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Non-invasive diagnostic tools for minimal residual disease monitoring in lymphoproliferative disorders

Candidato

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Abstract

IgM monoclonal gammopathies consist of a broad spectrum of diseases, ranging from apparently benign to malignant conditions, in which variable levels of serum monoclonal Immunoglobulin M (IgM) monoclonal proteins are detected. *MYD88*^{L265P} mutation has become increasingly important in the clinical management of IgM-gammopathies due to its role as prognostic and predictive biomarker of therapy response. *MYD88*^{L265P} detection is mainly performed by allele-specific quantitative PCR (ASqPCR), however recently, droplet digital PCR (ddPCR) has been proved to be more sensitive for screening and minimal residual disease monitoring (MRD), both in sorted and unsorted cells, as well as on liquid biopsy.

The aim of the doctoral project was to establish a sensitive and non-invasive diagnostic tool for screening and MRD monitoring of *MYD88*^{L265P} in IgM-gammopathies.

The milestones of this project included:

1) The *MYD88*^{L265P} assay validation by ddPCR and its comparison to the gold standard ASqPCR within different type of samples (bone marrow (BM) unsorted and sorted BM-CD19+ cells, peripheral blood (PB), and plasma-cfDNA (cfDNA)).

2) The corroboration of the previous data on a larger patient's series in the context of the multicentric FIL-BIOWM trial (NCT03521516). The primary endpoint of this observational, prospective and retrospective trial was to compare the levels of detectable *MYD88*^{L265P} by ddPCR in cfDNA from plasma samples with the gold standard tissue, the BM, to demonstrate a negligible difference between these two specimens.

Overall, a good concordance rate (74%) was observed between ASqPCR and ddPCR for MYD88 mutation detection, especially in BM samples, while discordances (26%), mostly in favour of ddPCR (ddPCR+ vs ASqPCR-), were particularly evident in samples with low mutational burden, such as PB and cfDNA. We also demonstrated that the selection of CD19+ sorted cells, despite enriching the sample in tumoral content, is dispensable for MYD88 mutational screening by ddPCR.

Subsequently, *MYD88*^{L265P} detection performed by ddPCR in paired BM, PB and cfDNA at diagnosis from 300 patients, with WM or IgM-MGUS, enrolled in the FIL-BIOWM trial shown that the median *MYD88*^{L265P} quantification in cfDNA was superimposable to BM, both in WM (1.4E-02 vs 3E-02) and in IgM-MGUS (1.5E-03 vs 2.6E-03), while PB values were always about 1 log lower (1.2E-03 and 8E-04, respectively). Concordance analysis in terms of mutation rates and levels confirmed a good agreement between BM and cfDNA (intercept=0.0107, slope =0.9).

In conclusion, these data are in favour of implementing *MYD88*^{L265P} ddPCR assay in plasmatic cfDNA for non-invasive and prompt mutational diagnostics in clinical practice for WM and IgM-MGUS patients. Further efforts to establish uniform guidelines regarding standardization procedures for samples collection and methodological validation are currently ongoing, both at the national and European context.

Introduction

IgM Gammopathies

IgM monoclonal gammopathies consist of a broad spectrum of diseases, ranging from apparently benign to malignant conditions, in which variable levels of serum monoclonal Immunoglobulin M (IgM) monoclonal proteins are detected [1,2].

WM is a lymphoplasmacytic lymphoma (LPL) characterized by a predominant bone marrow (BM) accumulation of small lymphocytes, plasma cells (PC) and plasmacytoid lymphocytes. In WM, these abnormal cells are responsible for the overproduction of the IgM paraprotein [3].

WM has an incidence of about 3.8 patients per million people per year, it represents 1–2% of all hematological malignancies and less than 5% of all non-Hodgkin lymphomas (NHLs). Predominantly affecting Caucasian males, its occurrence is two-fold higher in men than in women, 5.4 vs 2.7 per million, respectively [4]. The average age of diagnosis is approximately 63 years and the incidence increases with age, with 2.9 cases per 100.000, in patients older than 80 years.

Its etiology is still unknown, but it has been demonstrated that genetic factors play an important role in the development of the disease, and there is a high familial incidence, with about 18% of WM patients having at least a first degree relative with a B-cell neoplasm [5].

The most common manifestations of the disease are related both to BM infiltration, that causes cytopenia, anemia and resulting fatigue, and to hypersecretion of IgM paraprotein, that in solution forms a large pentameric immunoglobulin, responsible for the hyperviscosity symptoms, in 10-30% of WM patients, such as mucocutaneous bleeding, visual difficulties due to retinal bleeding, headache, dizziness, ataxia, deafness, and, rarely, stroke or cognitive impairment and altered mental status. Moreover, particularly in the elderly, heart failure can be aggravated due to increased blood viscosity, expanded plasma volume and anaemia [6].Despite the continuous advances in therapy, at present the WM remains manageable but not curable and the disease monitoring is essential to early drive the therapy.

The main recognized risk factor for WM, as well as for other lymphoplasmacytic lymphomas, is an history of IgM monoclonal gammopathy of undetermined significance (IgM-MGUS). IgM-MGUS is an asymptomatic, pre-neoplastic condition defined by the absence of BM infiltration by lymphoma cells, the presence of a serum IgM paraprotein and the absence of end-organ damage [7]. IgM-MGUS confers to patients higher relative risk of progression to WM or to other lymphoproliferative disorders (about 1.5-2% per year) [8].

Although it is plausible that multistep genetic and microenvironment changes lead to the transformation from IgM-MGUS to WM, the precise mechanism and the trigger underlying the progression between these two entities are still unknown [9].

Therefore, an appropriate diagnostic classification that can distinguish between these different entities is crucial. Different diagnostic criteria, for both WM and IgM-MGUS, have been updated

several times [10–16]. For instance, in 2003 the Second International Workshop Criteria (2 IWWM) formulated the following requirements for WM diagnosis: the presence of IgM monoclonal gammopathy of any size, a BM trephine biopsy with lymphoplasmacytic infiltration and an immunophenotype that excludes the possibility of other lymphoproliferative disorders [7]. While it defined the IgM-MGUS by the absence of BM infiltration by lymphoma cells and the presence of serum IgM paraprotein.

Important advances in understanding the biology of WM have been made by whole genome sequencing (WGS). In 2012, Treon et al. demonstrated a somatic mutation in myeloid differentiation primary response 88 gene (*MYD88*^{L265P}) in 90% of WM and 10% of IgM-MGUS cases [17]. These findings were soon reproduced in larger patient series, and increasingly sensitive methods confirmed the presence of *MYD88*^{L265P} in more than 95% of WM patients and in at least 50% of patients with IgM-MGUS. Moreover, WGS identified several other highly prevalent somatic mutations in CXCR4 (*CXCR4*^{WHIM} or *CXCR4*^{MUT}) and ARID1A genes [18,19]. Despite the discovery of these new diagnostic biomarkers, genetic factors have not yet been included in the current diagnostic and prognostic (International Prognostic Scoring System (IPSS)) criteria of WM described in 2016 [20,21].

Molecular biomarkers: MYD88 and CXCR4

The most noteworthy finding in WM has been the discovery of two activating somatic mutations affecting the MYD88 and CXCR4 genes [22].

MYD88^{L265P} is the most recurrent mutation in the genomic landscape of WM and is found in approximately 90% of the patients. This mutation is located at position 38182641 in chromosome 3p22.2, resulting in a single nucleotide change, T→C, with consequent switch of leucine to proline at amino acid position 265 (L265P) [17].

MYD88 is an adaptor protein that acts downstream of the Toll-like and interleukin-1 receptors (TLR/IL1R), which are both implicated in the innate immune response through a similar signaling cascade [23]. A common characteristic of innate immune receptor signaling is the self-clustering of proteins into oligomeric complexes, known as supramolecular organizing centers (SMOCs) [24,25]. TLR/IL1R activation, through the homotypic TIR domain interactions, triggers the oligomerization of MYD88 and the assembly of a multifunctional organizing center, named MYDDosome [26]. Although numerous structural conformations are possible, it has been observed that the size of the MYD88 oligomers (>4 MYD88s) is a decisive factor in the IL1R signal transduction and is crucial for recruiting and binding other post-receptor signal transducers, such as interleukin-1 receptor-associated kinases (IRAK4 and IRAK1) or bruton tirosine kinase (BTK), resulting in sustained NF-kB signaling [26,27]. Notably, it has been shown that the *MYD88*^{L265P} mutation, seated in the TIR domain, (and not the other no-L265P mutations) has an increased propensity to build extremely stable oligomers, compared to the wild-type protein, leading the MYDDosome formation and thereby constitutive NF-

kB activation, contributing to cell proliferation, cytokine secretion (i.e., TNF, IL-6, IL-1) and malignant cell survival [18,28,29].

In clinical practice, *MYD88*^{L265P} aids in supporting the diagnosis of WM and helps differentiate from other IgM-secreting lymphoid malignancies, such as marginal zone lymphoma (MZL) and IgM multiple myeloma (MM), where it is less frequently mutated or absent, respectively. Moreover, *MYD88*^{L265P} is detected in more than 50% of IgM-MGUS patients, 10% of whom can evolve to WM and has been observed that those with a higher mutated allele burden (mutant allele relative to wild type) have a greater risk to progress to WM [30,31].

The second most common somatic mutation, observed in up to 40% of WM patients, occurs in the CXCR4 gene [22,32,33]. CXCR4 is a chemokine receptor and member of the 7-transmembrane receptors family, that activates intracellular signaling pathways by binding to heterotrimeric G-proteins through its C-terminus segment [34]. More than 40 non-sense (NS) or frameshift (FS) mutations have been observed in the CXCR4 gene (CXCR4^{MUT}) [22,35]. The most common variant, representing over 50% of CXCR4 mutations, is a non-sense C > A or C > G transversion in a highly conserved region at nucleotide position 1013, responsible for the generation of a stop codon (S338X), resulting in the loss of 15 amino acids at the C-terminal region of the CXCR4 protein [36,37]. These *CXCR4*^{S338X} nonsense mutations affect the expression and activity of CXCR4 mainly through the PI3K-AKT-NF-kB and the MEK1/2 and ERK 1/2 pathways, involved in cell proliferation, migration, and survival [38,39]. Practically all *CXCR4*^{MUT} patients harbour *MYD88*^{L265P}, suggesting the subclonal nature of *CXCR4*^{MUT} with respect to *MYD88*^{L265P} acquisition, and only rare cases of *CXCR4*^{MUT}/*MYD88*^{WT} have been reported [22,35,40–42]. Moreover, *CXCR4*^{MUT} shows a highly variable clonal distribution in WM and IgM-MGUS patients and particularly *CXCR4*^{S338X}, as opposed to *CXCR4*^{FS} mutations, are associated with complex karyotypes [32,35]. In a recent case study in a *CXCR4*^{MUT} patient, WGS highlighted alterations in genes associated with DNA damage repair (DDR) (UVRAG gene), tumor suppression (BTG220, DAB2), chromosome instability (MACROD2, CCSER1), cell cycle regulation (SCAPER) and post-translational protein modifications (LNX1 and DCUN1D4). However, further analysis of 46 WM patients did not show a significantly different distribution of these mutations between *CXCR4*^{WT} vs. *CXCR4*^{MUT} patients [43].

MYD88^{L265P} and *CXCR4*^{MUT} have been initially assessed on CD19+ (CD19-selected cells) BM samples using allele-specific quantitative polymerase chain reaction (AS-qPCR) and Sanger sequencing. Subsequently, many studies analysed unselected BM samples with distinct assays and methods with different levels of sensitivity [44]. Moreover, has been described that, both mutations can be detected not only in BM, PB (of note, B-cell–depleting agents, particularly rituximab, can greatly decrease mutation detection rate in PB) but also in plasma, skin, cerebrospinal fluid (CSF) and pleural effusions [45–48].

As of today, a gold standard molecular method for MYD88 and CXCR4 mutation detection is lacking.

Several different assays and methods are available; however, there is a lack of consensus about the optimal specimens or technique, both in terms of operating procedures, test sensitivity and results interpretation. Despite mutational analysis for *MYD88*^{L265P} is mainly performed by ASqPCR, there are some technical limitations. Due to its low sensitivity (1E-03) [30,49], ASqPCR is not suitable for minimal residual disease (MRD) monitoring and it is suboptimal for testing specimens with low concentrations of circulating tumor cells, like unsorted BM or PB, (especially after chemo-immunotherapy), or for assessing cell-free tumor DNA (cfDNA), generally presents in small quantities in plasma, cerebrospinal fluid and pleural effusions [50]. Hence, it requires invasive BM biopsy procedure for the differential diagnosis between WM and IgM-MGUS, not well tolerated by the patient. Moreover, it requires CD19+ cells selection, not always convenient or not part of the routine practice in many diagnostic laboratories [30,40,51–56].
Recently, droplet digital PCR (ddPCR) has been described as a highly sensitive technique, among different PCR approaches and across different specimen types [45,57–59], suggesting that the implementation of ddPCR assay in routine diagnostic laboratories might be useful for mutational screening without the need for CD19+ selection.

Based on these observations, the aim of the doctoral project was to establish a sensitive and non-invasive diagnostic tool for screening and minimal residual disease monitoring of MYD88 in igM-gammopathies. The milestones of this project included: 1) the assay validation, a comparison to the gold standard and to different molecular method for MYD88 detection; 2) FIL-BIOWM trial (NCT03521516): the validation in a larger series of patients in the context of a multicentric study.

Assay development for *MYD88*^{L265P} detection by ddPCR

The assay for MYD88 detection by ddPCR has been described for the first time by our group in a previous study published in Haematologica in 2018 [45]. In this paper we describe a newly developed ddPCR assay for the identification of the *MYD88*^{L265P} mutation tested in a local series of 148 patients affected by WM or LPL. Overall, 291 samples (BM and PB) were collected at baseline and during the follow up, as well as a small cohort of 60 cfDNA samples were analysed.
142/148 (96%) patients scored *MYD88*^{L265P} mutated. All BM samples from 20 patients with multiple myeloma (MM), used as negative controls, were below the limit of detection. Moreover, to confirm the specificity of our assay for mutational screening, 15 patients with mantle cell lymphoma, 10 with follicular lymphoma and 10 with chronic lymphocytic leukemia were tested for the *MYD88*^{L265P} mutation. All samples from the patients with follicular lymphoma or mantle cell lymphoma were *MYD88*^{WT}, whereas only one out of 10 patients with chronic lymphocytic leukemia was mutated, as already described in this disease [30].
Moreover, in our study we observed that the ddPCR assay on unselected cells greatly improved the rate of *MYD88*^{L265P} detection compared to that achieved by ASqPCR. In fact, an analysis of 74 paired

BM/PB baseline samples showed an overall detection rate of 93% (69/74) in BM and 72% (53/74) in PB. Among 69 patients with detectable mutations in BM samples, the sensitivity for *MYD88*^{L265P} mutation detection by ddPCR on paired PB samples was 77% (53/69).

Finally, in order to investigate the feasibility and the sensitivity advantages of ddPCR-based *MYD88*^{L265P} mutation detection on plasma ctDNA vs PB gDNA, paired samples from 60 patients were analyzed. Interestingly, a higher median *MYD88*^{L265P} mutated/WT ratio was detected in plasma ctDNA (1.4E-02) than in PB (1E-03) (P<0.001), while no statistically significant difference was observed between ctDNA and BM samples from 32 patients (1.9E-02 vs 1.4E-02; P=0.2).

These results shows that ddPCR is a feasible and highly sensitive assay for *MYD88*^{L265P} mutational screening in WM, particularly in samples harbouring low concentrations of circulating tumor cells, as plasma ctDNA. This study represented an exploratory research, whose real advantages and values have been further validated in two studies, one focused on the laboratory recommendation for WM and IgM-MGUS marker screening *(Ferrante et al* in 2021) and the second one investigating the mutational status in patients enrolled in a multicenter, observational clinical trial (FIL_BIOWM, NCT03521596), both studies have been conducted during the doctoral period and are described below.

Assay validation and laboratory recommendation for marker screening

In spite of its diagnostic, prognostic, and predictive role, recognized by the European Society of Medical Oncology (ESMO) clinical guidelines [60], the detection of *MYD88*^{L265P} has not been standardized yet.

Due to the relevance of *MYD88*^{L265P} both in daily management of WM patients as well as in prospective clinical trial investigating the efficacy of novel agents, it is crucial to find an agreement among different diagnostic laboratories about the most sensitive, appliable and standardizable molecular technique for mutation detection.

Thus, the primary aim of this study was to directly compare the performance of ASqPCR and ddPCR in different tissues and at distinct time points in a wide case series. The objective was to establish useful recommendations on laboratory practice for WM and IgM-MGUS marker screening at diagnosis and for MRD analysis.

This study published on Diagnostics 2021, pointed out the reliability of ddPCR for *MYD88*^{L265P} detection in a cohort of samples collected in clinical routine, laying the premise for the further ongoing multicenter clinical FIL_BIOWM trial for non-invasive diagnostics and monitoring of MRD in WM and in IgM-MGUS patients as detailed below.

FIL-BIOWM trial (NCT03521516)

The FIL_BIOWM (NCT03521596) clinical trial, sponsored by the Fondazione Italiana Linfomi (FIL) and the International WM Foundation/Leukemia and Lymphoma Society, is an ongoing retrospective and prospective observational study, started in September 2018, including patients with Waldenström's Macroglobulinemia or IgM-MGUS.

The first goal of this project is to assess whether a reliable diagnosis of WM and IgM-MGUS is feasible on peripheral blood (PB) samples, overcoming the need for invasive procedures, by means of highly sensitive techniques, such as ddPCR and next-generation targeted sequencing (NGS). Therefore, the primary objective is to demonstrate that the rate of gene mutation detected in plasma show a negligible difference with the rate of mutations detected in bone marrow samples (the gold standard), comparing mutation analysis on genomic DNA with cell-free DNA to assess the most reliable source for mutation studies, as well as to assess whether WM condition might be differentiated from IgM-MGUS based on mutation analysis.

The second goal of this project is the assessment of minimal residual disease (MRD) after treatment, using ddPCR for MYD88, correlating clinical characteristics of patients and clinical response to treatment and conventional outcomes (progression-free and overall survival).

Materials and Methods

Patients and Samples for assay validation

BM and PB samples were collected at baseline and during follow-up (FU) from 227 patients affected by WM and IgM-MGUS. Three patient series, from hematological Italian centers routinely involved in management of IgM monoclonal gammopathies (Torino, Pavia and Varese), were tested for *MYD88*^{L265P} mutation by both ASqPCR and ddPCR. All patients provided written informed consent for sample collection and analysis.

Overall, 319 samples were analyzed including 53 MNC (mononuclear cells), 149 WBC (white blood cells), and 117 CD19+ selected cells (CD19+). Torino series included 62 patients: 60 WM, and 2 IgM-MGUS (105 WBC: 50 BM, 55 PB, (46 at diagnosis, 59 at FU)). Additionally, 64 cfDNA samples extracted from plasma were analyzed.

Pavia series included 67 patients. Samples from 46 patients were collected at diagnosis, 14 WM, and 32 IgM-MGUS (38 MNC: 31 BM, 7 PB, and 17 CD19+: 11 BM, 6 PB), while a small series of 21 WM patients' samples were collected before and post-treatment (42 samples: 27 BM-CD19+ and 15 BM-MNC). Varese series included 98 patients at baseline, 64 WM, 34 IgM-MGUS (117 samples: 73 CD19+ (52 BM, 21 PB) and 44 WBC (23 BM, 21 PB)).

A group of 60 samples, including 40 healthy subjects and 20 MM patients were used as negative control.

Patients and Samples in FIL-BIOWM trial

Overall, 300 patients have been enrolled: 63 were retrospectively enrolled and constituted the training cohort, while 237 prospective patients constituted the validation cohort. Samples have been collected from September 2018 to October 2020, from 14 Italian FIL centers and one Spanish center. Overall, 71 were IgM-MGUS (15 retrospective and 56 prospective) and 229 patients were WM (48 retrospective and 181 prospective). The study population included either asymptomatic WM or symptomatic WM patients requiring treatment at the diagnosis, according to recently published guidelines [61].

Diagnosis were defined according to criteria established at the second International Workshop on Waldenström's Macroglobulinemia [7]. For all patients paired BM, PB and plasma were collected at baseline (T0), after treatment (T2) (only for patients needed therapy), and at follow-up as reported below (Figure 1).

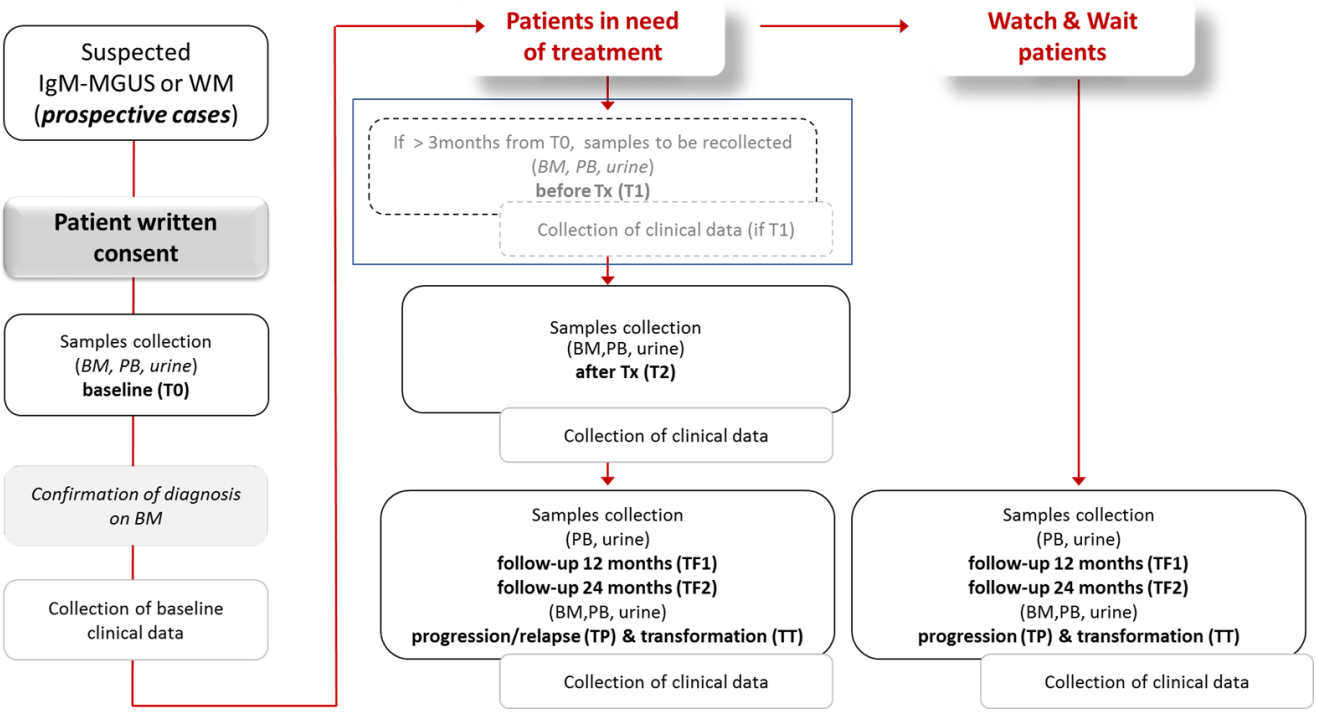


Figure 1. FIL-BIOWM study flow chart.

Sample processing

Sample processing is common in both studies. BM aspirate samples were collected into EDTA tubes [K2-EDTA (purple) Vacuette or BD Vacutainer].

In order to preserve the integrity of circulating tumour DNA, PB samples were collected in EDTA tube for Torino and Pavia (HUB) centers and were processed within 4 hours from the draw.

On the contrary, if patients were enrolled in the other FIL centers, PB samples were collected in Cell-Free DNA BCT tubes (Streck's black/beige vacutainers) that preserve the plasma for longer period

of time. The preservative reagent contained in the BCT-tube inhibits nuclease-mediated degradation of cfDNA, contributing to the overall stabilization of cfDNA for up to 14 days at room temperatures. For plasma recovery, PB samples (EDTA and BCT), were centrifuged at 1300× g for 13 min at room temperature. Plasma was transferred in separate tubes and centrifuged at 1800× g for 10 min at room temperature, before being stored at -80°C in 1ml aliquots.

BM (previously filtered through a 1ml syringe) and PB leftover (after plasma separation) were treated with erythrocytes lysis buffer (NH4Cl) (at 1:5, 1:2, and 1:2 dilution, respectively), left 15 min at room temperature (lying flat at dark), and centrifuged at 450xg for 10 min, at room temperature. The supernatant was discarded and the cell pellet was resuspended in 10–15 mL NH4Cl and centrifuged at 450× g for 10 min, at room temperature. The supernatant was discarded and the cell pellet was resuspended in 0.9% NaCl (q.s.), cells were counted, dispensed in 5–10 × 106 stocks, and stored as dry pellets at −80 °C, for further DNA extraction.

MNC B-cells were collected from BM and PB by lymphoprep standard density gradient centrifugation, while CD19+ cells were further isolated from MNCs by immunomagnetic adsorption on MiniMACS separation columns using an anti-CD19 antibody (Miltenyi Biotec GmbH), in accordance with the manufacturer recommendations. The purity of CD19+ separated cells was assessed by flow cytometry using anti-CD19 monoclonal antibodies (Becton Dickinson).

Nucleic Acid Extraction

Genomic DNA (gDNA) was extracted from WBC, MNC, and CD19+ cells by MaxWell RSC system with blood RSC kit (Promega) (Torino and FIL-BIOWM samples), by Puregene Blood DNA isolation kit (Qiagen) (Pavia samples), by Maxwell® 16 LEV Blood DNA Kit (Promega, Madison, WI, USA) (Varese samples), in accordance with the manufacturer recommendations. cfDNA was extracted by Maxwell RSC with LV ccfDNA kit (Promega), in accordance with the manufacturer instructions.

ASqPCR Assays for *MYD88*^{L265P} Detection

ASqPCR was performed by two assays, according to Jiménez et al., 2014 (Torino) [49] and Varettoni et al., 2013 [19] (Pavia and Varese). Two reverse primers were used by Torino: 5′-CCTTGTACTTGATGGGGATCA-3′ (wild-type-specific reverse primer) and 5′-CCTTGTACTTGATGGGGATGG-3′ (mutant-specific reverse primer) in combination with a common forward primer 5’-ACTTAGATGGGGGATGGCTG-3′ and a specific TaqMan probe (5′-TTGAAGACTGGGCTTGTCCCACC-3′). Pavia and Varese groups performed ASqPCR by SYBR green approach, with two different forward primers 5′-GTGCCCATCAGAAGCGCCT (wild-type-specific forward primer) and 5′-GTGCCCATCAGAAGCGCCC-3’ (mutant-specific forward primer) and a common reverse primer 5′-AGGAGGCAGGGCAGAAGTA-3′.

ddPCR Assay for *MYD88*^{L265P} Detection

ddPCR mutation detection assay was performed, as previously described [45].

A single set of primers (Forward FP 5'-CCTTGGCTTGCAGGT-3' and Reverse RP 5'-TCTTTCTTCATTGCCTTGT-3') was combined with two competitive probes, in two assays (CSTM DDPCR HEX/FAM ASSAY BIO-RAD) one for *MYD88*^{L265P} mutation (MUT 5'-TGGGGATCGGTCGC-3') labeled with FAM and one for *MYD88*^{L265P} wild type (WT 5'-TGGGGATCAGTCGCTT-3') labeled with HEX. At first, the ddPCR assay was optimized for DNA amount and PCR conditions using the QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA).

Briefly, for each replicate, 11 µl of 2X ddPCR Supermix for Probes with no dUTP (Bio-Rad Laboratories), 1.1 µl of each 20X mutation detection assay and 5.5 ul of gDNA (20 ng/ul) or cfDNA were mixed in a total 22 ul reaction volume.

For droplet generation, 20 µl of the reaction mix were transferred to the middle row of a DG8TM cartridge for the QX200 droplet generator (Bio-Rad Laboratories, Hercules, CA, USA). The DG8TM cartridge was already preloaded in the DG8 cartridge holder. After loading the PCR mix, 70 ul of droplet generation oil were loaded into the bottom wells of the DG8 cartridge. A gasket was attacked across the top of the DG8 cartridge. Finally, the DG8 cartridge was placed into the QX200 droplet generator. The droplet generator produces about 20,000 droplets per samples. Droplets were transferred to a 96-well PCR plate by pipetting gently. This was heat sealed using the Bio-Ra's PX1 PCR plate sealer. The PCR plate was transferred in a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) and end-point PCR was performed at following conditions: 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds, 55°C for 1 minute followed by 98°C for 10 minutes. Ramp rate was set at 2.5°C/second.

PCR products were loaded into the QX200 droplet reader and analyzed by QuantaSoft v1.6.6.0320 or QuantaSoft Analysis Pro 1.0.596 (Bio-Rad Inc.) Samples were tested in triplicate and results expressed as merge of wells.

Results was expressed as MUT/WT (a/b) ratio. The cut-off for mutation was set based on the highest *MYD88*^{L265P} level detected within the control group of healthy samples. A MUT/WT ratio of 3.4E-04 was established as cut off for negativity. All samples with a ratio below 3.4E-04 were considered as *MYD88*^{WT}.

Each experiment included a known highly mutated positive control sample (MUT/WT ratio 6.8E-01), a negative control (healthy donor or MM gDNA) and a no template control (NTC). Gate setting was performed based on the positive control results. The detection limit of this ddPCR assay is able to detect the mutation, with a sensitivity of 0.035% that means 10 mutated copies in 30000 WT.

Statistical Analysis

For methods comparison, ddPCR results were expressed as MUT/WT ratio, while ASqPCR as allele frequency/100. To calculate the correlation and agreement between the methods, we evaluated the test-retest reliability for continuous variables by a single measurement, consistent, 2-way mixed-effects model, Inter Class Correlation (ICC) analysis, with a 95% confident interval (CI) [62].

Qualitative variables were described as counts and percentage. Quantitative variables were summarized as median and Interquartile Range (IQR). The association between two categorical variables was tested via Fisher's exact test. The qualitative concordance between specimens was evaluated using the Cohen's Kappa coefficient while for the quantitative concordance Passing-Bablok method was used. P-values lower than 0.05 were considered significant. All statistical analyses were performed using Stata 17 (StataCorp. 2021. Stata Statistical Software: Release 17. College Station, TX: StataCorp LLC). Correlation analyses and their representation plots were performed using IBM SPSS Statistics (version 25.0. Armonk, NY, USA: IBM Corp.) or GraphPad5 Software (GraphPad Software Inc., San Diego, CA, USA).

Results

1-Assay Validation

Overall, 319 samples (239 WM, 80 IgM-MGUS) from 227 patients (159 WM and 68 IgM-MGUS) were tested, in order to define: (1) the most suitable technique, for *MYD88*^{L265P} mutation detection between ASqPCR and ddPCR; (2) the most informative specimen for *MYD88*^{L265P} screening and disease monitoring. To this purpose, different WM and IgM-MGUS tissue samples, such as CD19+ BM sorted cells, WBC, and MNC from BM and PB, were compared by measuring mutation level by both methods. Additionally, a group of 60 samples, including 40 healthy subjects and 20 MM were used as negative controls for both ASqPCR (median ΔCT = 10 (range ΔCT = 8.4–10.7)), setting the cut-off for negativity to ΔCT=8 and ddPCR (median MUT/WT ratio: 1.75E-04 (range 3.1E-04 –2.7E-05)), setting the cut-off for negativity to 3.4E-04 [45,49].

MYD88^{L265P} was quantified by both ddPCR and ASqPCR on 117 CD19+ selected cells (90 BM, 27 PB), 53 MNC (46 BM, 7 PB), 149 WBC (73 BM, 76 PB). Among them, results from 237/319 samples (74%) were concordant between the two methods: 182 positively concordant and 55 negatively concordant (Figure 2). Among the 182 ddPCR+/ASqPCR+ samples, 141 were BM (106 WM bluish circles, 35 IgM-MGUS reddish circles) and 41 PB (bluish squares: 38 WM, 3 IgM-MGUS), showing a major concordance rate between BM (77%) rather than PB samples (22%). Concordances between methods for each subtypes of samples (BM vs PB, CD19+ vs unsorted) are reported in Figure 3.

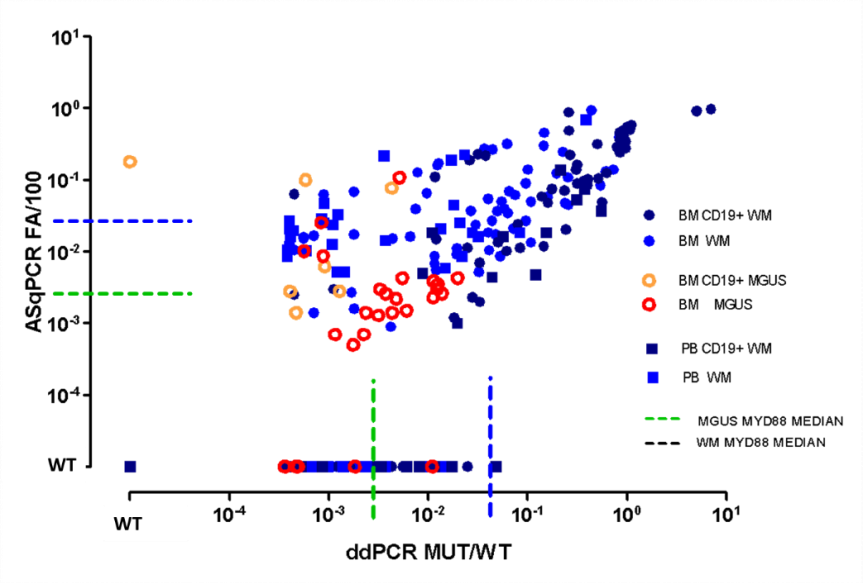


Figure 2. ASqPCR vs ddPCR. Comparison between ASqPCR and ddPCR for MYD88^{L265P} detection in terms of ratio between MUT and WT. BM: bone marrow; PB: peripheral blood; MGUS: IgM-monoclonal gammopathy of undetermined significance; WM: Waldenström; WT: Wildtype (quantitative value outside the limit of blank. Limit of blank was calculated based on healthy subjects as the mean value +1 SD); 10^{-4}: 1E-04.

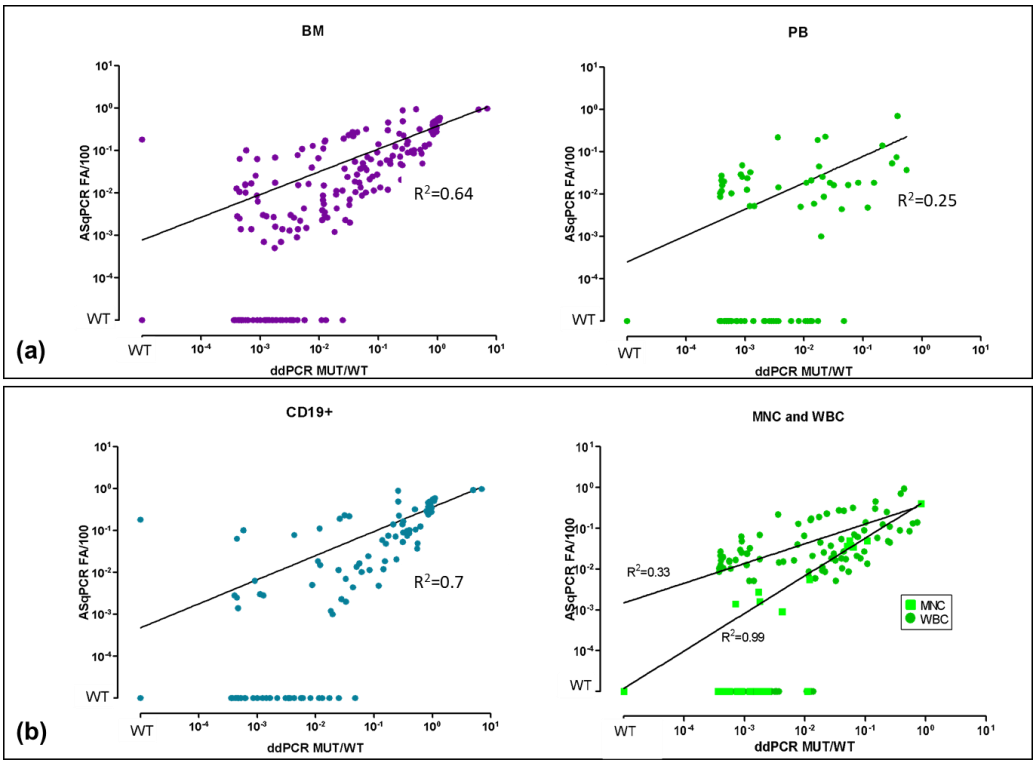


Figure 3. ASqPCR vs ddPCR in different specimens. Comparison between ASqPCR and ddPCR for MYD88^{L265P} detection in terms of ratio between MUT and WT in (**a**) BM vs PB and (**b**) CD19+ vs MNC and WBC. BM: bone marrow; PB: peripheral blood; WT: Wildtype (quantitative value outside the limit of blank. Limit of blank was calculated based on healthy subjects as the mean value +1 SD); MNC: mononuclear cells; WBC: white blood cells; 10^{-4}: 1E-04.

Interestingly, no statistically significant differences were detected between WBC (149) and MNC (53) samples, both in terms of concordance between methods (Figure 3b) and tissues. Overall, 58% of WBC (86/149) and 51% of MNC (27/53) were ddPCR+/ASqPCR+, while 21% of WBC (32/149) and 19% of MNC (10/53), were ddPCR-/ASqPCR-. Moreover, to further investigate the differences between WBC and MNC mutation levels, we selected 18 patients whose WBC and MNC samples were collected from the same blood sample showing no discordances in mutation detection between WBC and MNC samples and with a comparable median amount of mutation (3E-02 WBC, 3.9 E-02 MNC). Of note, *MYD88*^{L265P} levels by ddPCR, seemed to show a superior correlation between sample type compared to ASqPCR. Indeed, among WM samples, BM CD19+ and PB CD19+ (dark blue symbols) showed, as expected, higher mutation levels (MUT/WT > 1 x 10^{-2}), compared to BM WBC/MNC and PB WBC/MNC (light blue symbols). Specific clusters between BM and PB were not detectable, however, a high proportion of PB WBC/MNC (light blue squares), showing a lower mutation level compared to BM, clustered around 1E-03 MUT/WT ratio level, by ddPCR. Finally, IgM-MGUS samples showed a comparable distribution to WM but with 1 log lower mutation levels (Figure 2, dashed green line vs dashed blue line).

Within the 55 samples scored negative by both techniques, 21 (38%) were BM (14 WM, 7 IgM-MGUS), while 34 (61%) were PB (26 WM, 8 IgM-MGUS), highlighting the lower mutational load of PB.

Concerning the discordant samples, 82/319 (26%), 81 were ddPCR+/ASqPCR- (35 PB (28 WM, 7 IgM-MGUS) and 46 BM (27 WM, 19 IgM-MGUS)), while only one IgM-MGUS sample was ddPCR-/ASqPCR+. Regretfully it was not possible to replicate the analysis to verify whether it was the case of an ASqPCR false positivity or a ddPCR false negativity (Figure 2). Of note, discordances between ddPCR and ASqPCR were mostly related to samples analyzed by SYBR green approach.

ddPCR vs ASqPCR in Different Tissues (BM vs PB)

Considering BM and PB separately, we observed 162/209 (78%) concordant BM: 141 were ddPCR+/ASqPCR+ (median 4.1E-02 (range: 4E-04, 7.1 E-01)), 21 ddPCR-/ASqPCR- and 47 (22%) were discordant. Among discordant BM, 46 were ddPCR+/ASqPCR-, while only one (CD19+) was ddPCR-/ASqPCR+ (median 1.2 E-03 (range: 3.6 E-04, 2.5 E-02)) (Figure 3a).

Within PB, 75/110 (68%) were concordant: 41 ddPCR+/ASqPCR+ (median 1.1 E-02 (range: 3.8 E-04, 5.5 E-01)) and 34 ddPCR-/ASqPCR-, while 35 (32%) were discordant (all ddPCR+/ASqPCR-) (median 8.6E-04 (range: 3.7E-04, 4.8E-02)) (Figure 3a). Notably, ddPCR quantification in concordantly positive samples (ddPCR+/ASq CR+) showed a higher mutation load in BM and PB compared to the ddPCR+/ASqPCR- (median by ddPCR: 4.1 E-02 vs 1.2 E-03 for BM and 1.1E-02 vs 8.6 E-04 for PB).

ddPCR vs ASqPCR in CD19+ Sorted vs Unsorted Samples

Focusing on CD19+ selected samples, 82/117 (70%) were concordant: 69 ddPCR+/ASqPCR+ (median 2.2E-01; range: 4.1E-04, 7.1E-01) and 13 ddPCR-/ASqPCR-; while 35 (30%) were discordant, 34 ddPCR+/ASqPCR- (median 1.5E-03; range: 3.6E-04, 4.8E-02) with only one ddPCR-/ASqPCR+ (Figure 3b).

Since no difference in terms of mutation level was observed between WBC and MNC, we considered both samples in the analysis. Among 202 WBC/MNC samples 155 (77%) were concordant 113 ddPCR+/ASqPCR+ and 42 ddPCR-/ASqPCR- while 47 (23%) were discordant and all ddPCR+/ASqPCR-. Of note, *MYD88*^{L265P} level was lower in WBC/MNC compared to CD19+ selected cells (median 3.65E-03 vs 3.15E-02) (Figure 3b).

ddPCR vs ASqPCR in WM Compared to IgM-MGUS

As already described in literature, 93/97 (96%) BM WM and 54/62 (87%) BM IgM-MGUS at diagnosis were mutated by ddPCR, while 79/97 (81%) WM and 36/62 (58%) IgM-MGUS by ASqPCR [19,45,63].

Overall, considering BM, PB, diagnostic and FU samples, ddPCR detected MYD88^{L265P} in 83% (199/239) of WM and 80% (64/80) of IgM-MGUS, while ASqPCR in 60% (144/239) and 48% (39/80), respectively.

Interestingly IgM-MGUS, showed a lower median mutational level (Figure 2 dashed green line, Figure 4 dashed blue lines) by ddPCR 5.2E-03 (range: 3.6E-04, 2.6 E-01) compared to WM (Figure 2 dashed blue line, Figure 4 dashed orange lines) 1.7E-02 (range: 3.6E-04 – 7.1E-01) ($p < 0.0001$).

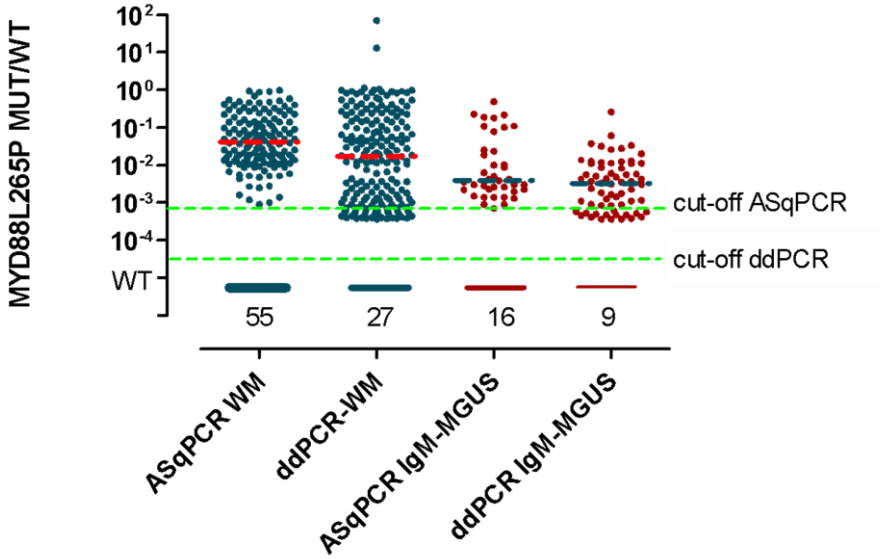


Figure 4. Mutational load comparison between WM and IgM-MGUS. The dashed orange and blue lines show, respectively, the median of *MYD88*^{L265P} mutational values in WM and IgM-MGUS patients. WT: Wildtype (quantitative value outside the limit of blank. Limit of blank was calculated based on healthy subjects as the mean value +1 SD); 10^{-4}: 1E-04.

ddPCR vs ASqPCR in Plasma-cfDNA

Finally, in order to test the sensitivity of ddPCR on liquid biopsy analysis, we evaluated 64 cfDNA from plasma (47 at diagnosis and 17 at FU) in 32 WM, 4 IgM-MGUS and 28 other B-lymphoproliferative disorders (i.e., central nervous system lymphoma, marginal zone lymphoma, chronic lymphocytic leukemia) (Figures 5). *MYD88*^{L265P} was detected in 66% (42/64) of samples by ddPCR (25 WM, 3 IgM-MGUS and 14 other B-lymphoproliferative disorders), while only 36% (23/64) of samples scored positive by ASqPCR, confirming the higher sensitivity of ddPCR compared to ASqPCR even in plasma-cfDNA samples.

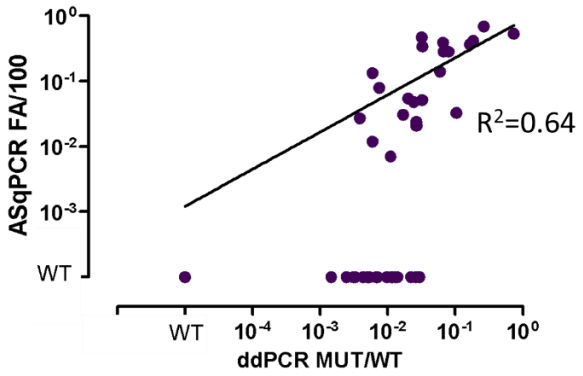


Figure 5. ASqPCR vs ddPCR in cfDNA from plasma samples. Comparison between ASqPCR and ddPCR for MYD88^{L265P} detection in terms of ratio between MUT and WT in 32 WM, 4 IgM-MGUS, and 28 Lymphomas, 47 at diagnosis and 17 at FU. WT: Wildtype (quantitative value outside the limit of blank. Limit of blank was calculated based on healthy subjects as the mean value +1 SD); 10-4: 1E-04.

This study highlighted ddPCR as a feasible approach for *MYD88*^{L265P} detection, more sensitive than ASqPCR across different specimen types (including plasma-cfDNA) and in distinct diseases. *MYD88*^{L265P} detection on PB samples, especially with ASqPCR, is suboptimal for *MYD88*^{L265P} screening and MRD analysis. CD19+ selection, despite enriching the sample tumor content, is dispensable for mutational screening, suggesting that the implementation of ddPCR assay in routine diagnostic laboratories might avoid the need of CD19+ selection.

Based on the results described above, we finally propose an algorithm for the best use of the most convenient PCR methods for MYD88L265P detection, based on the available method and type of specimens (Table 1) in order to establish a uniform testing approach for determining the MYD88 mutational status among different laboratories. The purpose of this study was to set up useful recommendations, that will improve routine clinical practice within different laboratories with the intention to standardize protocols and procedures for the management of both clinical routine and multicenter clinical trials.

WM	BM DIA		BM FU	PB DIA		PB FU	PLASMA
	WBC	CD19+	WBC	WBC	CD19+	WBC	cfDNA
ddPCR	++	++	++	++	++	+	++
ASqPCR	+	++	+	+	++	+/−	+/−
IgM-MGUS	BM DIA		BM FU	PB DIA		PB FU	PLASMA
	WBC	CD19+	WBC	WBC	CD19+	WBC	cfDNA
ddPCR	++	++	++	+	+	+	+
ASqPCR	+/−	++	+/−	+/−	+	+/−	+/−

Method sensitivity and detection rate: ++ 5×10^{-5}, + $>1 \times 10^{-3}$, +/− $\leq 1 \times 10^{-3}$, bolded ++ Recommended sample, ☐ Not recommended method.

Table 1. Sensitivity and capability of ASqPCR and ddPCR methods for MYD88L265P detection inWM and IgM-MGUS in different specimens. Comparison between ASqPCR and ddPCR for MYD88L265P detection in terms of sensitivity based on the type of sample analyzed and recommendations for using one of the methods based on the type of sample available. Of note, regardless of the sensitivity of the method, PB samples show a lower MYD88L265P mutational load compared to BM. BM: bone marrow; PB: peripheral blood; WBC: white blood cells; CD19+: CD19 selected cells; DIA: diagnosis; FU: Follow-up; cfDNA: cell free DNA.

2- FIL-BIOWM

FIL-BIOWM enrollment has been successfully completed in 2020 and clinical data were available for all 300 patients at baseline and for 59 post-treatment patients.

Clinical characteristics of WM and IgM-MGUS patients enrolled in the retrospective/training (63 patients) and prospective/validation (237 patients) series are reported in Table 2 and Table 3.

Variable		WM N=48	IgM-MGUS N=15	*p-value* *
		Median (interval 25-75°)		
Age	years	68 (63-72)	69 (56-74)	*0.914*
Hemoglobin	g/dL	12.1 (10.2-13.2)	13.6 (12.6-14.1)	*0.031*
Albumin	g/dL	3.9 (3.6-4.2)	4.3 (4.1-4.4)	*0.018*
s-Creatinine	mg/dL	0.86 (0.75-1.07)	1.03 (0.80-1.12)	*0.179*
WBC	10^9/L	6.90 (4.94-9.46)	5.60 (4.90-7.00)	*0.149*
ALC	10^9/L	1.80 (1.19-2.60)	1.59 (1.20-2.00)	*0.226*
ANC	10^9/L	3.95 (2.92-5.54)	3.50 (2.61-4.40)	*0.283*
Platelets	10^9/L	227 (166-326)	193 (156-268)	*0.230*
AST	UI	16 (13-22)	22 (17-30)	*0.038*
Monoclonal IgM	g/dL	1.48 (1.00-2.74)	0.74 (0.41-1.72)	*0.048*
Factor		**n (%)**	**n (%)**	***p-value***
Gender	F	20 (42)	5 (33)	*0.764*
Spleen	Not Inv.	31 (65)	11 (73)	*1.00*
Nodal sites	>1	12 (32)	0	*0.023*
LDH	>ULN	21 (12)	8 (14)	*0.642*
B2M	>ULN	23 (57)	3 (23)	*0.054*
Bence-Jones	-	9 (19)	6 (40)	*0.135*
	+	21 (44)	4 (27)	
	ND	18 (37)	5 (33)	
BMB	-	-	15 (100)	-
	+	48 (100)	-	
% infiltration BM	Median (25-75°)	50 (18-70)	-	
Treated at Diagn.	Yes	30 (62)	0 (0)	-

Table 2. Clinical features of retrospective series.

Variable		WM N=181	IgM-MGUS N=56	*p-value **
		Median (interval 25-75°)		
Age	years	68 (60-75)	67 (60-73)	*0.785*
Hemoglobin	g/dL	12.5 (10.8-13.8)	13.7 (12.6-14.7)	*<0.001*
Albumin	g/dL	3.9 (3.6-4.2)	4.2 (4.0-4.5)	*<0.001*
s-Creatinine	mg/dL	0.86 (0.71-1.04)	0.84 (0.70-0.96)	*0.246*
WBC	10^9/L	6.82 (5.39-8.16)	6.45 (5.25-7.31)	*0.086*
ALC	10^9/L	1.92 (1.50-2.44)	1.86 (1.40-2.28)	*0.205*
ANC	10^9/L	3.72 (3.00-4.90)	3.68 (3.06-4.24)	*0.505*
Platelets	10^9/L	253 (206-321)	203 (177-251)	*<0.001*
AST	UI	18 (15-22)	20 (17-24)	*0.020*
Monoclonal IgM	g/dL	1.50 (0.78-.2.58)	0.50 (0.30-0.66)	*<0.001*
Factor		**n (%)**	**n (%)**	***p-value***
Gender	F	69 (38)	30 (53)	*0.045*
Spleen	Not Inv.	130 (73)	47 (84)	*0.041*
Nodal sites	>1	18 (12)	2 (4)	*0.171*
LDH	>ULN	21 (12)	8 (15)	*0.642*
B2M	>ULN	77 (50)	18 (35)	*0.077*
Bence-Jones	-	67 (37)	32 (57)	*0.009*
	+	69 (38)	12 (22)	
	ND	45 (25)	12 (22)	
BMB	-	-	56 (100)	-
	+	181 (100)	-	*<0.001*
% infiltration BM	Median (25-75°)	30 (10-50)	-	
Treated at Diagn.	Yes	49 (27)	1 (2)	*<0.001*

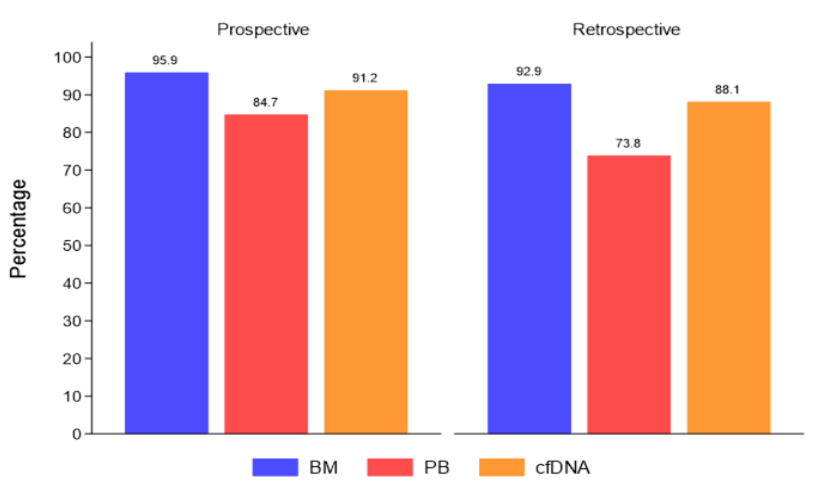
Table 3. Clinical features of prospective series.

Clinical features were equally distributed between WM and IgM-MGUS patients in both series. Of note, four clinical features showed a statistically significant difference ($p<0.05$ in retrospective and $p<0.001$ in prospective series) between WM and IgM-MGUS patients. Indeed, focusing on the prospective series (Table 3), hemoglobin levels were lower in WM (median 12.5 g/dL [range 10.8 – 13.8]) compared to IgM-MGUS (median 13.7 [range 12.6 – 14.7]) as well as albumin levels, that show a median of 3.9 g/dL [range 3.6- 4.2]) vs 4.2 [range 4 -4.5], respectively. Finally, platelets and monoclonal IgM values were higher in WM compared to IgM-MGUS: platelets median 253 x10^9/L (range: 206 - 321 x10^9/L) vs 203 x10^9/L (177 - 251 x10^9/L) and monoclonal IgM median 1.5 g/dL (range 0.78 – 2.58) vs median 0.5 g/dL (range 0.30 – 0.66), respectively.

MYD88^{L265P} detection at baseline

In 274 (91%) cases out of 300 patients, paired BM, PB and plasma samples (for cfDNA analysis) were collected: 218 prospective (170 WM, 48 IgM-MGUS) and 56 retrospective (42 WM, 14 IgM-MGUS) patients.

MYD88^{L265P} rates in prospective patients were: 96% in BM, 85% in PB and 91% in cfDNA in WM. Of note, PB samples showed 11% of false negative results compared to BM. On the contrary, cfDNA showed only 5% of false negatives, mirroring the frequency of BM. Similar results were observed in the retrospective cohort of WM with MYD88^{L265P} rates of 93% in BM vs 74% in PB vs 88% in cfDNA (Figure 6).



	Qualitative – Tissue, n (%)		
Prospective	BM	PB	cfDNA
Negative	7 (4)	26 (15)	15 (9)
Positive	163 (96)	144 (85)	155 (91)
Total	**170**	**170**	**170**

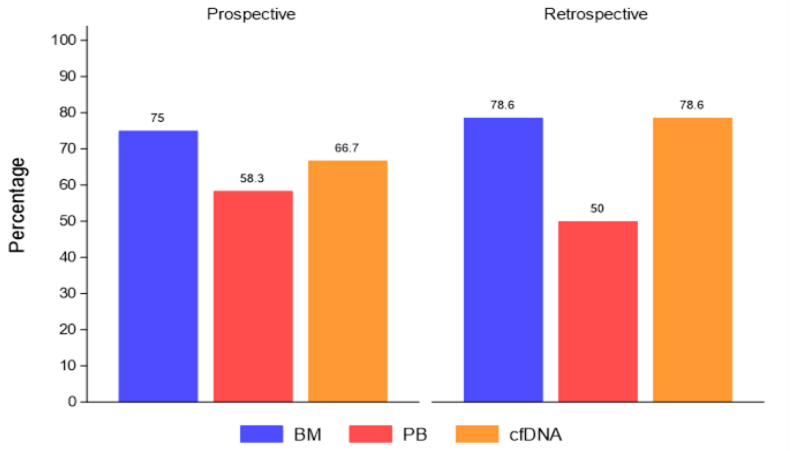
	Qualitative – Tissue, n (%)		
Retrospective	BM	PB	cfDNA
Negative	3 (7)	11 (26)	5 (12)
Positive	39 (93)	31 (74)	37 (88)
Total	**42**	**42**	**42**

Figure 6. Frequency of MYD88^{L265P} mutation in WM (prospective and retrospective)

MYD88^{L265P} rates in IgM-MGUS prospective patients were 75% in BM, 58% in PB and 66.7% in cfDNA (Figure 7).

The lower levels of PB samples were observed also in these patients, showing 17% of false negatives compared to BM. Of note, cfDNA data showed an intermediate rate of mutation (67%) that stays in the middle between BM (75%) and PB (58%), with 8% of false negatives compared to BM.

As observed in WM, similar results were observed in the retrospective cohort with MYD88L265P rates of 79% in BM, 50% in PB and 79% in cfDNA (Figure 7).



	Qualitative – Tissue, n (%)		
Prospective	BM	PB	cfDNA
Negative	12 (25)	20 (42)	16 (33)
Positive	36 (75)	28 (58)	32 (67)
Total	**48**	**48**	**48**

	Qualitative – Tissue, n (%)		
Retrospective	BM	PB	cfDNA
Negative	3 (21)	7 (50)	3 (21)
Positive	11 (79)	7 (50)	11 (79)
Total	**14**	**14**	**14**

Figure 7. Frequency of MYD88 mutation in IgM-MGUS (prospective and retrospective).

The comparison of the MYD88 mutation rate in WM and IgM-MGUS highlight lower mutational rates in all the 3 specimens of IgM-MGUS compared to WM (Figure 8).

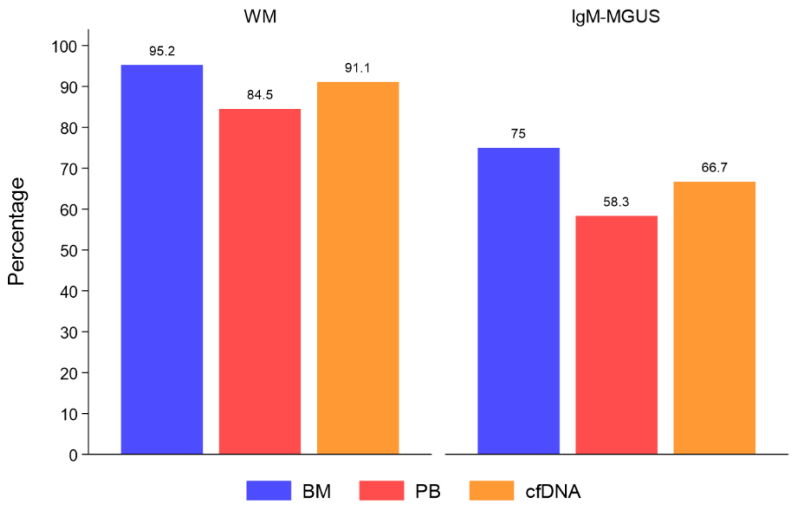


Figure 8. *MYD88*^{L265P} rate in WM and IgM-MGUS patients from the prospective series only.

Quantitative analysis performed by ddPCR allowed to perform a mutational level comparison between specimens, showing a median *MYD88*^{L265P} level in cfDNA superimposable to BM, both in WM (1.4E-02 vs 3E-02) and in IgM-MGUS (1.5E-03 vs 2.6E-03). On the contrary the median PB value was almost 1 log lower (1.2E-03 and 8E-04, respectively) than BM. Of note, mutational levels in IgM-MGUS patients were significantly lower than WM, in all specimens (Figure 9).

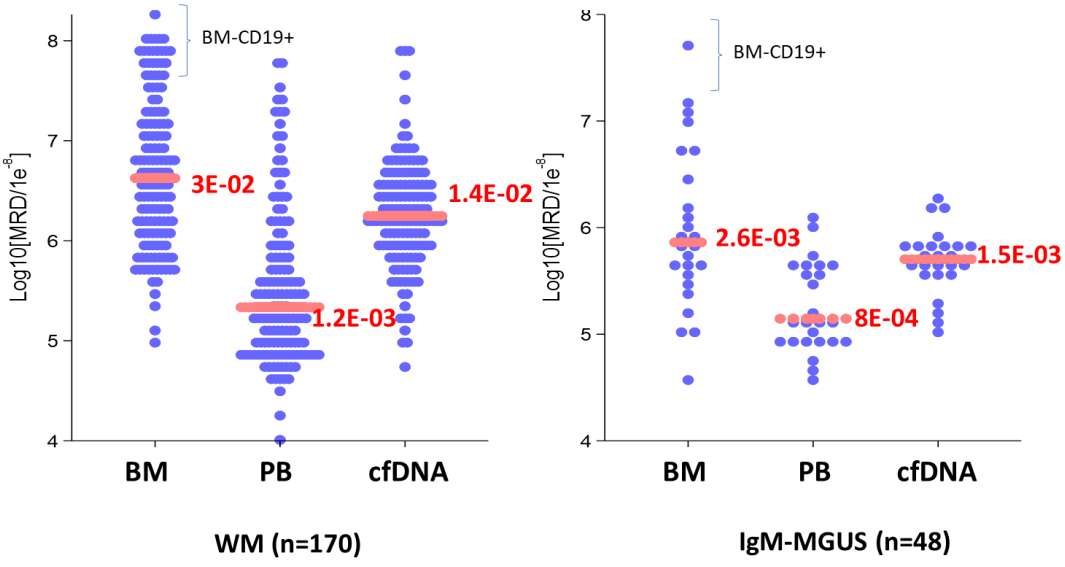


Figure 9. *MYD88*^{L265P} mutational levels in WM and IgM-MGUS patients.

As expected, BM-CD19+ cells recorded the highest median mutational level recorded among 68 BM-CD19+ cells analyzed. The median mutational level of these samples was 6.7E-01 (range 6.6E-04 – 1.9E+01) in 48 WM and 5.6E-02 (range 3.8E-03 – 4.9E-01) in 20 IgM-MGUS patients, as previously observed in the training series.

Moreover, among the 68 patients with BM-CD19-sorted cells, no difference in mutation rate was found between BM and plasma. Indeed, 60/68 (88%) patients scored *MYD88*^{L265P} in BM-CD19+ and 59/68 (87%) in paired plasma cfDNA. On the contrary, paired PB samples showed the lowest percentage of mutation (49/68, 72%) (Figure 10).

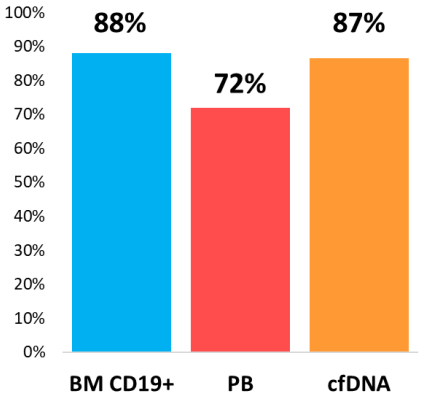


Figure 10. Frequency of *MYD88*^{L265P} in BM-CD19+ sorted cells.

Interestingly, the smoothed density plot reveals superimposable results of BM and cfDNA, while PB samples showed the lower levels and the BM-CD19+ the highest levels of mutation, both for WM and IgM-MGUS (Figure 11).

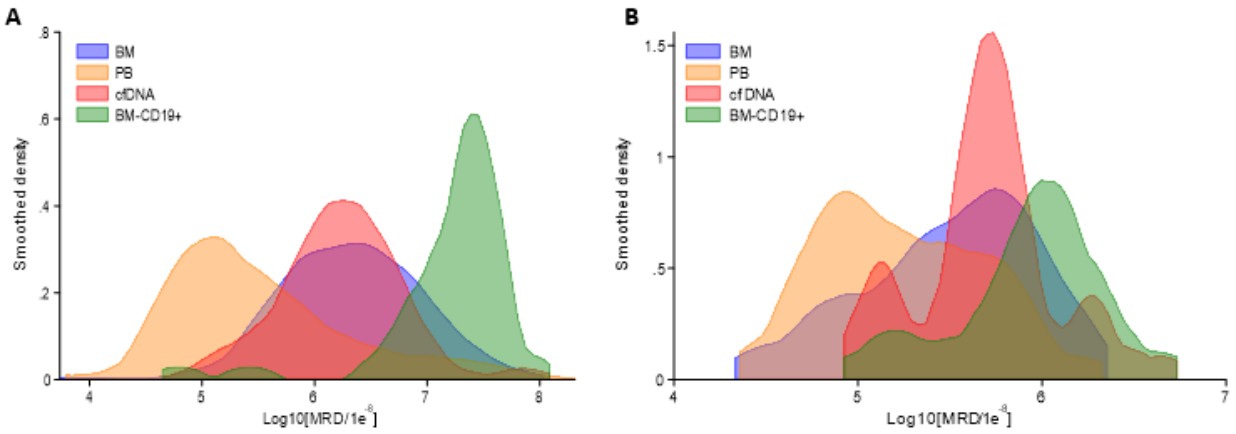


Figure 11. Comparison of *MYD88*^{L265P} quantitative values among different specimens in (A) WM and (B) IgM-MGUS in bone marrow (BM), peripheral blood (PB), BM-CD19+ selected cells and plasma cfDNA.

Concordance analysis of qualitative and quantitative results confirmed a good agreement and a negligible difference between BM and cfDNA. The qualitative concordance was excellent between BM and cfDNA both in WM (agreement 95.3%, K-statistic: 0.615) and IgM-MGUS (agreement 91.7%, K-statistic: 0.800) while only a fair concordance was observed between PB and BM (88%, 0.384 and 83.3%, 0.636 respectively) (Figure 12).

WM

	PB n (%)		
BM	**Negative**	**Positive**	**Total**
Negative	7 (27)	0	7 (4)
Positive	19 (73)	144 (100)	163 (96)
Total	26	144	170

Agreement 88.7%, K-statistic: 0.384

IgM-MGUS

	PB n (%)		
BM	**Negative**	**Positive**	**Total**
Negative	12 (60)	0	12 (25)
Positive	8 (40)	28 (100)	36 (75)
Total	20	28	48

Agreement 83.3%, K-statistic: 0.636

	PLASMA n (%)		
BM	**Negative**	**Positive**	**Total**
Negative	7 (47)	0	7 (4)
Positive	8 (53)	155 (100)	160 (96)
Total	15	155	170

Agreement **95.3%,** K-statistic: 0.615

	PLASMA n (%)		
BM	**Negative**	**Positive**	**Total**
Negative	12 (75)	0	12 (25)
Positive	4 (25)	32 (100)	36 (75)
Total	16	32	48

Agreement **91.7%,** K-statistic: 0.800

Figure 12. Qualitative concordance in WM & IgM-MGUS patients.

From the quantitative point of view, the concordance measured by Passing-Bablok regression plot showed a negligible difference in the quantitative dosage of cfDNA compared to BM (intercept=0.0107, slope =0.9) (Figure 13).

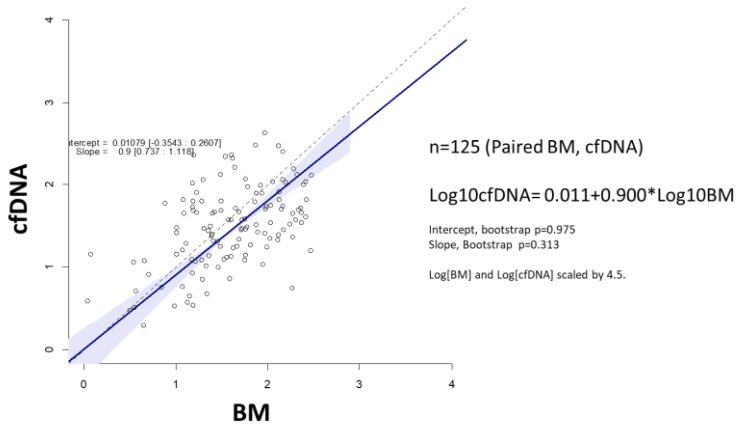


Figure 13. Passing-Bablok method for the quantitative concordance between cfDNA and BM in WM & IgM-MGUS patients.

MYD88^{L265P} detection for MRD purpose

As previously mentioned, the second aim of this project was to assess the MRD after treatment. So far, 59 WM patients have been analyzed both at baseline (T0) and post-treatment (T2). All these patients collected at least a sample of BM, PB or cfDNA before and after treatment, for totally 57 BM, 59 PB and 55 cfDNA samples at T0 and 50 BM, 56 PB and 52 cfDNA samples at T2.

The *MYD88*^{L265P} mutation rate in BM samples at the baseline 54/57 (95%) decrease after treatment where only 33/50 (66%) patients at T2 showed *MYD88*^{L265P} mutation. This reduction was more evident in PB samples were mutation rate dropped from 81% (48/59) to 9% (5/56), while the rate in plasma cfDNA samples decreased from 90% (50/55) to 44% (23/52) (Figure 14).

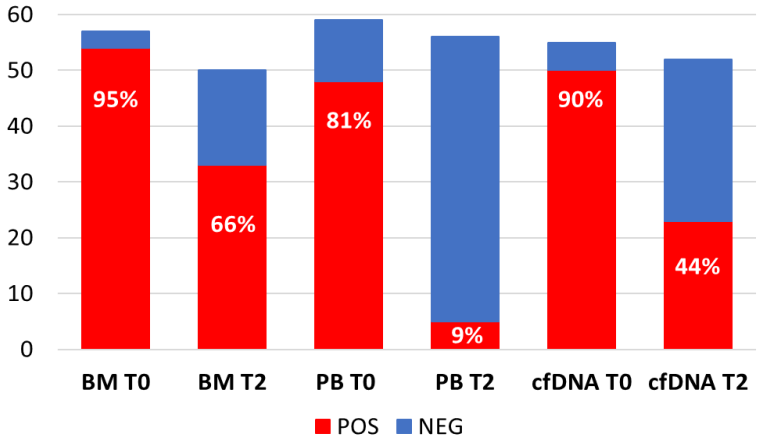


Figure 14. Frequency of *MYD88*^{L265P} before (T0) and post-treatment (T2) in WM patients.

The higher reduction rate in PB samples compared to BM and cfDNA, can be explained by the level of mutation at baseline that is 1 log lower compared to BM and closer to the cut-off of positivity.

The comparison of quantitative mutational levels before (pre) and after (post) therapy (timepoints T0 and T2), clearly showed the effects of therapy on the 3 specimens as represented in Figure 15 where on the y axis is reported the mutational level of each patient (expressed as log10[MRDvalue/1E-08]) before and post therapy.

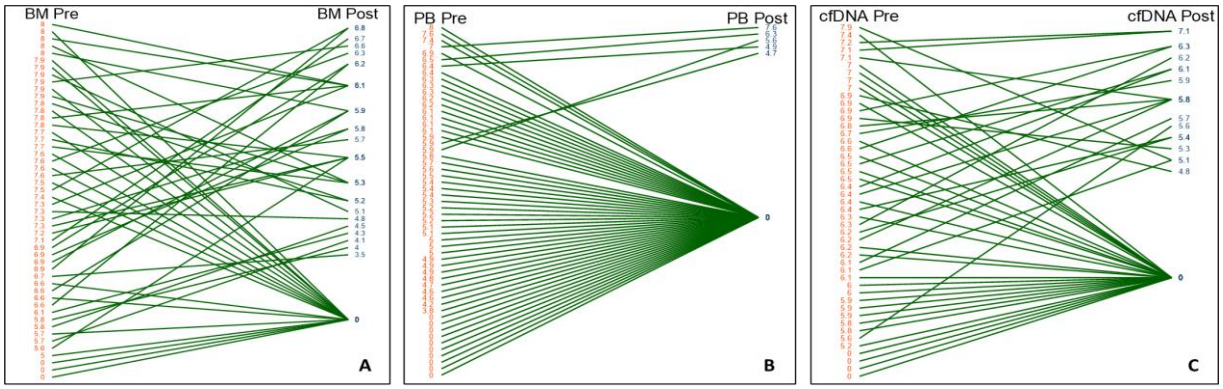


Figure 15. *MYD88*^{L265P} values (y axis) of each patient at T0 (pre-therapy) and T2 (post-therapy) in BM (A), PB (B) and cfDNA (C).

Conclusion

This thesis describes the evolution of a research project that began with the development of the *MYD88*^{L256P} assay by ddPCR on a first local series of samples collected at the hematology unit of the Torino Hospital that has been subsequently validated on a multicentric series involving three Italian hematological centers (Torino, Pavia ad Varese) to directly compare the novel described ddPCR assay with the well-known and standardized method of ASqPCR. Different ASqPCR assays and ddPCR were compared in order to define the abilities and aptitudes of each method for *MYD88*^{L265P} mutation detection and quantification in different type of samples, such as BM unsorted or sorted BM-CD19+ cells, PB, and plasma-cfDNA, collected from WM and IgM-MGUS patients. Overall, the analysis showed a good concordance rate (74%) between the two methods, especially in BM samples (78%), while discordances (26%) were mostly in favor of ddPCR (ddPCR+ vs. ASqPCR-). Notably, ddPCR showed a higher sensitivity in detecting *MYD88*^{L265P}, especially in samples with low mutational burden, such as PB, characterized by a low median mutational level. Moreover, by selecting BM-CD19+ cells no differences in frequency of patients carrying the *MYD88*^{L265P} were observed in comparison to WBC or MNC, even if the mutation level was as expected different.

Concordantly to published literature, in our series ASqPCR detected *MYD88*^{L265P} in diagnostic BM samples in 81% of WM and 58% of IgM-MGUS, however ddPCR detected the mutation in 96% of WM and 87% of IgM-MGUS. Finally, the analysis of plasma-cfDNA confirmed the role of ddPCR for mutational screening and MRD monitoring on a less-invasive way, representing an attractive alternative to BM biopsy particularly in asymptomatic patients, such as those affected by IgM-MGUS. These preliminary results on an internal series of patients and on multicenter national cohort allowed the development of a multicenter clinical trial for non-invasive diagnostics and monitoring of MRD in WM and in IgM-MGUS patients. The Fondazione Italiana Linfomi BIOWM trial (NCT03521516) is currently ongoing, with the primary aim of demonstrating that the *MYD88*^{L265P} mutation rate detected in plasma by ddPCR is superimposable to the rate detected in BM.

The trail enrollment is concluded, and the results obtained so far demonstrated that the primary endpoint of the trial is achieved: *MYD88*^{L265P} mutation rate and levels detected in plasma cfDNA are superimposable to the rate and levels detected in BM. Moreover, our data show that PB samples, even using the highly sensitive ddPCR assay, are still suboptimal for the mutational screening, evidence confirmed by the rate of false negative observed (11%).

Furthermore, the selection of CD19+ sorted cells, despite enriching the sample in tumoral content, is dispensable for MYD88 mutational screening, as the rate of mutation in these specimens was 88%. This makes the ddPCR assay ideal for diagnostic use in clinical routine, avoiding the costs and technical requirements for CD19+ cell sorting.

Interestingly, significantly different *MYD88*^{L265P} mutational levels observed between WM and IgM monoclonal gammopathy of undetermined significance patients suggest the need for further studies

in order to identify possible correlations between mutational levels and risk of progression to Waldenström.

Finally, the most innovative aspect of this study is the impact that ctDNA might have for *MYD88*^{L265P} mutation detection. We showed that baseline ctDNA mirrors the BM mutational burden much better than gDNA obtained from PB. These prospective data are in favor of implementing *MYD88*^{L265P} ddPCR assay on plasmatic cfDNA as less invasive and "patient-friendly" tissue source for mutational analysis and eventually MRD, especially attractive for screening and monitoring of asymptomatic WM and IgM-MGUS patients.

Besides its potential diagnostic role, *MYD88*^{L265P} can effectively and easily be used for MRD monitoring in WM. The percentage of mutated cases after treatment decrease in all the specimens analyzed. This reduction is more evident in PB samples that show a reduction of the mutational rate from 81% to 9%, further suggesting a faster clearance of MRD from PB than from BM (from 95 to 66%). The clearance in cfDNA was about 50% suggesting a behavior in between BM and PB. However, at the present the median follow-up of the trial is relatively short (34 months) and a longer follow-up will better clarify the clinical implications of these MRD values in our series. Indeed, as recently reported by the Pavia group (Varettoni Hematol Oncology 2022), the progression free survival was significantly longer for patients with undetectable MRD values compared with patients with detectable MRD values at the end of therapy [64].

Moreover, consensus recommendations and laboratory requirements for the diagnosis of WM have been currently published on behalf of the European Consortium of Waldenström's Macroglobulinemia (ECWM). The paper describes the procedures for multiparametric flow cytometry, fluorescence in situ hybridization and molecular tests offering a guidance for a standardized diagnostic work-up and methodological workflow of patients with IgM-MGUS and WM [65]. Furthermore, at national level a standardization quality assessment program has been recently launched among laboratories that used different techniques and different type of samples for clinical routine mutational detection. The primary aim of this project called "WM Labs" is to directly verify the performance of ASqPCR and ddPCR in distinct routine laboratories, on different tissues, and time points, to establish consensus on laboratory practice for marker screening and MRD analysis. At this scope, a group of laboratories around Italy have been involved through quality-assessment programs for *MYD88*^{L265P} and CXCR4 mutation detection by both ddPCR and AsqPCR methods in order to define a common data interpretation and report.

The main goal of this project thesis was the technical description of the *MYD88*^{L265P} ddPCR assay and the illustration of its wide potential applications, comprising those on liquid biopsy, highlighting how the technique is feasible and highly sensitive for *MYD88*^{L265P} mutational screening and MRD monitoring in WM. As mentioned, further efforts to establish uniform guidelines regarding standardization procedures for samples collection and methodological validation are currently ongoing, both at national and European context.

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