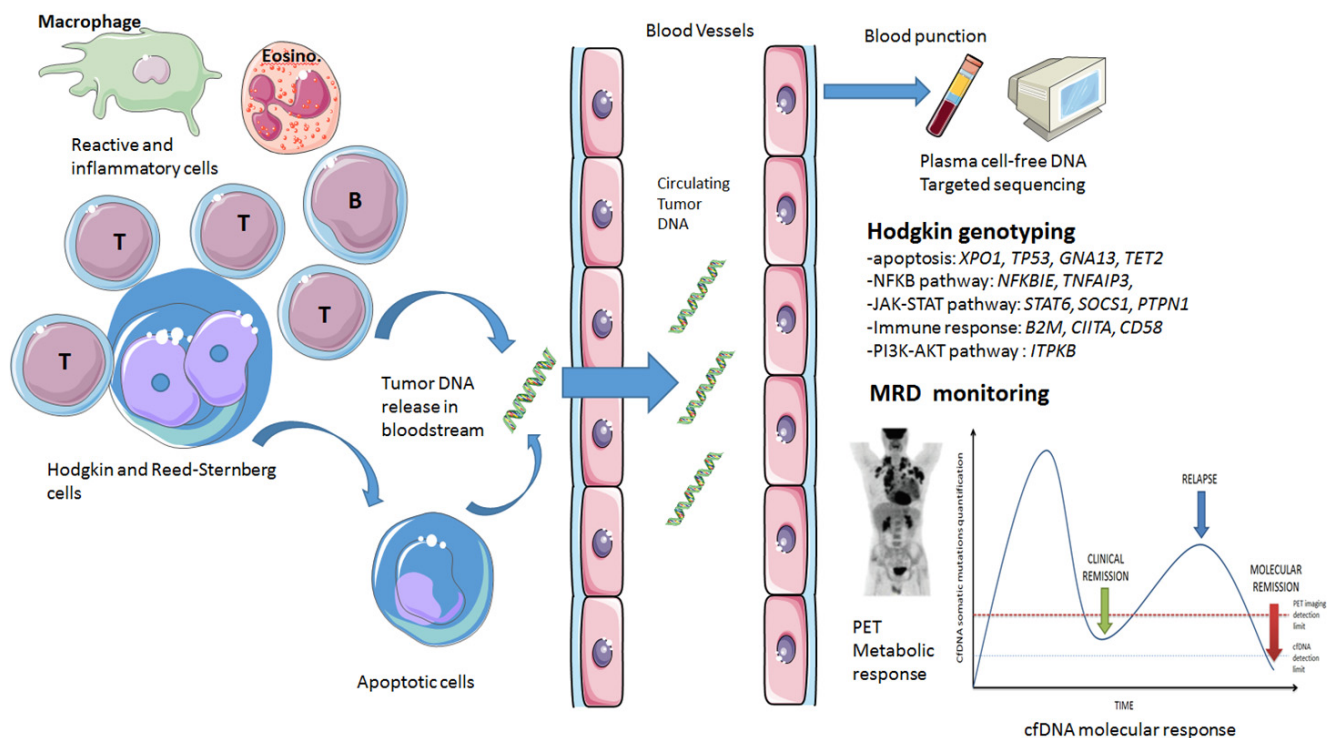
with these tumor DNA access difficulties, several teams hypothesized that discovering tumor specific somatic alterations would be more appropriate in the bloodstream, i.e., in cell-free DNA (cfDNA) extracted from plasma. Indeed, various proof-of-concept studies have recently shown that cHL molecular analysis is feasible using cfDNA and highly sensitive methods [7–11]. Despite the scarcity of HRS cells and a typical lower tumor volume, many studies have indicated that success rates for cfDNA detection in cHL are close to those achieved for diffuse large B cell lymphoma (DLBCL). This finding suggests that cHL seems to display a higher trend to release cfDNA than DLBCL. This finding may be related to an increased fraction of tumor cells in apoptosis but also related to the nature of the alterations in the nuclear DNA of reed Sternberg cells [12].

### 1.2. Cell-Free DNA Physiopathology

cfDNA was discovered in the bloodstream several years ago and was first assessed in solid tumors. Previous interesting works in solid tumors settings established that cfDNA fragments may invade distant cells in other tissues, modifying the biology of these cells and contributing to the onset of metastases [13,14]. For cHL, no similar data exist. cfDNA is predominantly freed in plasma by cells in apoptosis and is in addition actively secreted by certain tumor cells or liberated during necrosis phenomenon (see Figure 1). These facts are established by the existing broad spectrum of cfDNA fragment sizes (from 0.150 to several kilobases) and the demonstration of similar genetic profiles in tumor genomic DNA (gDNA) and cfDNA [15–17]. Of note, cfDNA exist in healthy subjects and mainly derived from apoptosis of hematopoietic cells. This normal cfDNA is typically detectable in very small quantities in blood [18]. However, these levels increase 15-fold 30 min postexercise and return to normal levels thereafter [19]. The physiological role of cfDNA remains undoubtedly elusive, but a twofold increase in cfDNA levels after the psychosocial stress test and five-fold increase after exhaustive treadmill exercise were recently demonstrated, suggesting that cfDNA is a biomarker of molecular stress [20]. Biological and environmental variables may also modulate cfDNA release, including sex, body composition, age, smoking, exercise, autoimmune disorders, comorbidities, infectious disease, inflammatory conditions, oxidative stress, and pregnancy [21,22]. In addition, cfDNA clearance is also complex and may imply DNase I activity [23], renal clearance [24], and uptake by the liver and spleen followed by macrophage elimination [25]. Several teams measured that the cfDNA half-life in blood is comprised in a range of 16 min to 2.5 h [26] but is largely dependent on several patient settings: healthy subjects vs. cancer patients, before surgery/chemotherapy/radiotherapy or after, and at rest vs. after physical activity [22]. Furthermore, cfDNA clearance is also influenced by binding to cell-surface receptors [27] and several serum proteins [28] (albumin, fibrinogen, prothrombin, and C-reactive protein), the levels of which may considerably vary during the cancer course. All these features complicate biological studies on this topic. It is important to note that cfDNA combined both “normal/non-tumoral” cfDNA and circulating tumor DNA (ctDNA) fragments [18,29], and there is no tool to separate cfDNA arisen from cancer cells and cfDNA liberated by normal cells, which begs the question of background noise and sensitivity of the technologies used in liquid biopsy works to detect somatic variants. Of note, several research teams in solid tumors detected cfDNA in other human fluids, such as cerebrospinal fluid (CSF) in primary central nervous system lymphomas (PCNSLs) with *MYD88* L265P mutation assessment [30,31], urine [32] in the bladder cancer, sputum [33] in lung cancer patients, and uterine lavage fluid in patients with endometrial cancers [34]. In addition, stool DNA may also be valuable in patients with colorectal carcinoma with improved detection rates and a commercially available tool (Cologuard™ assay) [35]. However, these sources of cfDNA seems irrelevant for cHL with no published data to date and no possibility to determine their value for disease burden assessment or genotyping at the time of diagnosis.

Finally, relevant advantages of plasma cfDNA testing in cHL include: (i) simple venous puncture to obtain sample, (ii) measurable tool which may be performed at any

time during patient's journey (iii) dynamic assessment of clonal evolution, and (iv) less spatial heterogeneity than tissue biopsy genotyping [36–38].



**Figure 1.** Schematic overview of cell-free DNA in classical Hodgkin lymphoma. Abbreviations: MRD: minimal residual disease.

### 1.3. Cell-Free DNA Molecular Tools

We know that the optimal way for noninvasive liquid biopsy testing is to extract cfDNA from plasma after blood puncture with nucleic acid preservation tubes (for example, Roche or Streck cfDNA BCT<sup>®</sup> [39]). These tubes should then be promptly processed (within 6 h after venous puncture) with consecutive low- and high-speed [40] centrifugations to reduce leukocyte lysis. The intensity of low temperature storage room (−20 °C or −80 °C) remains controversial, but the relevance of leukocyte stabilization tubes is clearly established for easier use [41], especially in multicentric studies. Research teams may experience altered sample quality if they do not satisfy the optimal preanalytical requirements [42]. cfDNA extraction from plasma samples is easy and feasible in most academic laboratories using commercial kits [43,44]. As mentioned above, in cHL, cfDNA comes from rare lymphoma cells and normal cells, thereby necessitating highly sensitive methods for accurate measurement of somatic alterations.

It was previously established that tumor cells in cHL arise from B-cells [45], and clono-specific B-cells are detectable in cHL patients' blood samples [46]. Normal C-cells and lymphoma cells both expressed B-cell receptor (BCR). BCR variety is a consequence of variable-diversity-joining (VDJ) genes rearrangement during lymphopoiesis. This mechanism provides specific clonotypes, and so each tumor-specific VDJ profile may be considered as a "barcode" for noninvasive tracking of lymphoma in liquid biopsy. Indeed, using ClonoSEQ technology (Adaptive Biotechnologies, Seattle, WA, USA) Oki et al. described a small proof of concept series of seventeen patients, of whom eleven harbored a detectable lymphoma-specific clonotype in tumor biopsies, 8/11 (73%) displayed the same clonotypes in plasma cfDNA, and 33% exhibited the same clonotypes in PBMCs [47]. Using universal VDJ and IdK primers instead of tumor-specific primers, it is possible to detect clonospecific sequences in a single cfDNA sample. This may grant to disclose exhaustive patients' immunoglobulin repertoire and monitor individual subclones. This ClonoSEQ assay is the sole FDA-cleared minimal residual disease (MRD) tool in lymphomas. Nevertheless, to our

knowledge, ClonoSEQ technology results in cHL patients have not been reproduced by other teams, and the sensitivity and specificity of this technique in cHL remain unclear at the moment. Moreover, this technique requires the initial tumor biopsy material for exact assessment of the VDJ profile before tracking it in the blood. In addition, VDJ rearrangements may be unproductive or abortive [48], so the ClonoSEQ method may not work for in this situation, restricting the informativeness of this tool.

In contrast, next-generation sequencing (NGS) gene panel tests may detect concordant potentially “actionable” somatic mutations in the patients’ plasma (cfDNA) and biopsy (gDNA) of lymphoma patients [49] and may contribute to decide appropriate salvage treatments in relapsed/refractory aggressive B-cell lymphoma using new target therapies currently in development. For example, CAncer Personalized Profiling by deep Sequencing (CAPP-seq) is a powerful method for cfDNA measurement that allows deep DNA sequencing and grants an easy detection and quantification of ultralow abundance genetic alterations [50]. CAPP-seq relevance was well described in non-Hodgkin and Hodgkin lymphoma patients. This technology is able to measure disease burden, detect early relapse before radiological progression, perform cell of origin (COO) classification, separate indolent follicular lymphomas and those at risk for high grade transformation, and monitor variants’ clearance in chemosensitive patients versus non-responders patients who display persistent genetic alterations in plasma after treatment [8,51–53]. However, such results are only possible at high cost given the elevated number of genes included in the panels and in trained research teams with experienced bioinformaticians able to combine barcoding and unique molecular identifiers (UMIs) with integrated digital error suppression [54]. To date, CAPP-seq is not commercially available. Another report recently assessed cfDNA using real-time PCR in an impressive cohort of 155 pediatric cHL. In this work, the authors showed that baseline cfDNA level is higher in cHL than in healthy subjects, and that higher cfDNA concentration is linked to B-symptoms and inflammatory syndrome. The authors also established that the augmentation of cfDNA concentration after one cycle of chemotherapy led to unfavorable outcome [55].

Finally, digital PCR (dPCR) is a quick, simple and barely costless tool which only needs a small amount of plasma cfDNA. dPCR process dilutes and partitions DNA samples into thousands microcompartments (i.e., microscopic PCR reactors) with each one including a single copy or no copies of the target region [56]. It is then easy to quantify the exact normal or mutated DNA copies number by counting the number of positive compartments with the fluorescent probe corresponding to the wild-type or mutated region. The advantages of dPCR include rapid implementation, the lack of a need for a bioinformatics pipeline and high sensitivity ( $10^{-5}$  detection limit), making it a relevant tool when used independently and in addition to NGS for hotspot single-nucleotide variant (SNV) detection, such as *XPO1* E571K (primary mediastinal B-cell lymphoma and cHL) [44]. Indeed, dPCR is based on single-point mutations quantification, and so is not designed to provide the complete molecular landscape of the patient’s lymphoma and, therefore, probably not suitable for molecular response assessment given frequent subclonal evolution. In addition, treatment sensitivity could remove those subclones from cfDNA profiles, so other clones could arise or survive with a false negative dPCR assay. A solution could be to multiplexed dPCR assays to test several hotspot mutations in the same experience, and indeed new dPCR tools can do so (including RainDance<sup>®</sup> or Biorad<sup>®</sup>) [57–60]. Furthermore, low amount of cfDNA in some plasma samples impairs the capability to measure molecular response given insufficient haploid genome equivalent quantities. Of note, false positives/background noise issues and technical limits of variant detection are still debated [61,62]. In addition, to date, no published multicenter study of dPCR MRD approaches exists in cHL patients.

Finally, it is important to pinpoint that there is a need for peripheral blood mononuclear cell (PBMC) collection at diagnosis when performing liquid biopsy because hematopoietic clone-derived variants may be detectable in cfDNA samples of both healthy subjects and lymphoma patients and may likely lead to false positive results in cfDNA monitoring

experiments [63]. In this situation, gDNA from PBMC may help researchers discriminate polymorphisms from true somatic alterations.

All these molecular biology techniques (summarized in Table 1) are nevertheless interesting and complimentary in the field of cfDNA testing. In this review, we will focus on landmark works in cfDNA assessment in the setting of cHL and we will present clinical practice impact of this tool.

**Table 1.** Minimal residual disease (MRD) assessment and “liquid biopsy” methods in patients with classical Hodgkin lymphoma (cHL).

Plasma Cell-Free DNA			
Characteristics	Advantages	Disadvantages	
cfDNA source	<ul style="list-style-type: none"> <li>Blood collection: Plasma (Two-step centrifugation process)</li> <li>Standard EDTA or cell-free DNA preservative leukocyte stabilization tubes</li> <li>Well-defined circuit to handle the tubes</li> <li>Less than three years before cfDNA extraction</li> <li>Avoid &gt;2 free-thaw cycles</li> </ul>	<ul style="list-style-type: none"> <li>Easily accessible: Simple venipuncture</li> <li>Extensive data in the literature on cfDNA extracted from plasma</li> <li>Commercial kits for plasma cfDNA extraction</li> </ul>	<ul style="list-style-type: none"> <li>Less than 6 h between venipuncture and storage if EDTA tubes</li> <li>Intensity of low temperature storage room (−20 °C or −80 °C) remains controversial</li> </ul>
cfDNA applications	<ul style="list-style-type: none"> <li>Hodgkin lymphoma genotyping</li> </ul>	<ul style="list-style-type: none"> <li>Non-invasive “liquid biopsy” tool</li> <li>Disease risk assessment; potential interest in the differential diagnosis with other lymphomas (gray-zone, primary mediastinal B cell lymphoma)</li> <li>Potential theranostic interest</li> <li>Excellent mirror of the tumor cell genetic panorama;</li> </ul>	<ul style="list-style-type: none"> <li>Fresh frozen tissue led to a better concordance rate between genomic DNA and cfDNA</li> <li>Tissue biopsy remains “gold standard” for diagnosis</li> </ul>
	<ul style="list-style-type: none"> <li>Minimal residual disease (MRD) monitoring</li> </ul>	<ul style="list-style-type: none"> <li>Dynamic assessment: baseline, during treatment, after treatment, with repeated serial blood samples</li> </ul>	<ul style="list-style-type: none"> <li>Lack of validation to date in large clinical trials</li> <li>No standardized MRD measurement criteria</li> <li>Concerns about background noise and detection limit</li> </ul>
	<ul style="list-style-type: none"> <li>Therapeutic “molecular” response assessment in complement to positron emission tomography (PET)</li> </ul>	<ul style="list-style-type: none"> <li>Add relevant information to PET data, for example in cases of suspected PET false positives or suspected relapse</li> </ul>	<ul style="list-style-type: none"> <li>No standardized technique</li> <li>No multicenter randomized data available for clinical validation</li> </ul>
cfDNA tools	<ul style="list-style-type: none"> <li>High-throughput Ig-VDJ rearrangement sequencing (ClonoSEQ)</li> </ul>	<ul style="list-style-type: none"> <li>Real-time dynamic assessment</li> <li>Commercially available</li> <li>FDA-cleared</li> </ul>	<ul style="list-style-type: none"> <li>Calibration failures (frozen tissue vs. FFPE)</li> <li>Not suitable to tailor targeted therapy</li> <li>No detection of drug-resistant clone during therapy</li> <li>Sensitivity and specificity are unclear</li> </ul>
	<ul style="list-style-type: none"> <li>Panel-directed Next-Generation Sequencing (CAPP-seq and other targeted panels)</li> </ul>	<ul style="list-style-type: none"> <li>Measure disease burden, detect early relapse before radiological progression</li> <li>Monitor variants’ clearance in chemo sensitive patients versus non-responders patients who display persistent genetic alterations in plasma after treatment</li> </ul>	<ul style="list-style-type: none"> <li>No standardized technique</li> <li>Not commercially available</li> <li>high cost due to elevated number of genes included in the panel</li> <li>Need trained research teams with experienced bioinformaticians able to combine barcoding and unique molecular identifiers (UMIs) with integrated digital error suppression</li> </ul>

Table 1. Cont.

Plasma Cell-Free DNA		
Characteristics	Advantages	Disadvantages
<ul style="list-style-type: none"> <li>• Droplet digital PCR</li> </ul>	<ul style="list-style-type: none"> <li>• Short turnaround time</li> <li>• Low cost</li> <li>• Detection of “hotspot” targetable activating mutations</li> <li>• Easy serial testing</li> <li>• 10<sup>-5</sup> detection limit</li> </ul>	<ul style="list-style-type: none"> <li>• Not commercially available</li> <li>• Insufficient data to verify the reproducibility</li> <li>• False-positive and detection limit concerns</li> </ul>

Abbreviations: Ig: immunoglobulin; CAPP-seq: Cancer Personalized Profiling by deep Sequencing; FDA: Food and Drug administration; PCR: polymerase chain reaction; cfDNA: cell-free DNA.

## 2. Genotyping Classical Hodgkin Lymphoma Using cfDNA

### 2.1. Mutational Landscape Obtained by cfDNA Sequencing

A notable NGS study with low-pass sequencing on cfDNA of ten newly-diagnosed localized and advanced stage nodular sclerosis cHL patients revealed genomic imbalances in HRS cells in nine patients at baseline and a rapid clearance after frontline treatment (within a month), revealing cfDNA as a promising tool for molecular response monitoring [11]. In a retrospective proof-of-concept study from our group, including 94 patients with all stages of cHL homogeneously treated with standard frontline chemotherapy, *XPO1* E571K mutations were found using dPCR and NGS experiments in 24.2% of patients. We noted that 29% of all *XPO1* E571K mutations were only discovered in cfDNA, which may be explained by HRS cell scarcity in cHL. Our group was then able to develop a multigene panel allowing the detection of several somatic alterations of genes involved in the lymphomagenesis of cHL or frequently mutated in this disease. By dPCR and NGS, we found an average of 2.13 mutations per case of cHL; in particular, 30.5% of patients were mutated in the DNA binding domain of *STAT6* [9]. However, this panel was only informative for 50% of the patients using cfDNA sources, so we extended it to a nine-gene panel including *SOC51*, *XPO1*, *STAT6*, *NFKBIE*, *TNFAIP3*, *PTPN1*, *B2M*, *ITPKB*, and *GNA13*. Our team also led an observational prospective study based on cfDNA testing including 60 consecutive cHL cases treated by frontline ABVD and/or escalated BEACOPP. We observed somatic variants in 42/60 (70%) patients at baseline [10]. However, this gene panel was unable to disclose variants in all of the patients, probably because the panel was too restricted and sensitivity was insufficient to reveal ultra-low abundance subclones which are close to the sequencer limit of detection (variant allele frequency (VAF) 0.1%). The comprehension of cHL biology is growing quickly, and we should include additional genes in next panels. For example, *ATM*, *KMT2D*, *TP53*, *ARID1A*, and *CIITA* are interesting and frequently mutated in cHL.

In addition, in 2018, Spina et al. reported a major study establishing the genetic panorama of cHL patients using CAPP-seq on plasma-extracted cfDNA. The most commonly mutated genes encompassed *STAT6* (37.5%), *TNFAIP3* (35%), *ITPKB* (27.5%), *GNA13* (18.7%), *B2M* (16.2%), *ATM* (15%), *SPEN* (12.5%), and *XPO1* (11.2%) [8]. The predominance of *STAT6* alterations is a discovery that was never reported in past exome works [64] and clearly reflects the impact of cytokines signaling pathway in cHL [65].

### 2.2. Comparisons between cfDNA and Tumor DNA

In solid tumors [59,60], the similarity rate between variants found in paired tumors and cfDNA samples changed from 88.2% to 64.7% for time intervals of less than three weeks and >3 weeks between venous puncture and tissue biopsy, respectively [60], revealing a sampling time issue. Thompson et al. also demonstrated in lung cancer that increasing the time between tumor and blood collection from <14 days to >6 months highly reduced the similarity rate [61].

Using CAPP-seq with cfDNA, microdissected HRS-cell enriched areas from biopsies, paired tumor genomic DNA (gDNA) and paired normal gDNA, Spina et al. demon-

strated the tumor origin of cfDNA variants depicted in their cHL patients. The similarity ( $R^2 = 0.978$ ) of mutational profiles from paired gDNA/cfDNA samples favors the capability of CAPP-seq to precisely detect low burden variants in cfDNA. In our experience, comparability between gDNA and cfDNA profiles with an NGS-limited gene panel is close to 85% [10] at the level variant. Of note, median VAF appears to be higher in cfDNA than in biopsies probable due to the common scarcity of tumor cells in cHL biopsies [10]. In the study by Desch et al. [66] in pediatric cHL patients, the average VAFs were 1.1% for tumor DNA (from whole tissue sections) and 11.1% for cfDNA, but all 30 variants discovered in cfDNA were then confirmed in macrodissected HRS-cell rich regions of paired tumor biopsies, confirming the reliability of cfDNA-obtained mutational profiles. Of note, fresh frozen tissue led to a better concordance rate between genomic DNA and cfDNA (57.1% vs. 66.7% for FFPE tissue) given DNA alterations induced by the FFPE process, particularly for amplicon-based amplification assays [67–69]. Nevertheless, this issue could be largely fixed by FFPE DNA repair methods [70] before NGS sequencing. Notwithstanding, tumor subclones are probably dynamically dispersed between various anatomical sites (spatial heterogeneity), which may prevent exhaustive discovery of all possibly existing variants mutations in a unique lymph node resection or fine-needle biopsy. In our opinion, this issue may be surmounted by assessing paired tumor biopsy/plasma cfDNA samples. It now seems established that cfDNA is an excellent mirror of the HRS cell genetic panorama (see Figure 1).

### 2.3. Comparisons between cfDNA Results and cHL Histological Subtypes

In the WHO classification, four distinct cHL subtypes are described [71]: Nodular sclerosis cHL (NSCHL), which is the most common, mixed cellularity (MCCHL), lymphocyte-depleted cHL (LDCHL), and lymphocyte-rich cHL (LRCHL) [72]. Clinical characteristics, overall prognosis, HRS cells phenotype and treatments are similar but the transcriptome and microenvironment show substantial differences. HRS cells typically express *MYC*, *NOTCH1*, and *IRF4* in all cHL histologic subtypes [73,74].

Nevertheless, gene expression profiling studies demonstrated at the transcriptome level [75,76] that the histologic subtypes of cHL are also biologically distinct. According to Reichel et al., *B2M* mutations are exclusively found in the nodular sclerosis subtype [64]. The work published by Spina et al. [8] confirms these data and indicates that these subtypes are distinct at the genetic level. In particular, NSCHL and EBER-negative cHL are associated with more frequent *STAT6* and *TNFAIP3* cfDNA somatic mutations than other subtypes. Nevertheless, *XPO1* E571K recurrent mutations are detectable in all subtypes and so are not a pathognomonic feature of a particular subtype [7]. Plasma cfDNA concentrations at baseline and genetic profiles from tumor biopsies at diagnosis were also assessed in the 4 cHL subtypes, and no differences were observed in another retrospective study [9].

### 2.4. Potential Interest in the Differential Diagnosis with Other Lymphomas (Gray-Zone, Primary Mediastinal B Cell Lymphoma)

cHL is sometimes hard to diagnose and can therefore be mistaken for several differential diagnoses, including DLBCL, primary mediastinal large B-cell lymphoma (PMBL), anaplastic large cell lymphoma (ALCL), and mediastinal gray-zone lymphoma (MGZL), both of which may display CD30 positivity [71]. In particular, PMBL and NSCHL pathological features are overlapping, so several authors estimated that these two entities are derived from thymic B cells [77,78]. The data demonstrating that the *XPO1* E571K, *STAT6*, and *SOCS1* mutations are frequent PMBL and NSHL but rare in DLBCL [7,8,10,79] support the idea of a shared oncogenic origin between cHL and PMBL. In our opinion, we may use *XPO1* E571K detection by cfDNA analysis to help pathologists to orient between NSCHL, MGZL and DLBCL, especially in the relapse setting if this variant was already present at diagnosis, despite the lack of specificity of this hotspot mutation [79–81]. In addition, *STAT6* mutations are easily detectable by cfDNA analysis, are not observed in nodular lymphocyte predominant Hodgkin lymphoma [82] (NLPHL) and are frequent in cHL [8]. Thus, *STAT6* mutations may be useful to differentially diagnose these two entities.

### 3. Association of cfDNA Level with Clinical Features

The amount of cfDNA appears to be related to tumor mass and aggressive disease presentation in cHL, making it an interesting prognostic tool starting from diagnosis. Indeed, in a recent prospective study [10], pre-treatment elevated cfDNA concentration was linked to adverse clinical characteristics: age  $\geq 45$  years, presence of anemia, albuminemia  $< 40$  g/L, sedimentation rate  $\geq 50$  mm, stage III–IV disease, lymphocyte count  $< 0.6$  g/L, presence of B symptoms, International Prognostic Index  $\geq 3$ , and elevated lactate dehydrogenase. Patients with mutations in *ITPKB* and *B2M* had more disseminated disease than patients with “negative” plasma. *XPO1* mutated patients were predominantly female with no apparent biological reason for this finding. These results are consistent with those reported in the DLBCL setting [52,53] where higher stage, International Prognostic Index (IPI), and tumor metabolic volume (TMV) are linked to elevated cfDNA levels.

In another study in pediatric cHL patients, augmented cfDNA concentrations were observed in patients aged  $> 10$  year (age  $\leq 10$ : cfDNA range 0–58 ng/mL; age  $> 10$ : cfDNA range of 10–1650 ng/mL,  $p = 0.01$ ) [83]. In addition, in the study by Spina et al. [8] *STAT6* alterations were more frequently observed in young patients ( $< 60$  years), maybe due to a higher proportion of mixed cellularity and EBER-positive cases in elderly cHL patients.

### 4. cfDNA and Pediatric cHL Specificities

The number of publications concerning circulating DNA in pediatric forms of LH is relatively limited. Simona Primerano et al. [55] determined the pertinence of cfDNA assessment in a series of 155 children with cHL by a Taq-Man-based real-time PCR assay for the *POLR2* with serial venous punctures: at baseline and during frontline chemotherapy. The authors established a control group with 15 pediatric healthy individuals to assess the “normal” (background noise) range of cfDNA in plasma. Median age of the patients was 13.6 years (2.7–19.7) and the authors observed that cfDNA levels were strongly increased in comparison with healthy subjects (mean value 112 ng/mL in cHL cases versus 5.5 ng/mL in controls,  $p = 0.002$ ). In this cohort, pre-treatment cfDNA levels were linked to B symptoms presence ( $p = 0.027$ ) and high erythrocyte sedimentation rate ( $p = 0.049$ ). cfDNA kinetics was studied in a subgroup of patients. Interestingly, after one cycle of chemotherapy, an increase in cfDNA was observed in HL with mediastinal bulky disease in 45% of cases. In comparison, only 17% of no bulky disease patients had elevated cfDNA levels after C1. This finding suggests that cfDNA concentration dynamic assessment represent a promising noninvasive tool, especially in bulky mediastinal disease. However, the cfDNA measure performed in this work was not able to distinguish circulating tumor DNA from other sources of circulating DNA, such as myeloid-derived suppressor cells or hematopoiesis cells, during chemotherapy, therefore limiting the meaning of the data.

More recently, Desch and colleagues performed an extensive analysis of tumor circulating DNA in pediatric cHL patients using a hybrid capture-targeted next-generation sequencing assay allowing the detection of single nucleotide variants, insertions/deletions, translocations, and VH-DH-JH rearrangements [66]. Ninety-six pretherapeutic plasma samples from children enrolled in the German cohort of the EuroNet PHL-C2 study were analyzed. Genetic variants were revealed at baseline in cfDNA samples of 72/96 patients (75%), a rate similar to those observed in DLBCL. Variants ranged from 1 to 21 with alleles with frequencies from 0.6 to 42%. Genes implicated in *JAK/STAT*, *NF-kB* and *PI3K* pathways and antigen presentation signaling were most frequently affected.

Sixty percent of all cases displayed *SOCS1* alterations, which is an AID target, and represented the most common variant. The authors add that 118 (29%) of all 399 disclosed somatic mutations affected *SOCS1*. Indeed, *SOCS1* mutations were typically present in multiple copies, deleterious and classified as major mutation events (stops and all indels). Importantly, the authors observed nine translocation breakpoints with various partners, including the *IGH* locus but also *TBLXR1* or *IRF4*. These results indicated that *SOCS1* inactivation is a major and highly selected genetic event in pediatric HL. Consistent with the results obtained in adults, cfDNA levels at baseline were significantly associated with



higher TMV. In addition, cfDNA kinetics correlated with the early response assessment by qPET, a quantitative extension of the Deauville scale. Altogether, these studies showed that cfDNA provided a considerable amount of information to genetically characterize HL and monitor therapy response in children with cHL. This technology may offer the possibility of reducing the toxicity of therapies, particularly radiotherapy. However, as in adults, homogenization and validation of analysis techniques are necessary.

### 5. cfDNA as A MRD Biomarker in cHL

cfDNA levels at baseline in newly-diagnosed cHL may predict the patient's overall outcome (see Figure 1). Indeed, it was recently demonstrated that significantly higher pretreatment median cfDNA quantities at diagnosis were correlated with adverse baseline clinical characteristics, as mentioned above [10]. This finding suggested a link between cfDNA concentration and disease burden and the lymphoma-generated inflammatory syndrome. Although it is not possible to define a reproducible cfDNA concentration threshold predictive of patient survival and their response to treatment, these first results nevertheless indicate that the baseline cfDNA level is a tool for assessing the overall risk of patients in addition to standard clinical, biological, and metabolic imaging prognostic markers. Indeed, in the same series of patients [10], higher MTV at diagnosis is associated with higher cfDNA median VAF. This finding is common in DLBCL [84] and follicular lymphoma [85] settings. Tumor burden in terms of MTV is, therefore, correlated with the quantity of cfDNA released and the overall prognosis of lymphoma. However, this correlation is slight, indicating that several distinct features may confound these two ways of lymphoma burden assessment. The total metabolic tumor surface (TMTS), representing the tumor-host contact, was significantly associated with cfDNA levels in cHL, and the TumBB parameter, representing the dispersion volume of the tumor, was also correlated with cfDNA concentration [86]. It is particularly interesting to understand the association between these new PET parameters and cfDNA because TMTS represents the "battlefront" between the tumor and the microenvironment at the tumor surface where cytolysis and apoptosis occur [87], whereas TumBB is correlated to disease extent and so could be a surrogate marker of Ann Arbor staging. These recent parameters may probably be soon easier to perform because lymphoma imaging will be, in the future, automatically segmented [88]. This will certainly settle the debate whether cfDNA is complimentary to PET [86] or is sufficient itself for disease monitoring.

Although cHL patients respond well to treatment with high metabolic CR rates, a very low number of cHL cells can persist and may cause disease to return. This MRD may be measurable with highly sensitive methods.

In a preliminary retrospective work, it was reported that patients with undetectable *XPO1* mutations after frontline chemotherapy had a trend toward a better PFS than those having end of treatment positive cfDNA [7]. We may suggest that the variants clearance in blood is a reliable biomarker of good therapeutic response. In a landmark report, Spina et al. [8] retrospectively analyzed several blood samples in 24 advanced stage cHL patients homogeneously treated with standard ABVD and demonstrated that a 2-log drop in cfDNA between diagnosis and after two chemotherapy courses was linked to complete metabolic response and cure. This threshold was already established in DLBCL [89] and is likely a relevant cutoff to predict disease progression. Therefore, cfDNA assessment adds relevant data to interim PET in determining the therapeutic response. Indeed, cured patients with interim PET positivity had a greater than 2-log reduction in cfDNA. In contrast, relapsed/refractory patients with interim PET negativity had a less than 2-log reduction in cfDNA [8]. In a prospective setting, it was also established that liquid biopsy may provide overall genetic landscape of newly diagnosed and relapsing cHL and may evaluate disease response during and at end of treatment, in compliment to standard PET [10].

In addition, cfDNA may also serve as a tool to identify the clonal evolution of cHL. In the study by Spina et al. [8], a clonal shift between baseline and relapse paired samples

was established, which can be tracked by NGS cfDNA analysis. The clonal and subclonal evolution that has been reported by this team is, interestingly, very different depending on whether the patient is receiving treatment by chemotherapy and/or antibody-drug conjugates or treatment by immunotherapy. In the first case, the mutations of the ancestral clone remained detectable in the clone on relapse with new point mutations (*STAT6*, *GNA13*, *ITPKB*); in the second case, the ancestral mutations disappeared completely, and the tumor tried to escape immunotherapy by generating completely different mutations. In any case, cfDNA is an excellent tool to follow this MRD and this clonal evolution (see Figure 1).

However, points should be clarified concerning the strategy to be adopted in the event of infraclinical molecular relapse. It is not certain that early retreatment for infraclinical relapse will be positive for patients, will ensure better disease response or that therapeutic intensification in case of a detectable MRD after standard conventional therapy will be associated with better survival. It will therefore certainly take several more years and the establishment of interventional clinical trials based on MRD to be able to integrate this tool into everyday clinical practice.

## 6. Current Challenges and Future Perspectives

Many uncertainties still weigh on our ability to transfer into routine practice the promising data observed with cfDNA in cHL. Remaining issues consist in: (i) lack of sensitivity due to scarcity of HRS cells and low cfDNA quantity in peripheral blood; (ii) limit of detection concerns in plasma cfDNA sequencing background noise (PCR or sequencing errors); (iii) lack of multicenter reproducibility; (iv) lack of uniformity of preanalytical requirements (including storage temperature) and various targeted gene panel; and (v) lack of consensus on the attitude to adopt in the event of evidence of a subclinical relapse, with no localization of the site of the relapse in the event of a positive cfDNA. In addition, NGS requires trained bioinformaticians, robust analysis pipelines and time-consuming dedicated computational validation methods to exclude false-positive artifacts. In our opinion, the central issue lasts the medical significance of “molecular relapse detection” which remains unclear in cHL, instead of acute leukemia settings. Interventional clinical trials based on molecular relapse or positive MRD events are to be imagined to increase the transferability of these recent data into clinical practice.

Finally, it seems important to pinpoint recent research work carried out on alternative liquid biopsy sources to cfDNA plasma. Indeed, platelets have a well-described pivotal role in the immune response to cancer progression [90]. Tumor-educated platelets (TEP) participated in the growth and dissemination of numerous solid tumors and spliced TEP RNA “signatures” (intraplatelet RNA splicing) can grant data on the existence and location of a tumor and also provide molecular genotyping [91]. TEPs were described as promising with several interesting features as compared to standard plasma cfDNA. A great amount of undamaged TEP RNA [92] is accessible in the equivalent of one drop of blood and TEPs grants early pan-cancer detection. Of note, a recent report described a standardized analysis pipeline [93] to enhance reproducibility. Despite the lack of published data in cHL, TEPs will probably be useful in the next future in compliment with other liquid biopsy tools. Finally, extracellular vesicles (EVs) are also an innovative area of research in liquid biopsy. EVs are secreted by normal and tumoral cells, have a size ranging from 30–2000 nm, and have several biological functions, including intracellular communication and tumor microenvironment remodeling [94]. EVs. are not confined to the tumor microenvironment in cancer patients and can be isolated by several yet not standardized methods from blood as well as from other human fluids and so are able to provide good quality tumor RNA and miRNAs [95,96]. For example, *IDH1* somatic alterations in glioblastoma are detectable in mRNA extracted from EVs. using droplet digital PCR sequencing [97]. However, to date there is no published data in cHL regarding EVs. and TEPs and much work remains to be done on these non-invasive exploratory sources of tumor genetic material.

## 7. Conclusions

cfDNA testing is clearly a rising technology in the setting of cHL and is relevant at every step of the patient's journey. cfDNA is useful to provide an overview of the genomic panorama of the tumor, to assess prognostic stratification and to monitor disease response as a complement to PET. This tool should now be systematically integrated as an endpoint into future clinical trials for cHL at diagnosis and relapse.

**Funding:** This research received no external funding.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

- Diehl, V.; Thomas, R.K.; Re, D. Part II: Hodgkin's lymphoma—diagnosis and treatment. *Lancet Oncol.* **2004**, *5*, 19–26. [[CrossRef](#)]
- Hasenclever, D.; Diehl, V. A prognostic score for advanced Hodgkin's disease. International Prognostic Factors Project on Advanced Hodgkin's Disease. *N. Engl. J. Med.* **1998**, *339*, 1506–1514. [[CrossRef](#)]
- Casasnovas, R.-O.; Bouabdallah, R.; Brice, P.; Lazarovici, J.; Ghesquieres, H.; Stamatoullas, A.; Dupuis, J.; Gac, A.-C.; Gastinne, T.; Joly, B.; et al. PET-Adapted Treatment for Newly Diagnosed Advanced Hodgkin Lymphoma (AHL2011): A Randomised, Multicentre, Non-Inferiority, Phase 3 Study. *Lancet Oncol.* **2019**, *20*, 202–215. [[CrossRef](#)]
- Hutchings, M.; Loft, A.; Hansen, M.; Pedersen, L.M.; Buhl, T.; Jurlander, J.; Buus, S.; Keiding, S.; D'Amore, F.; Boesen, A.-M.; et al. FDG-PET after Two Cycles of Chemotherapy Predicts Treatment Failure and Progression-Free Survival in Hodgkin Lymphoma. *Blood* **2006**, *107*, 52–59. [[CrossRef](#)] [[PubMed](#)]
- Schmitz, R.; Stanelle, J.; Hansmann, M.-L.; Küppers, R. Pathogenesis of classical and lymphocyte-predominant Hodgkin lymphoma. *Annu. Rev. Pathol.* **2009**, *4*, 151–174. [[CrossRef](#)] [[PubMed](#)]
- VanderLaan, P.A. Fine-needle aspiration and core needle biopsy: An update on 2 common minimally invasive tissue sampling modalities: FNA Versus CNB. *Cancer Cytopathol.* **2016**, *124*, 862–870. [[CrossRef](#)]
- Camus, V.; Stamatoullas, A.; Mareschal, S.; Viailly, P.-J.; Sarafan-Vasseur, N.; Bohers, E.; Dubois, S.; Picquenot, J.M.; Ruminy, P.; Maingonnat, C.; et al. Detection and Prognostic Value of Recurrent Exportin 1 Mutations in Tumor and Cell-Free Circulating DNA of Patients with Classical Hodgkin Lymphoma. *Haematologica* **2016**, *101*, 1094–1101. [[CrossRef](#)]
- Spina, V.; Brusca, A.; Cuccaro, A.; Martini, M.; Di Trani, M.; Forestieri, G.; Manzoni, M.; Condoluci, A.; Arribas, A.; Terzi-Di-Bergamo, L.; et al. Circulating Tumor DNA Reveals Genetics, Clonal Evolution, and Residual Disease in Classical Hodgkin Lymphoma. *Blood* **2018**, *131*, 2413–2425. [[CrossRef](#)] [[PubMed](#)]
- Bessi, L.; Viailly, P.-J.; Bohers, E.; Ruminy, P.; Maingonnat, C.; Bertrand, P.; Vasseur, N.; Beaussire, L.; Cornic, M.; Etancelin, P.; et al. Somatic Mutations of Cell-Free Circulating DNA Detected by Targeted next-Generation Sequencing and Digital Droplet PCR in Classical Hodgkin Lymphoma. *Leuk. Lymphoma* **2018**, *60*, 498–502. [[CrossRef](#)]
- Camus, V.; Viennot, M.; Lequesne, J.; Viailly, P.-J.; Bohers, E.; Bessi, L.; Marcq, B.; Etancelin, P.; Dubois, S.; Picquenot, J.-M.; et al. Targeted Genotyping of Circulating Tumor DNA for Classical Hodgkin Lymphoma Monitoring: A Prospective Study. *Haematologica* **2020**, *106*, 154–162. [[CrossRef](#)]
- Vandenbergh, P.; Wlodarska, I.; Tousseyn, T.; Dehaspe, L.; Dierickx, D.; Verheecke, M.; Uyttebroeck, A.; Bechter, O.; Delforge, M.; Vandecaveye, V.; et al. Non-Invasive Detection of Genomic Imbalances in Hodgkin/Reed-Sternberg Cells in Early and Advanced Stage Hodgkin's Lymphoma by Sequencing of Circulating Cell-Free DNA: A Technical Proof-of-Principle Study. *Lancet Haematol.* **2015**, *2*, e55–e65. [[CrossRef](#)]
- Righolt, C.H.; Knecht, H.; Mai, S. DNA Superresolution Structure of Reed-Sternberg Cells Differs Between Long-Lasting Remission Versus Relapsing Hodgkin's Lymphoma Patients: DNA Structure In Pre-Treatment Hodgkin's Lymphoma. *J. Cell Biochem.* **2016**, *117*, 1633–1637. [[CrossRef](#)] [[PubMed](#)]
- Wen, F.; Shen, A.; Choi, A.; Gerner, E.W.; Shi, J. Extracellular DNA in Pancreatic Cancer Promotes Cell Invasion and Metastasis. *Cancer Res.* **2013**, *73*, 4256–4266. [[CrossRef](#)]
- Bendich, A.; Wilczok, T.; Borenfreund, E. Circulating DNA as a Possible Factor in Oncogenesis. *Science* **1965**, *148*, 374–376. [[CrossRef](#)] [[PubMed](#)]
- Diaz, L.A.; Bardelli, A. Liquid biopsies: Genotyping circulating tumor DNA. *J. Clin. Oncol.* **2014**, *32*, 579–586. [[CrossRef](#)]
- Lowes, L.E.; Bratman, S.V.; Dittamore, R.; Done, S.; Kelley, S.O.; Mai, S.; Morin, R.D.; Wyatt, A.W.; Allan, A.L. Circulating Tumor Cells (CTC) and Cell-Free DNA (CfDNA) Workshop 2016: Scientific Opportunities and Logistics for Cancer Clinical Trial Incorporation. *Int. J. Mol. Sci.* **2016**, *17*, 1505. [[CrossRef](#)] [[PubMed](#)]
- Alizadeh, A.A.; Aranda, V.; Bardelli, A.; Blanpain, C.; Bock, C.; Borowski, C.; Caldas, C.; Califano, A.; Doherty, M.; Elsnér, M.; et al. Toward Understanding and Exploiting Tumor Heterogeneity. *Nat. Med.* **2015**, *21*, 846–853. [[CrossRef](#)]
- Stroun, M.; Lyauté, J.; Lederrey, C.; Olson-Sand, A.; Anker, P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clin. Chim. Acta Int. J. Clin. Chem.* **2001**, *313*, 139–142. [[CrossRef](#)]
- Fatouros, I.G.; Jamurtas, A.Z.; Nikolaidis, M.G.; Destouni, A.; Michailidis, Y.; Vrettou, C.; Douroudos, I.I.; Avloniti, A.; Chatzinikolaou, A.; Taxildaris, K.; et al. Time of Sampling Is Crucial for Measurement of Cell-Free Plasma DNA Following Acute Aseptic Inflammation Induced by Exercise. *Clin. Biochem.* **2010**, *43*, 1368–1370. [[CrossRef](#)] [[PubMed](#)]

20. Hummel, E.M.; Hesas, E.; Müller, S.; Beiter, T.; Fisch, M.; Eibl, A.; Wolf, O.T.; Giebel, B.; Platen, P.; Kumsta, R.; et al. Cell-Free DNA Release under Psychosocial and Physical Stress Conditions. *Transl. Psychiatry* **2018**, *8*, 236. [[CrossRef](#)]
21. Van der Vaart, M.; Pretorius, P.J. The Origin of Circulating Free DNA. *Clin. Chem.* **2007**, *53*, 2215. [[CrossRef](#)] [[PubMed](#)]
22. Bronkhorst, A.J.; Ungerer, V.; Holdenrieder, S. The emerging role of cell-free DNA as a molecular marker for cancer management. *BioMol. Detect. Quantif.* **2019**, *17*, 100087. [[CrossRef](#)] [[PubMed](#)]
23. Tamkovich, S.N.; Cherepanova, A.V.; Kolesnikova, E.V.; Rykova, E.Y.; Pyshnyi, D.V.; Vlassov, V.V.; Laktionov, P.P. Circulating DNA and DNase Activity in Human Blood. *Ann. N. Y. Acad. Sci.* **2006**, *1075*, 191–196. [[CrossRef](#)] [[PubMed](#)]
24. Botezatu, I.; Serdyuk, O.; Potapova, G.; Shelepov, V.; Alechina, R.; Molyaka, Y.; Ananév, V.; Bazin, I.; Garin, A.; Narimanov, M.; et al. Genetic Analysis of DNA Excreted in Urine: A New Approach for Detecting Specific Genomic DNA Sequences from Cells Dying in an Organism. *Clin. Chem.* **2000**, *46*, 1078–1084. [[CrossRef](#)]
25. Diehl, F.; Li, M.; Dressman, D.; He, Y.; Shen, D.; Szabo, S.; Diaz, L.A.; Goodman, S.N.; David, K.A.; Juhl, H.; et al. Detection and Quantification of Mutations in the Plasma of Patients with Colorectal Tumors. *Proc. Natl. Acad. Sci.* **2005**, *102*, 16368–16373. [[CrossRef](#)] [[PubMed](#)]
26. Yao, W.; Mei, C.; Nan, X.; Hui, L. Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. *Gene* **2016**, *590*, 142–148. [[CrossRef](#)]
27. Chelobanov, B.P.; Laktionov, P.P.; Vlasov, V.V. Proteins involved in binding and cellular uptake of nucleic acids. *Biochem. Mosc.* **2006**, *71*, 583–596. [[CrossRef](#)] [[PubMed](#)]
28. Thierry, A.R.; El Messaoudi, S.; Gahan, P.B.; Anker, P.; Stroun, M. Origins, structures, and functions of circulating DNA in oncology. *Cancer Metastasis Rev.* **2016**, *35*, 347–376. [[CrossRef](#)]
29. Jahr, S.; Hentze, H.; Englisch, S.; Hardt, D.; Fackelmayer, F.O.; Hesch, R.D.; Knippers, R. DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin from Apoptotic and Necrotic Cells. *Cancer Res.* **2001**, *61*, 1659–1665. [[PubMed](#)]
30. Fontanilles, M.; Marguet, F.; Bohers, E. Somatic Mutations Detected in Plasma Cell-Free DNA By Targeted Sequencing: Assessment of Liquid Biopsy in Primary Central Nervous System Lymphoma. *Blood* **2015**, *126*, 332. [[CrossRef](#)]
31. Rimelen, V.; Ahle, G.; Pencreach, E.; Zinniger, N.; Debliquis, A.; Zalmai, L.; Harzallah, I.; Hurstel, R.; Alamome, I.; Lamy, F.; et al. Tumor Cell-Free DNA Detection in CSF for Primary CNS Lymphoma Diagnosis. *Acta Neuropathol. Commun.* **2019**, *7*, 43. [[CrossRef](#)] [[PubMed](#)]
32. Streleckiene, G.; Reid, H.M.; Arnold, N.; Bauerschlag, D.; Forster, M. Quantifying cell free DNA in urine: Comparison between commercial kits, impact of gender and inter-individual variation. *BioTechniques* **2018**, *64*, 225–230. [[CrossRef](#)] [[PubMed](#)]
33. Fumagalli, C.; Bianchi, F.; Rafaniello Raviele, P.; Vacirca, D.; Bertalot, G.; Rampinelli, C.; Lazzeroni, M.; Bonnani, B.; Veronesi, G.; Fusco, N.; et al. Circulating and Tissue Biomarkers in Early-Stage Non-Small. *Ecancermedalscience* **2017**, *11*, 717. [[CrossRef](#)] [[PubMed](#)]
34. Nair, N.; Camacho-Vanegas, O.; Rykunov, D.; Dashkoff, M.; Camacho, S.C.; Schumacher, C.A.; Irish, J.C.; Harkins, T.T.; Freeman, E.; Garcia, I.; et al. Genomic Analysis of Uterine Lavage Fluid Detects Early Endometrial Cancers and Reveals a Prevalent Landscape of Driver Mutations in Women without Histopathologic Evidence of Cancer: A Prospective Cross-Sectional Study. *PLoS Med.* **2016**, *13*, e1002206. [[CrossRef](#)] [[PubMed](#)]
35. Imperiale, T.F.; Ransohoff, D.F.; Itzkowitz, S.H.; Levin, T.R.; Lavin, P.; Lidgard, G.P.; Ahlquist, D.A.; Berger, B.M. Multitarget Stool DNA Testing for Colorectal-Cancer Screening. *N. Engl. J. Med.* **2014**, *370*, 1287–1297. [[CrossRef](#)] [[PubMed](#)]
36. Chae, Y.K.; Davis, A.A.; Carneiro, B.A.; Chandra, S.; Mohindra, N.; Kalyan, A.; Kaplan, J.; Matsangou, M.; Pai, S.; Costa, R.; et al. Concordance between Genomic Alterations Assessed by Next-Generation Sequencing in Tumor Tissue or Circulating Cell-Free DNA. *Oncotarget* **2016**, *7*, 65364. [[CrossRef](#)]
37. Huang, A.; Zhang, X.; Zhou, S.-L.; Cao, Y.; Huang, X.-W.; Fan, J.; Yang, X.-R.; Zhou, J. Detecting Circulating Tumor DNA in Hepatocellular Carcinoma Patients Using Droplet Digital PCR Is Feasible and Reflects Intratumoral Heterogeneity. *J. Cancer* **2016**, *7*, 1907–1914. [[CrossRef](#)]
38. Liao, W.; Yang, H.; Xu, H.; Wang, Y.; Ge, P.; Ren, J.; Xu, W.; Lu, X.; Sang, X.; Zhong, S.; et al. Noninvasive Detection of Tumor-Associated Mutations from Circulating Cell-Free DNA in Hepatocellular Carcinoma Patients by Targeted Deep Sequencing. *Oncotarget* **2016**, *7*, 40481–40490. [[CrossRef](#)] [[PubMed](#)]
39. Parackal, S.; Zou, D.; Day, R.; Black, M.; Guilford, P. Comparison of Roche Cell-Free DNA collection Tubes to Streck Cell-Free DNA BCT s for sample stability using healthy volunteers. *Pract. Lab. Med.* **2019**, *16*, e00125. [[CrossRef](#)]
40. Merker, J.D.; Oxnard, G.R.; Compton, C.; Diehn, M.; Hurley, P.; Lazar, A.J.; Lindeman, N.; Lockwood, C.M.; Rai, A.J.; Schilsky, R.L.; et al. Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. *Arch. Pathol. Lab. Med.* **2018**, *142*, 1242–1253. [[CrossRef](#)]
41. Kang, Q.; Henry, N.L.; Paoletti, C.; Jiang, H.; Vats, P.; Chinnaiyan, A.M.; Hayes, D.F.; Merajver, S.D.; Rae, J.M.; Tewari, M. Comparative Analysis of Circulating Tumor DNA Stability In K3EDTA, Streck, and CellSave Blood Collection Tubes. *Clin. Biochem.* **2016**, *49*, 1354–1360. [[CrossRef](#)]
42. El Messaoudi, S.; Rolet, F.; Mouliere, F.; Thierry, A.R. Circulating cell free DNA: Preanalytical considerations. *Clin. Chim. Acta Int. J. Clin. Chem.* **2013**, *424*, 222–230. [[CrossRef](#)] [[PubMed](#)]

43. Murtaza, M.; Dawson, S.-J.; Tsui, D.W.Y.; Gale, D.; Forshew, T.; Piskorz, A.M.; Parkinson, C.; Chin, S.-F.; Kingsbury, Z.; Wong, A.S.C.; et al. Non-Invasive Analysis of Acquired Resistance to Cancer Therapy by Sequencing of Plasma DNA. *Nature* **2013**, *497*, 108–112. [[CrossRef](#)] [[PubMed](#)]
44. Camus, V.; Sarafan-Vasseur, N.; Bohers, E.; Dubois, S.; Mareschal, S.; Bertrand, P.; Viailly, P.-J.; Ruminy, P.; Maingonnat, C.; Lemasle, E.; et al. Digital PCR for Quantification of Recurrent and Potentially Actionable Somatic Mutations in Circulating Free DNA from Patients with Diffuse Large B-Cell Lymphoma. *Leuk. Lymphoma* **2016**, *57*, 2171–2179. [[CrossRef](#)]
45. Küppers, R.; Rajewsky, K.; Zhao, M.; Simons, G.; Laumann, R.; Fischer, R.; Hansmann, M.L. Hodgkin Disease: Hodgkin and Reed-Sternberg Cells Picked from Histological Sections Show Clonal Immunoglobulin Gene Rearrangements and Appear to Be Derived from B Cells at Various Stages of Development. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 10962–10966. [[CrossRef](#)] [[PubMed](#)]
46. Jones, R.J.; Gocke, C.D.; Kasamon, Y.L.; Miller, C.B.; Perkins, B.; Barber, J.P.; Vala, M.S.; Gerber, J.M.; Gellert, L.L.; Siedner, M.; et al. Circulating Clonotypic B Cells in Classic Hodgkin Lymphoma. *Blood* **2009**, *113*, 5920–5926. [[CrossRef](#)]
47. Oki, Y.; Neelapu, S.S.; Fanale, M.; Kwak, L.W.; Fayad, L.; Rodriguez, M.A.; Wallace, M.; Klinger, M.; Carlton, V.; Kong, K.; et al. Detection of Classical Hodgkin Lymphoma Specific Sequence in Peripheral Blood Using a Next-Generation Sequencing Approach. *Br. J. Haematol.* **2015**, *169*, 689–693. [[CrossRef](#)]
48. Daly, J.; Licence, S.; Nanou, A.; Morgan, G.; Mårtensson, I.-L. Transcription of productive and nonproductive VDJ-recombined alleles after IgH allelic exclusion. *EMBO J.* **2007**, *26*, 4273–4282. [[CrossRef](#)] [[PubMed](#)]
49. Bohers, E.; Viailly, P.J.; Dubois, S.; Bertrand, P.; Maingonnat, C.; Mareschal, S.; Ruminy, P.; Picquenot, J.-M.; Bastard, C.; Desmots, F.; et al. Somatic Mutations of Cell-Free Circulating DNA Detected by next-Generation Sequencing Reflect the Genetic Changes in Both Germinal Center B-Cell-like and Activated B-Cell-like Diffuse Large B-Cell Lymphomas at the Time of Diagnosis. *Haematologica* **2015**, *100*, e280–e284. [[CrossRef](#)]
50. Newman, A.M.; Bratman, S.V.; To, J.; Wynne, J.F.; Eclov, N.C.W.; Modlin, L.A.; Liu, C.L.; Neal, J.W.; Wakelee, H.A.; Merritt, R.E.; et al. An Ultrasensitive Method for Quantitating Circulating Tumor DNA with Broad Patient Coverage. *Nat. Med.* **2014**, *20*, 548–554. [[CrossRef](#)]
51. Kurtz, D.M.; Scherer, F.; Newman, A.M.; Lovejoy, A.F.; Klass, D.M.; Chabon, J.J.; Gambhir, S.; Diehn, M.; Alizadeh, A.A. Dynamic Noninvasive Genomic Monitoring for Outcome Prediction in Diffuse Large B-Cell Lymphoma. *Blood* **2015**, *126*, 130. [[CrossRef](#)]
52. Rossi, D.; Diop, F.; Spaccarotella, E.; Monti, S.; Zanni, M.; Rasi, S.; Deambrogi, C.; Spina, V.; Brusca, A.; Favini, C.; et al. Diffuse Large B-Cell Lymphoma Genotyping on the Liquid Biopsy. *Blood* **2017**, *129*, 1947–1957. [[CrossRef](#)]
53. Scherer, F.; Kurtz, D.M.; Newman, A.M.; Stehr, H.; Craig, A.F.M.; Esfahani, M.S.; Lovejoy, A.F.; Chabon, J.J.; Klass, D.M.; Liu, C.L.; et al. Distinct Biological Subtypes and Patterns of Genome Evolution in Lymphoma Revealed by Circulating Tumor DNA. *Sci. Transl. Med.* **2016**, *8*, 364ra155. [[CrossRef](#)] [[PubMed](#)]
54. Sater, V.; Viailly, P.-J.; Lecroq, T.; Prieur-Gaston, É.; Bohers, É.; Viennot, M.; Ruminy, P.; Dauchel, H.; Vera, P.; Jardin, F. UMI-VarCal: A New UMI-Based Variant Caller That Efficiently Improves Low-Frequency Variant Detection in Paired-End Sequencing NGS Libraries. *Bioinformatics* **2020**, *36*, 2718–2724. [[CrossRef](#)]
55. Primerano, S.; Burnelli, R.; Carraro, E.; Pillon, M.; Elia, C.; Farruggia, P.; Sala, A.; Vinti, L.; Buffardi, S.; Basso, G.; et al. Kinetics of Circulating Plasma Cell-Free DNA in Paediatric Classical Hodgkin Lymphoma. *J. Cancer* **2016**, *7*, 364–366. [[CrossRef](#)] [[PubMed](#)]
56. Baker, M. Digital PCR hits its stride. *Nat. Methods* **2012**, *9*, 541–544. [[CrossRef](#)]
57. Schiavon, G.; Hrebien, S.; Garcia-Murillas, I.; Cutts, R.J.; Pearson, A.; Tarazona, N.; Fenwick, K.; Kozarewa, I.; Lopez-Knowles, E.; Ribas, R.; et al. Analysis of ESR1 Mutation in Circulating Tumor DNA Demonstrates Evolution during Therapy for Metastatic Breast Cancer. *Sci. Transl. Med.* **2015**, *7*, 313ra182. [[CrossRef](#)] [[PubMed](#)]
58. Taly, V.; Pekin, D.; Benhaim, L.; Kotsopoulos, S.K.; Le Corre, D.; Li, X.; Atochin, I.; Link, D.R.; Griffiths, A.D.; Pallier, K.; et al. Multiplex Picodroplet Digital PCR to Detect KRAS Mutations in Circulating DNA from the Plasma of Colorectal Cancer Patients. *Clin. Chem.* **2013**, *59*, 1722–1731. [[CrossRef](#)] [[PubMed](#)]
59. Tone, A.A.; McConechy, M.K.; Yang, W.; Ding, J.; Yip, S.; Kong, E.; Wong, K.-K.; Gershenson, D.M.; Mackay, H.; Shah, S.; et al. Intratumoral Heterogeneity in a Minority of Ovarian Low-Grade Serous Carcinomas. *BMC Cancer* **2014**, *14*, 982. [[CrossRef](#)]
60. Laurent-Puig, P.; Pekin, D.; Normand, C.; Kotsopoulos, S.K.; Nizard, P.; Perez-Toralla, K.; Rowell, R.; Olson, J.; Srinivasan, P.; Le Corre, D.; et al. Clinical Relevance of KRAS-Mutated Subclones Detected with Picodroplet Digital PCR in Advanced Colorectal Cancer Treated with Anti-EGFR Therapy. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **2015**, *21*, 1087–1097. [[CrossRef](#)]
61. Dugo, N.; Padula, F.; Mobili, L.; Brizzi, C.; D’Emidio, L.; Cignini, P.; Mesoraca, A.; Bizzoco, D.; Cima, A.; Giorlandino, C. Six Consecutive False Positive Cases from Cell-Free Fetal DNA Testing in a Single Referring Centre. *J. Prenat. Med.* **2014**, *8*, 31–35. [[CrossRef](#)]
62. Ilie, M.; Hofman, V.; Long, E.; Bordone, O.; Selva, E.; Washetine, K.; Marquette, C.H.; Hofman, P. Current Challenges for Detection of Circulating Tumor Cells and Cell-Free Circulating Nucleic Acids, and Their Characterization in Non-Small Cell Lung Carcinoma Patients. What Is the Best Blood Substrate for Personalized Medicine? *Ann. Transl. Med.* **2014**, *2*, 107. [[CrossRef](#)]
63. Liu, J.; Chen, X.; Wang, J.; Zhou, S.; Wang, C.L.; Ye, M.Z.; Wang, X.Y.; Song, Y.; Wang, Y.Q.; Zhang, L.T.; et al. Biological Background of the Genomic Variations of Cf-DNA in Healthy Individuals. *Ann. Oncol.* **2019**, *30*, 464–470. [[CrossRef](#)] [[PubMed](#)]
64. Reichel, J.B.; McCormick, J.; Fromm, J.R.; Elemento, O.; Cesarman, E.; Roshal, M. Flow-sorting and Exome Sequencing of the Reed-Sternberg Cells of Classical Hodgkin Lymphoma. *J. Vis. Exp. JoVE* **2017**, *124*, 54399. [[CrossRef](#)] [[PubMed](#)]

65. Skinnider, B.F.; Elia, A.J.; Gascoyne, R.D.; Patterson, B.; Trumper, L.; Kapp, U.; Mak, T.W. Signal Transducer and Activator of Transcription 6 Is Frequently Activated in Hodgkin and Reed-Sternberg Cells of Hodgkin Lymphoma. *Blood* **2002**, *99*, 618–626. [[CrossRef](#)]
66. Desch, A.-K.; Hartung, K.; Botzen, A.; Brobeil, A.; Rummel, M.; Kurch, L.; Georgi, T.; Jox, T.; Bielack, S.; Burdach, S.; et al. Genotyping Circulating Tumor DNA of Pediatric Hodgkin Lymphoma. *Leukemia* **2020**, *34*, 151–166. [[CrossRef](#)]
67. Guo, Q.; Wang, J.; Xiao, J.; Wang, L.; Hu, X.; Yu, W.; Song, G.; Lou, J.; Chen, J. Heterogeneous Mutation Pattern in Tumor Tissue and Circulating Tumor DNA Warrants Parallel NGS Panel Testing. *Mol. Cancer* **2018**, *17*, 131. [[CrossRef](#)]
68. Wang, B.; Wu, S.; Huang, F.; Shen, M.; Jiang, H.; Yu, Y.; Yu, Q.; Yang, Y.; Zhao, Y.; Zhou, Y.; et al. Analytical and Clinical Validation of a Novel Amplicon-Based NGS Assay for the Evaluation of Circulating Tumor DNA in Metastatic Colorectal Cancer Patients. *Clin. Chem. Lab. Med. CCLM* **2019**, *57*, 1501–1510. [[CrossRef](#)] [[PubMed](#)]
69. Perdignes, N.; Murtaza, M. Capturing tumor heterogeneity and clonal evolution in solid cancers using circulating tumor DNA analysis. *Pharmacol. Ther.* **2017**, *174*, 22–26. [[CrossRef](#)]
70. McDonough, S.J.; Bhagwate, A.; Sun, Z.; Wang, C.; Zschunke, M.; Gorman, J.A.; Kopp, K.J.; Cunningham, J.M. Use of FFPE-Derived DNA in next Generation Sequencing: DNA Extraction Methods. *PLoS ONE* **2019**, *14*, e0211400. [[CrossRef](#)] [[PubMed](#)]
71. Swerdlow, S.H.; Campo, E.; Pileri, S.A.; Harris, N.L.; Stein, H.; Siebert, R.; Advani, R.; Ghielmini, M.; Salles, G.A.; Zelenetz, A.D.; et al. The 2016 Revision of the World Health Organization Classification of Lymphoid Neoplasms. *Blood* **2016**, *127*, 2375–2390. [[CrossRef](#)]
72. Diehl, V.; Sextro, M.; Franklin, J.; Hansmann, M.-L.; Harris, N.; Jaffe, E.; Poppema, S.; Harris, M.; Franssila, K.; van Krieken, J.; et al. Clinical Presentation, Course, and Prognostic Factors in Lymphocyte-Predominant Hodgkin's Disease and Lymphocyte-Rich Classical Hodgkin's Disease: Report From the European Task Force on Lymphoma Project on Lymphocyte-Predominant Hodgkin's Disease. *J. Clin. Oncol.* **1999**, *17*, 776–783. [[CrossRef](#)] [[PubMed](#)]
73. Carbone, A.; Gloghini, A.; Aldinucci, D.; Gattei, V.; Dalla-Favera, R.; Gaidano, G. Expression pattern of MUM1/IRF4 in the spectrum of pathology of Hodgkin's disease. *Br. J. Haematol.* **2002**, *117*, 366–372. [[CrossRef](#)] [[PubMed](#)]
74. Jiwa, N.M.; Kanavaros, P.; van der Valk, P.; Walboomers, J.M.; Horstman, A.; Vos, W.; Mullink, H.; Meijer, C.J. Expression of C-Myc and Bcl-2 Oncogene Products in Reed-Sternberg Cells Independent of Presence of Epstein-Barr Virus. *J. Clin. Pathol.* **1993**, *46*, 211–217. [[CrossRef](#)] [[PubMed](#)]
75. Tiacci, E.; Döring, C.; Brune, V.; van Noesel, C.J.M.; Klapper, W.; Mechttersheimer, G.; Falini, B.; Küppers, R.; Hansmann, M.-L. Analyzing Primary Hodgkin and Reed-Sternberg Cells to Capture the Molecular and Cellular Pathogenesis of Classical Hodgkin Lymphoma. *Blood* **2012**, *120*, 4609–4620. [[CrossRef](#)] [[PubMed](#)]
76. Devillard, E.; Bertucci, F.; Trepont, P.; Bouabdallah, R.; Llorion, B.; Giaconia, A.; Brousset, P.; Granjeaud, S.; Nguyen, C.; Birnbaum, D.; et al. Gene Expression Profiling Defines Molecular Subtypes of Classical Hodgkin's Disease. *Oncogene* **2002**, *21*, 3095–3102. [[CrossRef](#)]
77. Traverse-Glehen, A.; Pittaluga, S.; Gaulard, P.; Sorbara, L.; Alonso, M.A.; Raffeld, M.; Jaffe, E.S. Mediastinal Gray Zone Lymphoma: The Missing Link between Classic Hodgkin's Lymphoma and Mediastinal Large B-Cell Lymphoma. *Am. J. Surg. Pathol.* **2005**, *29*, 1411–1421. [[CrossRef](#)]
78. Giulino-Roth, L. How I treat primary mediastinal B-cell lymphoma. *Blood* **2018**, *132*, 782–790. [[CrossRef](#)] [[PubMed](#)]
79. Jardin, F.; Pujals, A.; Pelletier, L.; Bohers, E.; Camus, V.; Mareschal, S.; Dubois, S.; Sola, B.; Ochmann, M.; Lemonnier, F.; et al. Recurrent Mutations of the Exportin 1 Gene (XPO1) and Their Impact on Selective Inhibitor of Nuclear Export Compounds Sensitivity in Primary Mediastinal B-Cell Lymphoma: XPO1 Mutations in Primary Mediastinal B-Cell Lymphoma. *Am. J. Hematol.* **2016**, *91*, 923–930. [[CrossRef](#)]
80. Dunleavy, K.; Wilson, W.H. Primary mediastinal B-cell lymphoma and mediastinal gray zone lymphoma: Do they require a unique therapeutic approach? *Blood* **2015**, *125*, 33–39. [[CrossRef](#)]
81. Dunleavy, K.; Grant, C.; Eberle, F.C.; Pittaluga, S.; Jaffe, E.S.; Wilson, W.H. Gray zone lymphoma: Better treated like Hodgkin lymphoma or mediastinal large B-cell lymphoma? *Curr. Hematol. Malign. Rep.* **2012**, *7*, 241–247. [[CrossRef](#)] [[PubMed](#)]
82. Van Slambrouck, C.; Huh, J.; Suh, C.; Song, J.Y.; Menon, M.P.; Sohani, A.R.; Duffield, A.S.; Goldberg, R.C.; Dama, P.; Kiyotani, K.; et al. Diagnostic Utility of STAT6/CD30 Expression in Classical Hodgkin Lymphoma and Related Entities. *Mod. Pathol.* **2020**, *33*, 834–845. [[CrossRef](#)] [[PubMed](#)]
83. Mussolin, L.; Burnelli, R.; Pillon, M.; Carraro, E.; Farruggia, P.; Todesco, A.; Mascarini, M.; Rosolen, A. Plasma Cell-Free DNA in Paediatric Lymphomas. *J. Cancer* **2013**, *4*, 323–329. [[CrossRef](#)] [[PubMed](#)]
84. Bohers, E.; Viailly, P.-J.; Becker, S.; Marchand, V.; Ruminy, P.; Maingonnat, C.; Bertrand, P.; Etancelin, P.; Picquenot, J.-M.; Camus, V.; et al. Non-Invasive Monitoring of Diffuse Large B-Cell Lymphoma by Cell-Free DNA High-Throughput Targeted Sequencing: Analysis of a Prospective Cohort. *Blood Cancer J.* **2018**, *8*, 74. [[CrossRef](#)] [[PubMed](#)]
85. Delfau-Larue, M.-H.; van der Gucht, A.; Dupuis, J.; Jais, J.-P.; Nel, I.; Beldi-Ferchiou, A.; Hamdane, S.; Benmaad, I.; Laboure, G.; Verret, B.; et al. Total Metabolic Tumor Volume, Circulating Tumor Cells, Cell-Free DNA: Distinct Prognostic Value in Follicular Lymphoma. *Blood Adv.* **2018**, *2*, 807–816. [[CrossRef](#)] [[PubMed](#)]
86. Decazes, P.; Camus, V.; Bohers, E.; Viailly, P.-J.; Tilly, H.; Ruminy, P.; Viennot, M.; Hapdey, S.; Gardin, I.; Becker, S.; et al. Correlations between Baseline 18F-FDG PET Tumour Parameters and Circulating DNA in Diffuse Large B Cell Lymphoma and Hodgkin Lymphoma. *EJNMMI Res.* **2020**, *10*, 120. [[CrossRef](#)]

87. Rachinel, N.; Salles, G. The host-tumor interface in B-cell non-Hodgkin lymphoma: A new world to investigate. *Curr. Hematol. Malig. Rep.* **2009**, *4*, 196–201. [[CrossRef](#)] [[PubMed](#)]
88. Hu, H.; Decazes, P.; Vera, P.; Li, H.; Ruan, S. Detection and segmentation of lymphomas in 3D PET images via clustering with entropy-based optimization strategy. *Int. J. Comput. Assist. Radiol. Surg.* **2019**, *14*, 1715–1724. [[CrossRef](#)] [[PubMed](#)]
89. Kurtz, D.M.; Scherer, F.; Jin, M.C.; Soo, J.; Craig, A.F.M.; Esfahani, M.S.; Chabon, J.J.; Stehr, H.; Liu, C.L.; Tibshirani, R.; et al. Circulating Tumor DNA Measurements As Early Outcome Predictors in Diffuse Large B-Cell Lymphoma. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **2018**, *36*, 2845–2853. [[CrossRef](#)]
90. Best, M.G.; Wesseling, P.; Wurdinger, T. Tumor-Educated Platelets as a Noninvasive Biomarker Source for Cancer Detection and Progression Monitoring. *Cancer Res.* **2018**, *78*, 3407–3412. [[CrossRef](#)] [[PubMed](#)]
91. Best, M.G.; Vancura, A.; Wurdinger, T. Platelet RNA as a circulating biomarker trove for cancer diagnostics. *J. Thromb. Haemost.* **2017**, *15*, 1295–1306. [[CrossRef](#)] [[PubMed](#)]
92. In 't Veld, S.G.J.G.; Wurdinger, T. Tumor-educated platelets. *Blood* **2019**, *133*, 2359–2364. [[CrossRef](#)] [[PubMed](#)]
93. Best, M.G.; In 't Veld, S.G.J.G.; Sol, N.; Wurdinger, T. RNA sequencing and swarm intelligence-enhanced classification algorithm development for blood-based disease diagnostics using spliced blood platelet RNA. *Nat. Protoc.* **2019**, *14*, 1206–1234. [[CrossRef](#)] [[PubMed](#)]
94. Santiago-Dieppa, D.R.; Steinberg, J.; Gonda, D.; Cheung, V.J.; Carter, B.S.; Chen, C.C. Extracellular vesicles as a platform for 'liquid biopsy' in glioblastoma patients. *Expert Rev. Mol. Diagn.* **2014**, *14*, 819–825. [[CrossRef](#)]
95. Rekker, K.; Saare, M.; Roost, A.M.; Kubo, A.-L.; Zarovni, N.; Chiesi, A.; Salumets, A.; Peters, M. Comparison of Serum Exosome Isolation Methods for MicroRNA Profiling. *Clin. Biochem.* **2014**, *47*, 135–138. [[CrossRef](#)]
96. Tauro, B.J.; Greening, D.W.; Mathias, R.A.; Ji, H.; Mathivanan, S.; Scott, A.M.; Simpson, R.J. Comparison of Ultracentrifugation, Density Gradient Separation, and Immunoaffinity Capture Methods for Isolating Human Colon Cancer Cell Line LIM1863-Derived Exosomes. *Methods* **2012**, *56*, 293–304. [[CrossRef](#)] [[PubMed](#)]
97. Chen, W.W.; Balaj, L.; Liau, L.M.; Samuels, M.L.; Kotsopoulos, S.K.; Maguire, C.A.; LoGuidice, L.; Soto, H.; Garrett, M.; Zhu, L.D.; et al. BEAMing and Droplet Digital PCR Analysis of Mutant IDH1 mRNA in Glioma Patient Serum and Cerebrospinal Fluid Extracellular Vesicles. *Mol. Ther. Nucleic Acids* **2013**, *2*, e109. [[CrossRef](#)] [[PubMed](#)]