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Preface and acknowledgements

This thesis is based upon work we needed to conduct in order for a larger project to be fulfilled. The main project for which the work from this thesis was a prerequisite, is run by medical student Even Holth Rustad as the project leader. I want to express gratitude to Even for letting me in on his projects, and for teaching me my way around in the lab. Even has been essential for me to learn all laboratory techniques used in this thesis. He has also contributed a great deal to the thesis itself by way of fruitful discussions.

I want to thank my supervisor professor Anders Waage, who contributed with both wisdom, support and care in our travel towards a better understanding of why we really did what we were doing. Furthermore, I want to thank my second supervisor, Dr. Hong Yan Dai for enduring endless emails from Even and myself. She has been an expert to truly rely on both in the lab and in discussions about our methods. Hong Yan, you made me a better scientist. Additionally, I would like to thank Eivind Coward, for his wonderful help with analysis and making our figures.

Lastly, I want to thank my husband Erlend, who showed patience, consideration and care at a time when I tried to juggle being a fulltime medical student while doing research on my own field of Neuroscience, working part time in the psychiatry ward, and familiarizing myself in a lab within a field of science completely novel to me. I could not have had a better friend and partner.

Delimitation of the project

This project is part of a bigger study where we used circulating tumor DNA (ctDNA) as a marker for monitoring tumor dynamics in 20 patients with multiple myeloma, and up to 52 sequential serum samples were analyzed. Quantification of circulating tumor DNA with specific mutations in the malignant cells may give valuable information about the development of the disease, response to treatment and clonal dynamics. My “master thesis in medicine” is limited to optimization and validation of the assays used in the main project. My work in the main project exceeds that of this thesis. However, delimitation was necessary in order for me to make a project of reasonable size for a master thesis and, furthermore, not to overlap too much with the main project which might preclude publication of the work. The main project has recently been accepted for publication with me as third author, to the journal *Haematologica*. The paper describes the application of the actual ddPCR assays in monitoring of patients and is entitled *Monitoring of Multiple Myeloma by quantification of recurrent mutations in serum*, with authors Even H Rustad, Eivind Coward, Emilie R Skytøen, Kristine Misund, Toril Holien, Therese Standal, Magne Børset, Vidar Beisvåg, Ola Myklebost, Leonardo A Meza-Zepeda, Hong Yan Dai, Anders Sundan, and Anders Waage (see Appendix).

Abstract

Circulating tumor DNA (ctDNA) has been shown as a promising biomarker for monitoring several tumor types. We want to study the use of ctDNA in monitoring multiple myeloma, by use of digital droplet PCR (ddPCR). The objective of this thesis, was to perform optimization and validation of 14 ddPCR assays for further use in the main project on monitoring multiple myeloma with cfDNA. We found all assays to be highly sensitive and specific, with a false positive rate and limit of detection fluctuating between ~0.00 and 0,07 copies/ μ L of reaction volume, and with a linearity displaying a good dynamic range. The assays were used in our main project which culminated in a paper published in the journal *Haematologica*.

1. Introduction

1.1 Multiple Myeloma

Multiple myeloma (MM) is an incurable neoplastic disorder of plasma cells in the bone marrow.¹ Plasma cells are differentiated B-cells which main function is to produce and secrete large quantities of specific antibodies.² Normally, we can detect these antibodies in the serum as *polyclonal* immunoglobulins. Multiple myeloma displays a *monoclonal* proliferation of plasma cells. This is due to the fact that most malignant plasma cells originate from one single B-cell, with the same Ig gene rearrangement during the B-cell development. Therefore, these cells are characterized by secretion of antibodies with a *single* specificity. The monoclonal immunoglobulins produced by the malignant cells can be detected as M-protein in serum and urine in myeloma patients. The quantity of M-protein reflects the total tumor mass, and is therefore widely used as a biomarker for both diagnosing and monitoring multiple myeloma.^{3,4} However, in a specific subtype of MM, called non-secretory MM, no M-protein can be detected, neither in serum, plasma nor urine. Bone marrow sampling and observations for clinical manifestations of organ damage are the only means of monitoring the disease and its response to treatment for patients suffering of this subtype.^{5,6} These patients are in need of a better substitute for the lack of M-protein.

1.2 Circulating cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA)

Circulating cell-free DNA is released into the bloodstream by mechanisms like active secretion, spontaneous release, cellular turnover, apoptosis and necrosis. These mechanisms can be triggered by both physiologic and pathologic conditions, such as homeostasis, exercise, pregnancy, infections, trauma and tumors.⁷⁻¹⁰ CfDNA from tumor cells is called ctDNA. These fragments of nucleic acid display the same molecular characteristics as the tumor itself.¹¹⁻¹³ This makes ctDNA a potential tumor marker, and qualitative and quantitative analysis of ctDNA may become a method for rapid, sensitive and accurate diagnosis and monitoring of malignancies.

Many tumor markers existing today are non-specific proteins, also found in lower concentrations in healthy individuals.¹⁴⁻¹⁶ Furthermore, patients may have normal biomarker levels even if the malignancy is advanced.¹⁷ These aspects highlight the need for more sensitive and specific cancer biomarkers. Cancer genome sequencing studies have described that essentially all types of cancer show somatic genetic mutations.¹⁸ These mutations occur

only rarely in normal cell populations, and thus may function as specific tumor markers.^{19,20} ctDNA as a tumor marker may enable a simple blood draw to give a low-cost, low-risk “liquid biopsy”.²¹

1.3 cfDNA in sickness and health

It is important that we can distinguish between normal cfDNA, also found in healthy individuals, and ctDNA, which derives from tumor cells. One acknowledged distinction between cfDNA in healthy individuals and cancer patients is related to the size of cfDNA fragments. Fragments of approximately 180 base pairs (bp) have generally been associated with apoptosis, while fragments of circa 10,000bp have been observed with necrosis.^{22,23} In normal, healthy individuals fragments of the size related to apoptosis is dominant, while cancer patients show both fragments of apoptotic and necrotic origin, albeit often with necrosis as the predominant process.²²⁻²⁴ This, and other differences, are essential factors to consider in order for cfDNA to be used as a specific biomarker.

1.4 ctDNA in multiple myeloma

In multiple myeloma, M-protein has been the standard for diagnosis and monitoring of the disease. Comparing the detection and quantification of ctDNA to the detection and quantification of M-protein in patients with the secretory form of MM may provide insights into how well ctDNA reflects tumor burden. If it reflects it well, ctDNA may offer a useful substitute for patients with non-secretory MM. M protein is one of the better performing protein markers of tumor mass when it comes to cancer in general, thus, a comparison of ctDNA and M protein may be of interest in other cancers where no suitable tumor mass biomarker is available for comparison.

1.5 Digital droplet PCR as a method for detecting ctDNA

PCR is a biotechnical procedure allowing a defined DNA fragment to be copied and amplified in large amounts. There are three principle steps in this procedure. First, the DNA must be denatured by way of heating. Secondly, annealing takes place where specific primers are bound to their complementary nucleotides on the single-stranded DNA made in the first step. Third, DNA synthesis is initiated by a polymerase, such that two new DNA helixes are made from the original template.²⁵ In real-time PCR, a fluorescently labeled probe specifically targeting the amplified region allows quantification of the target. Somatic mutations and their

wild type (non-mutated) counterparts can be identified in this way, using different specific fluorescently labeled probes.²⁶ (Figure 1)^{27,28}

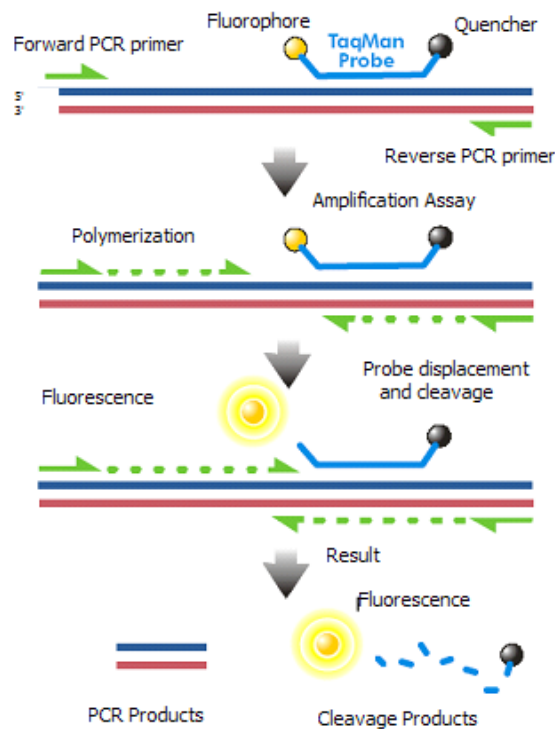


Figure 1: Principles of real-time PCR using TaqMan probes.²⁵

The TaqMan probe is an oligonucleotide with a fluorophore attached to its 5' end and a quencher dye attached to its 3' end. As long as the probe is intact, the proximity of the quencher decreases the fluorescence emitted by the fluorophore. The quenching is not total even when the probe is intact, so some background fluorescence will be observed. A significant fluorescent signal will only be emitted when the probe is cleaved. During PCR, the polymerase cleaves the probe and separates the fluorophore from the quencher, increasing the fluorescence. The cleavage also removes the probe from the target strand, so the extension of primer can continue to the end of the template strand.

The fraction of tumor-derived cfDNA is small^{29,30} and can even be less than 0,01% of the total amount of cfDNA.³¹ The low concentrations of ctDNA found in a complex mixture of similar molecules offers a challenge for detection and quantification. One promising way to solve this issue is to make use of digital droplet polymerase chain reactions (ddPCR), which allow for enumeration of rare mutant variants in a complex background.³² ddPCR builds on the basic PCR principles, but additionally allows for high sensitivity quantification. The high sensitivity may be achieved by compartmentalization of the sample, such that the sample is partitioned into multiple replicate reactions.^{33,34} ddPCR partitions the reaction into many emulsion-based droplets. (Figure 2)^{35,36}. Both amplification and analysis are done separately for each droplet, and the droplets are scored as either positive or negative for the template based on their fluorescence amplitude.³⁷ The partitioning allows estimation of the quantity of different molecules at a high sensitivity and precision by the assumption that the population of molecules is distributed randomly, following a Poisson distribution. Poisson statistics is used to measure the DNA concentration, relying on the droplets being either negative or positive for the template.^{33,37} ddPCR has already proven itself useful in detection and quantification of ctDNA in several types of cancers.^{30,38}



Figure 2: Biorad QX100/QX200 ddPCR system workflow^{31,32}

1. Reaction mix is loaded into the cartridge for droplet generation. 8 wells can each generate 20000 droplets from each run in the droplet generator. Target DNA and background DNA distribute randomly into the droplets.
2. Droplets are transferred to a PCR plate of 96 wells and the plate is sealed. PCR protocol is run.
3. Plate is loaded into the droplet reader. Positive and negative droplets are read, and we analyze the results with Quantasoft software.

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2. Objectives

In order for ddPCR to be used to measure ctDNA in the main project we needed to make sure that the assays were optimally functioning and valid to use. The objective of this project was to perform optimization and validation of 14 ddPCR mutation detection assays to be used in the main project. *Optimization* and *validation* are terms broadly defined in the literature, and there exists no universally accepted consensus for what specific procedures the different terms comprise. Digital droplet PCR is a new technology and its use is not yet standardized. In this thesis, optimization refers to steps undertaken to determine the optimal running conditions for each assay. In principle, optimization could involve changing a range of conditions including the pH and concentrations of Mg²⁺, primers and probes in the reaction mix. However, as recommended from the manufacturer of our ddPCR platform, Bio-Rad laboratories, we limit the optimization to determining the optimal annealing temperature for each assay.

Once the optimal running conditions for each ddPCR assay was determined, we performed a series of experiments to determine the properties of the assay under these conditions. These experiments, which we refer to as validation of the assays, comprise finding the optimal limit of detection (LoD), the false positive rate (FPR) and the assays' linearity. The test results would be used later in interpretation of the results from running the assays on patient samples. Optimization and validation are essential steps for the use of ddPCR, in order to obtain the optimal sensitivity and specificity needed for the main project.

3. Materials and methods

3.1 Approvals

This project did not require any ethical approvals. However, the main project was approved by the Regional Committee for Medical and Health Research Ethics, with reference numbers 2016/821.

3.2 Limit of detection (LOD), false positive rate (FPR) and linearity

Limit of detection, false positive rate and linearity are parameters that helped us judge the performance of the assays and find their sensitivity and the specificity. The LOD of an assay describes how well it can separate true positive samples from false positives. It is expressed as the lowest concentration of mutant DNA in a sample that can be confidently called mutant positive. One of the primary determinants of the LOD is the copy number of template screened in the reaction volume. A practical consequence of this is that patient sampling volume, dilutions etc. will influence the LOD. A statistically founded rule of thumb of unknown origin³⁹ is that in order to be 95% confident that at least 1 mutant allele will be screened in the sample, three times the number of expected wild-type alleles must be screened.

Several parameters affect the LOD, one of them being FPR, which is the concentration of mutant DNA measured in wells of negative controls. If there is no sign of contamination, any mutation-positive droplet in our negative controls will by definition be falsely positive. In addition to contamination, other sources of false positives are cross-reactivity of probes, primer dimers, de-novo mutations and polymerase wrong-reading, all of which one should attempt to prevent and control for.⁴⁰ For a highly specific assay the FPR will be close to 0.

Linearity measures the variability within a dilution range and whether the efficiency of amplification is similar for different start-point copy numbers. Ultimately this defines the assay's dynamic range, i.e. the range of DNA concentrations within which the relationship between input DNA concentration and the measured concentration remains constant. We want the measured concentration to be strictly linear to the input DNA concentration of the target molecule. We can find assay linearity by running a serial log dilution and calculate the R^2 .

3.3 Controls

All PCR-based mutation detection experiments require a specific set of controls. When setting up the tests we needed the following:

Non-template controls (NTCs):

ddPCR is able to detect very low levels of DNA template, it is thus important to control for contamination and nontarget amplification in all reagents. In NTCs, amplifiable DNA was substituted with H₂O. These controls indicate contamination when they are positive for either WT or mutant DNA. NTCs with positive droplets likely indicate poor laboratory routines.

Negative controls:

We used non-digested genomic DNA from peripheral blood leukocytes of healthy donors as negative control DNA. They provided us with a way to measure our false positive rate and thus determine our LoD. False positives may indicate contamination or poor primer-probe design.

Positive controls:

These controls were either from a specific cell line or they were preordered gene-fragments positive for the mutation (Table 1). Positive controls helped us to verify the negative results, and to check that the conditions we used were able to amplify our template.

The three above-mentioned controls were used to aid gating of droplets into double negative, single-positive or double positive clusters. For each assay, the same type and amount of control-DNA was used in each experimental setup to assure that the assay performed reliably over time.

Dilution series of positive controls:

This series helped us to assess the assays' linearity.

3.4 Mutations examined

We investigated 14 different mutations detected in one or more patients.

- BRAF V600E
- NRAS: Q61K, Q61R, G12A, G12D
- KRAS: Q61R, Q22K, Q61H, A146P, G12S
- IRF4 K123R
- FAM46C S272Y
- DIS3 H788R
- TP53 Y236N

3.5 Assays

We used pre-validated primer/probe sets (PrimePCR ddPCR Mutation Assays, BioRad) commercially available or designed on demand by the manufacturer (Table 1).

3.6 Workflow

Preparing and mixing reagents:

First we provided a clean working environment by wiping down the working surfaces with RNase/DNase to prevent contamination. We diluted the DNA or gene fragments with a logarithmic dilution series. The master mix was prepared in a pre-PCR lab in order to prevent contamination from DNA in the PCR lab. The master mix contained the following reagents:

- 10 µl Supermix for Probes (no dUTP) (BioRad)
- 1 µl primer and probe with associated fluorophore for wild-type DNA. (PrimePCR ddPCR Mutation Assays, BioRad)
- 1 µl primer and probe with associated fluorophore for mutant DNA. (PrimePCR ddPCR Mutation Assays, BioRad)

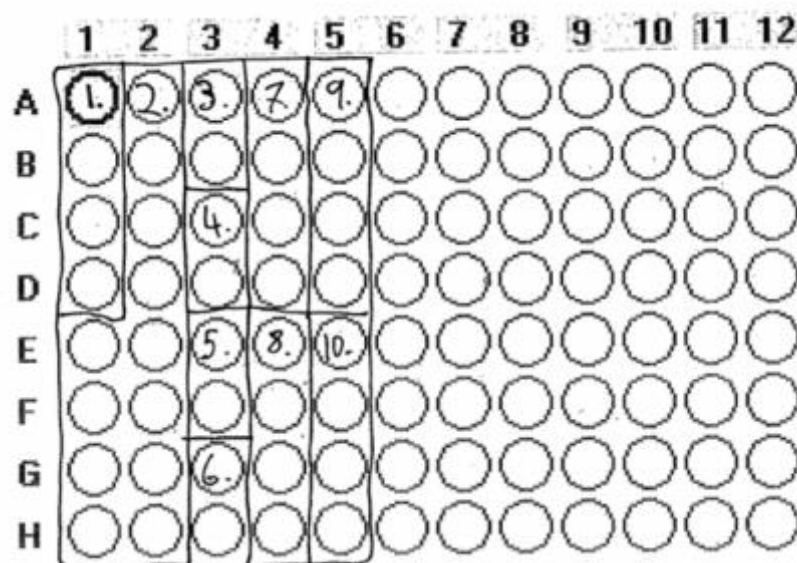
The reaction mix was made in the PCR lab by adding 8 µl DNA to each master mix. For multiplex reactions we used 4 µl wild-type DNA + 4 µl mutant DNA. For four of the mutations (NRAS G12A, NRAS Q61R, KRAS Q61R and IRF4 K123R) we used gBlocks at a concentration of 0,0125 pg/µl as mutant DNA. For NTCs we substituted the DNA sample with water. For negative controls we only used wild-type DNA.

Droplet generation:

The reaction mixes were distributed on a 96-well PCR plate. We transferred 22 µl reaction mix to each well in the following pattern:

1. 4 replicates of NTC, reaction mix with water in multiplex.
2. 12 replicates of negative controls, reaction mix with wild-type DNA in multiplex.
3. 2 replicates of positive controls, reaction mix with wild-type and mutant DNA in multiplex, no dilution (1:1).
4. 2 replicates of reaction mix with wild-type probe in monoplex.
5. 2 replicates of reaction mix with mutant probe in monoplex.
6. 2 replicates of reaction mix with mutant DNA and wild-type DNA 1:1.
7. 2 replicates of reaction mix with mutant DNA diluted 1:10 in a constant background wild-type DNA.
8. 4 replicates of reaction mix with mutant DNA diluted 1:100 in a constant background of wild-type DNA.
9. 4 replicates of reaction mix with mutant DNA diluted 1:1000 in a constant background of wild-type DNA.

10. 4 replicates of reaction mix with mutant DNA diluted 1:10000 in a constant background of wild-type DNA.



We transferred 20 μ l of each reaction mix from the PCR plate to a cartridge for droplet generation, 8 wells at a time, using a multichannel pipette. Then we added 70 μ l droplet generator oil to oil-specific wells in the cartridge, using a multichannel pipette. The cartridge was placed in QX100 Droplet Generator (BioRad), which partitioned the samples into droplets. 40 μ l from droplet-specific wells in the cartridge were transferred to a new PCR-plate and when all wells were partitioned and transferred, the PCR-plate was heat sealed.

Thermal cycling:

Thermal cycling for amplification of DNA was performed with an initial 10 minutes activating step at 95°C, followed by 40 cycles of denaturation at 94°C for 30 seconds and annealing for 1 minute at the optimum temperature found for each assay, and lastly a 10 minutes stabilizing step at 98°C.

Droplet reading and data analysis:

Droplets were read with QX200 Droplet Reader, (BioRad). The analysis was carried out using QuantaSoft v. 1.7. Droplets cluster into four distinct groups, given that the assay is performing ideally (figure 3):

1. Double negative droplets. These don't contain any DNA.
2. Single positive droplets for wild-type DNA.
3. Single positive droplets for mutant DNA.
4. Double positive droplets. These contain both wild-type and mutant DNA.

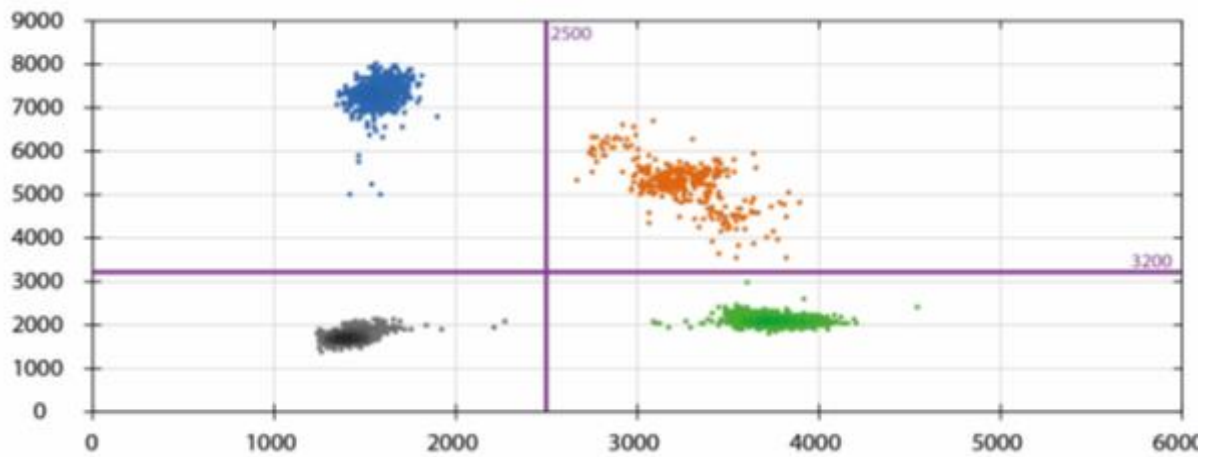


Figure 3: Two-dimensional scatterplot based on mutant and wild type probe fluorescence amplitude. Fluorescence is measured in arbitrary fluorescence units (FU). Each dot represents a droplet. A purple cross is drawn at 3200 mutant FU and 2500 wild type FU, representing a manual gating threshold set to separate droplets into color coded clusters. Gray dots represent droplets not containing any amplifiable DNA, green dots represent droplets containing only wild type DNA, blue dots represent droplets containing only mutant DNA, and orange dots represent droplets containing both wild type and mutant DNA.

4. Results

4.1 Annealing temperature

For optimization of the annealing temperature we ran each assay with a temperature gradient in order to find which temperature that provided the optimal PCR efficiency, as judged by fluorescence amplitude and separation of positive and negative droplets into clusters. This test was run separately from the other tests. The temperature gradient was centered around the manufacturer's suggested annealing temperature of 55,0°C for all assays. In a PCR plate the gradient would be represented in the following manner: well A: 58,6 °C; B: 58,3 °C; , C: 57,6 °C; D: 56,4°C; E: 55,0 °C; F: 53,8 °C; G: 53,0 °C; H: 52,6 °C. Out of 14 assays, 7 had an annealing temperature of 55,0 °C, 4 assays had an annealing temperature of 53,8 °C, one of 53,0 °C and one of 54,6 °C. (Table 2, example in figure 4 a and b)

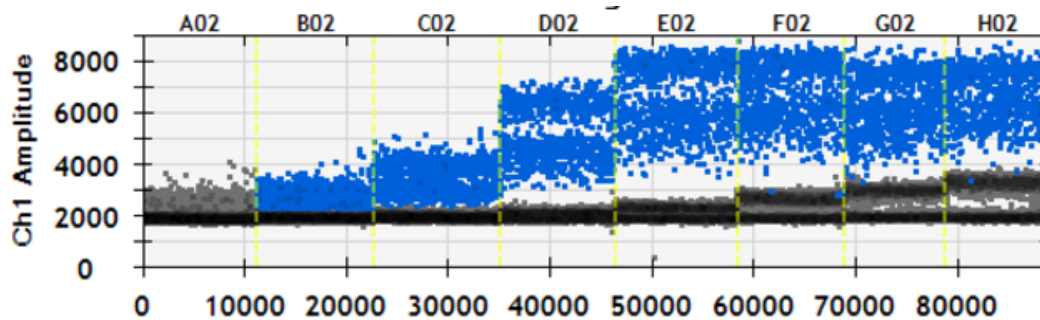


Figure 4 a):

1D fluorescence amplitude plot showing the temperature gradient for BRAF V600E. Well E02 represents a temperature of 55°C, and is showing the best separation between template positive droplets (blue) and template negative droplets (grey). Well D02 (56,3°C) is the second best option.

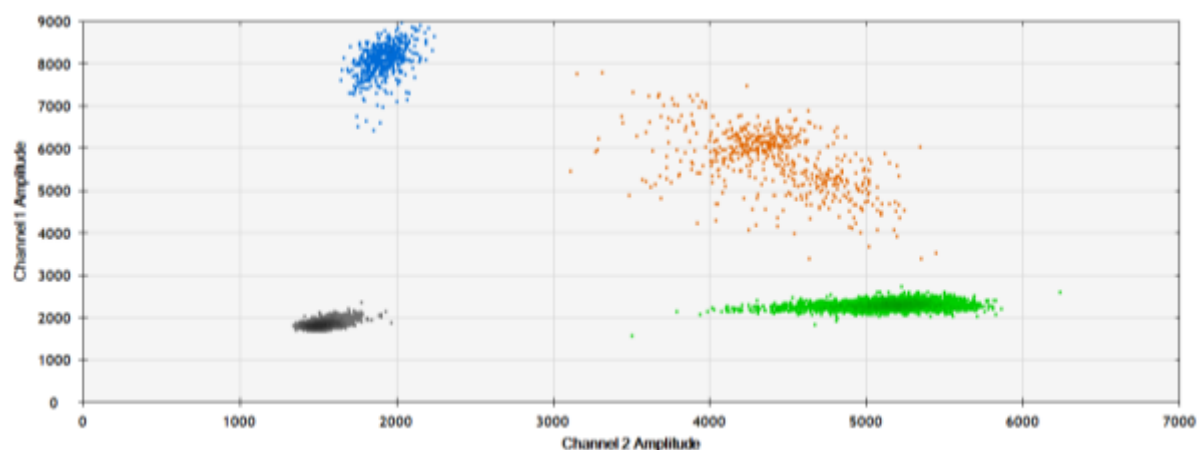


Figure 4 b):

2D fluorescence amplitude plot of BRAF V600E with the optimal temperature of 55°C, shown in 4a. Note the nice separation between all four clusters. Lower left NTC, lower right positive for WT, upper left mutation positive droplets, upper right double positive droplets for WT and the mutation.

4.2 FPR and LOD

We determined LOD empirically by calculating it based on the frequency of mutation positive droplets in a series of negative controls. We ran a set of test experiments on each assay where the LOD was determined as the upper value of the 95% confidence interval of the false positive rate. (Figure 5 and 7, Table 2).

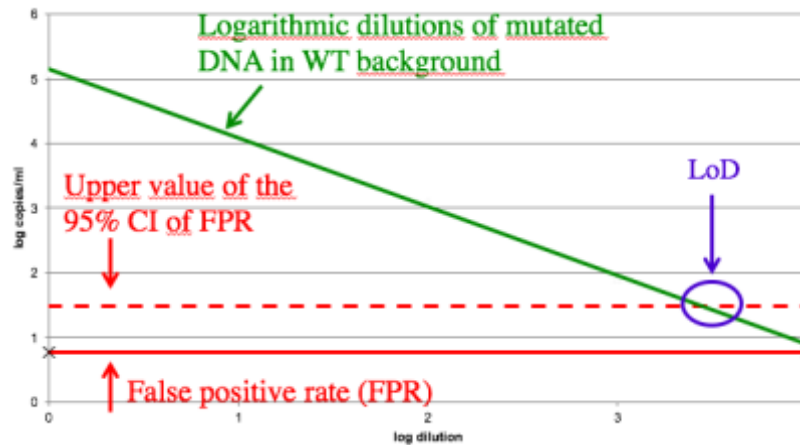


Figure 5: LOD as the average number of falsely detected copies of mutated DNA per μL of negative control, here shown by a log dilution series, and with units of log copies/ml. Note that the LOD strongly depends on the amount of WT DNA in the background.

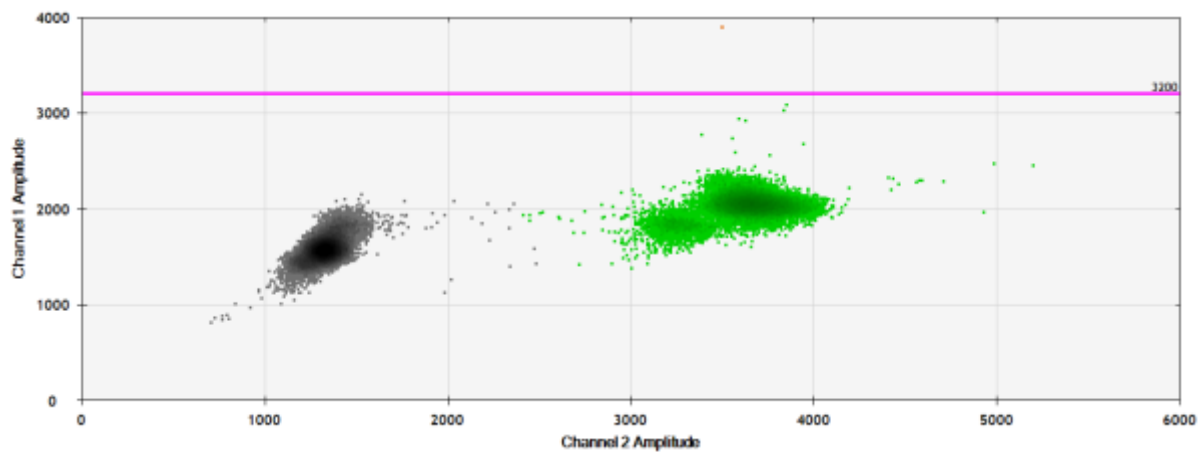
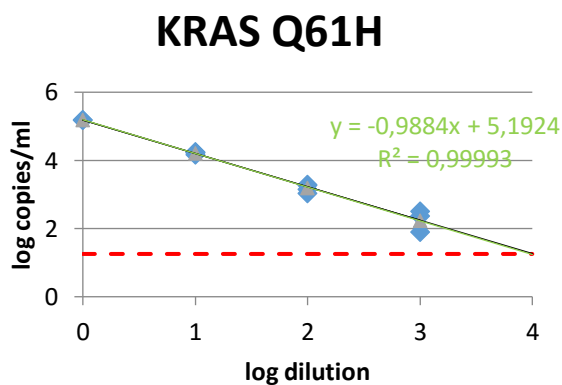
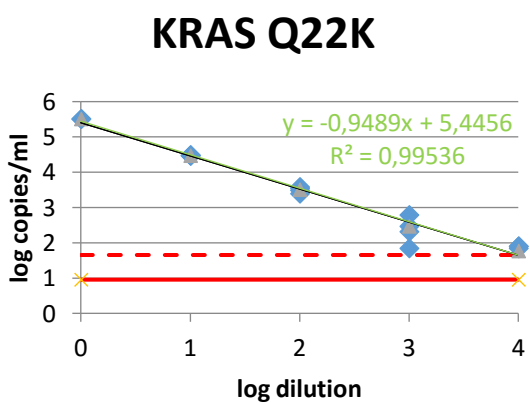
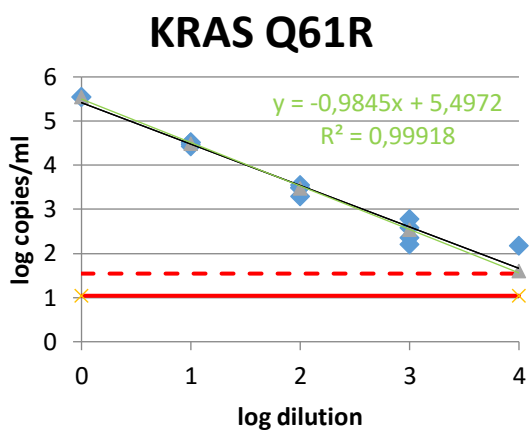
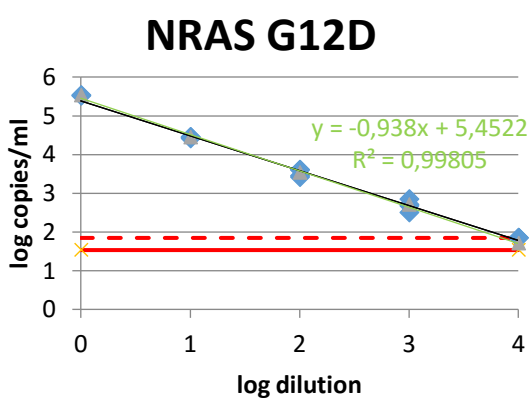
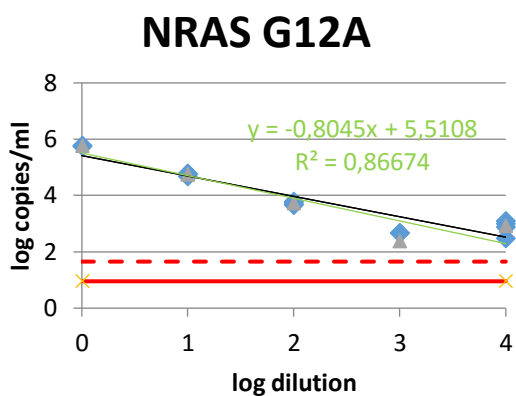
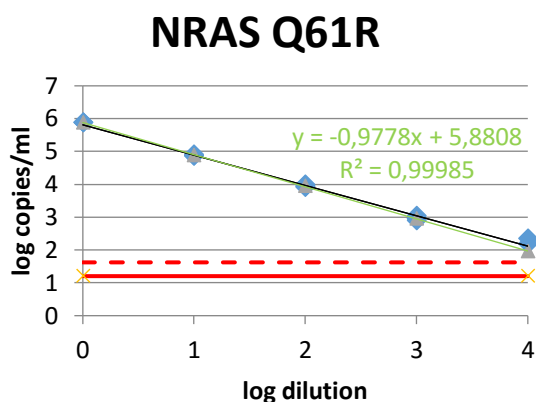
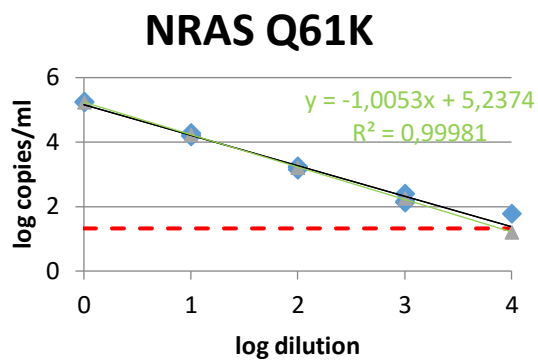
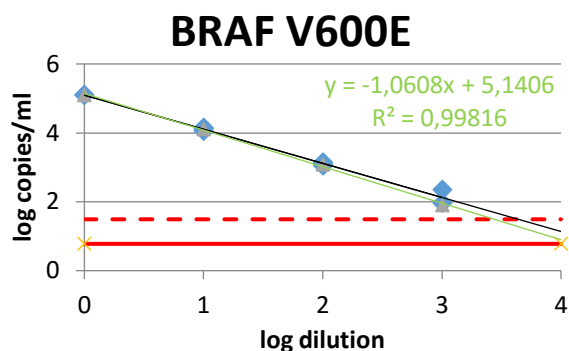


Figure 6: 2D plot of all 12 negative control wells for assay BRAF V600E as an example. Note one orange dot above the purple threshold, representing a droplet positive for both WT and the mutation.



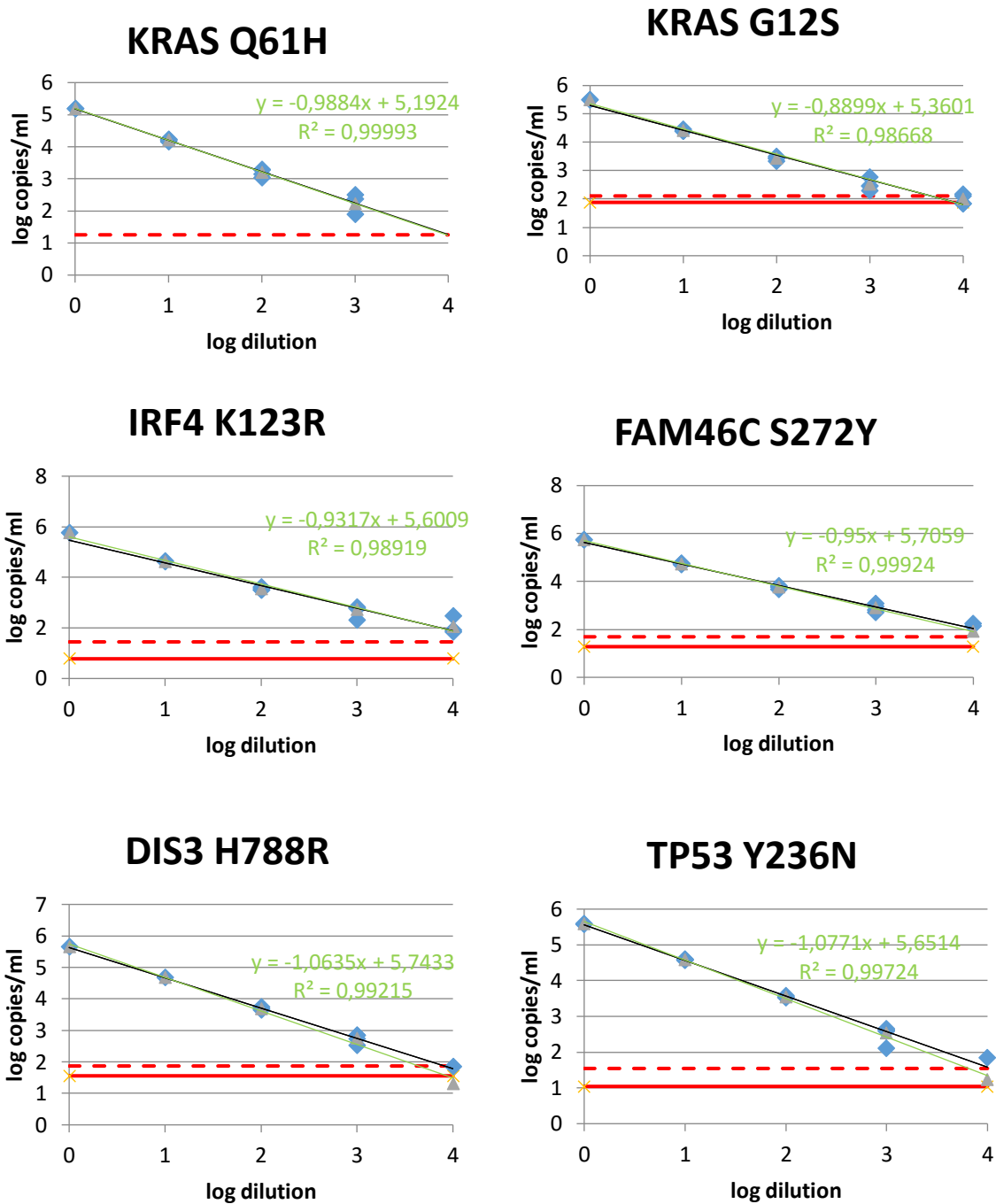


Figure 7: Dilution series for all assays. Red dashed line represents upper 95 confidence interval for FPR. Blue squares represent measured values in log concentration at different dilution steps. Grey triangles represent average concentrations for each dilution step, with a green solid line showing the linear regression between them. The black solid line represents linear regression based on every *positive* value measured.

4.3 Linearity

We used an experimental setup with four 1:10 dilutions of mutated DNA in a background concentration of wild type DNA comparable to the concentration in serum samples. We ran 2-4 replicate wells for each dilution step. Additionally, we ran 14-16 negative control wells, and 4 non-template control wells for each assay. We found that all assays showed sufficient linearity within the dilution range, with a correlation coefficient of $R^2 > 0,98$ (Figure 7, Table 2).

Assay	Annealing temperature, °C	FPR, copies/ μ L	LOD, copies/ μ L	Linearity, R^2
BRAF V600E	55,0	0,006	0,031	0,9982
NRAS Q61K	55,0	0,000	0,021	0,9998
NRAS Q61R	55,0	0,016	0,042	0,9999
NRAS G12A	53,8	0,013	0,040	0,9985
NRAS G12D	53,8	0,034	0,070	0,9981
KRAS Q61R	55,0	0,011	0,035	0,9992
KRAS Q22K	55,0	0,009	0,045	0,9954
KRAS Q61H	55,0	0,000	0,018	0,9999
KRAS A146P	53,8	0,000	0,022	0,9935
KRAS G12S	53,8	0,076	0,128	0,9866
IRF K123R	55,0	0,006	0,028	0,9892
FAM46C S272Y	53,0	0,019	0,049	0,9992
DIS3 H788R	53,8	0,036	0,074	0,9922
TP53 Y236N	56,4	0,011	0,035	0,9972

Table 2: Annealing temperature found for all assays. FPR defined as the average number of mutated copies falsely detected in negative controls. LOD was set to the upper value of the 95% confidence interval of FPR. R^2 , correlation coefficient of dilution series based on average values of replicate wells.

5. Discussion

ddPCR is a method with remarkable sensitivity and precision. However, as all PCR methods, it is susceptible to poor assay design and sub-optimal PCR conditions. To ensure optimal assay performance and correct interpretations of the results, it is important to plan experiments with optimization and validation of assays included. Doing this for 14 unique ddPCR mutation detection assays was the scope for this thesis. We found all assays performing satisfactorily for further use in the main project regarding ctDNA in multiple myeloma.

Digital droplet PCR is a rather new technology. To our knowledge, ddPCR has not been put to systematic use at our institution prior to this work. Thus, there were no local traditions or procedures to build on in this study. Furthermore, there is no international consensus on planning or interpreting ddPCR experiments, including the definitions of terms such as LoD or FPR. A set of guidelines for reporting of ddPCR experiments has been published, but it has not been widely adopted.⁴¹ Due to the immature status of this field, the present work has relied heavily on the manufacturers' instructions, our own reasoning and the scarce available literature.

According to the assay manufacturer, an optimal annealing temperature could be defined as when the mutant probe exhibits no false positive droplets in the wild type control, and the relative distance, i.e. the difference in fluorescence amplitude, between the mutant-only and the wild type-only clusters is maximal.⁴² Gating of droplets into clusters was done manually by eye, which is a crude method, particularly when involving "spray" of droplets outside of the cluster core. Additionally, several of the assays showed quite similar performance at multiple annealing temperatures, which made the decision-making even more complicated. (See figure 4a). However, often we found the range of acceptable annealing temperatures to be large, which generally indicates solid assays.

When investigating a tests' validity, we essentially want to find if the test measures what we want it to measure. We also want to find our test's sensitivity and specificity. Without comparing it to a gold standard however, this is not feasible in the strict sense. It is therefore prudent to clarify what use we can make of our validations. In practical terms, we had decided to define a patient sample as positive when the concentration of mutant DNA was larger than

the assays' limit of detection. Thus, we needed to find the false positive rate and the limit of detection of our assays.

The high sensitivity of ddPCR is mostly desirable, but it also presents the undesirable drawback of amplifying nucleic acids other than the intended target molecule. Contamination, de novo mutations, cross-reactivity of probes and the concentration of wild type DNA all affects FPR. In addition to prevent contamination, we decided to use slightly more wild type DNA than what to expect in healthy donors, yielding a probable overestimation of the FPR rather than an underestimation. This might stop us from making a type I error, by calling a sample positive when it is in fact not.

We ran 12 negative control wells per each assay test. When considering exceptionally rare events, we need numerous replicates to provide accurate estimation. Several of our assays showed false positives in negative controls, see figure 6 for an example. When considering 12 replicate wells with about 12000-19000 droplets each, a few falsely detected positives will still yield an acceptable FPR. Furthermore, when the false positive droplets' fluorescence amplitudes were compared to the amplitudes of the positive controls, they would often differ to a large extent.

In some of our assays, even under optimized conditions, we found WT single positive droplets and droplets positive for both WT and the mutation to be partially overlapping in the two-dimensional scatterplots. WT-only droplets seemed to have a higher fluorescence amplitude on the mutant probe, which is likely due to unspecific binding. This implies that double-positive droplets are more prone to be false positives than mutant-only droplets. In almost all instances of detected false positives, we found no mutant-only droplets.

Digital droplet PCR is completely based on the idea that all target DNA is distributed randomly into droplets. The partitioning process can generate a maximum of 20.000 droplets in each well, although the actual droplet number often is around 14-16000. Some droplets will be lost during handling of the sample. Reading only a subset of the total amount of droplets should principally not affect the concentration measurement, as the concentration is calculated on the base of the fraction of droplets not containing the target molecules. Nevertheless, when concentrations become extremely small elimination of too many droplets will lead to larger

error bars.⁴² Our data presented some inter-well droplet variation, but generally all wells were above 12.000 droplets, which is acceptable.

To sum up, all assays were found to be highly sensitive and specific, with FPR and LOD fluctuating between ~0.00 and 0,07 copies/ μ L reaction volume, and with a linearity displaying a good dynamic range. The assays were used in the main project to measure ctDNA in 251 archived samples from patients with multiple myeloma. M protein had been measured in the same samples and a major finding was a close co-variation between concentrations of M protein and ctDNA. Such a co-variation is meaningful and cannot be random. (See Appendix for details). We take it as a validation of the ctDNA measurements and a confirmation of the pre-analytical testing of the 14 assays. In the process of accomplishing our objectives, we have established a relatively simple procedure that may be used by others in the future.

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7. Appendix

Attached is the paper for our main project.



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Monitoring multiple myeloma by quantification of recurrent mutations in serum

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ABSTRACT

Circulating tumor DNA (ctDNA) is a promising biomarker to monitor tumor load and genome alterations. We explored the presence of circulating tumor DNA in multiple myeloma patients and its relation to disease activity during long-term follow-up. We used digital droplet polymerase chain reaction analysis to monitor recurrent mutations, mainly in mitogen activated protein kinase pathway genes *NRAS*, *KRAS* and *BRAF*. Mutations were identified by next-generation sequencing or polymerase chain reaction analysis of bone marrow plasma cells, and their presence analyzed in 251 archived serum samples obtained from 20 patients during a period of up to 7 years. In 17 of 18 patients, mutations identified in bone marrow during active disease were also found in a time-matched serum sample. The concentration of mutated alleles in serum correlated with the fraction in bone marrow plasma cells ($r=0.507$, $n=34$, $P<0.002$). There was a striking covariation between circulating mutation levels and M protein in ten out of 11 patients with sequential samples. When relapse evaluation by circulating tumor DNA and M protein could be directly compared, the circulating tumor DNA showed relapse earlier in two patients (3 and 9 months), later in one patient (4 months) and in three patients there was no difference. In three patients with transformation to aggressive disease, the concentrations of mutations in serum increased up to 400 times, an increase that was not seen for the M protein. In conclusion, circulating tumor DNA in myeloma is a multi-faceted biomarker reflecting mutated cells, total tumor mass and transformation to a more aggressive disease. Its properties are both similar and complementary to M protein.

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Introduction

Multiple myeloma is caused by proliferation of monoclonal plasma cells in the bone marrow and is the second most common hematologic malignancy.¹ The treatment options have improved markedly in recent years and led to prolonged survival.² However, the disease is still considered to be incurable. The typical course of multiple myeloma is repeated treatment responses followed by increasingly aggressive relapses. Ultimately, the disease becomes refractory to all treatment and the patient dies.

To assess disease progression and treatment response, clinicians rely on monitoring of the monoclonal immunoglobulin (M protein) secreted by the tumor cells as a biomarker for tumor mass.^{3,4} However, some patients escape the traditional monitoring. Between 1-3% of patients have non-secretory multiple myeloma and no detectable M protein.^{5,6} Furthermore, 10% of newly diagnosed myeloma patients

have oligo-secretory disease, defined as a baseline level of M protein that is too low to evaluate treatment response reliably by traditional methods.^{3,7} These patients are challenging to monitor and are, therefore, often denied access to clinical trials.⁷

A promising new cancer biomarker is circulating tumor DNA (ctDNA), which may be extracted from serum or plasma.⁸ DNA fragments are released from cancer cells as well as normal cells in the body during apoptosis and necrosis.^{9,10} The cancer-derived fragments may be identified if they contain tumor-specific mutations or other genetic aberrations.⁸ In studies of solid tumors, ctDNA has provided information about tumor mass and residual disease, as well as information about the tumor genome that could otherwise only have been obtained by a tumor biopsy.¹¹⁻¹⁵ Information about ctDNA in multiple myeloma lags behind as only a single study has so far been published.¹⁶

The somatic mutational landscape of multiple myeloma has been described in several studies.¹⁷⁻²¹ Out of more than 6,000 genes in which coding mutations have been identified, 13 are mutated more frequently than predicted from the background mutation rate, suggesting that they are implicated in the development of the disease.^{17,18} Among these recurrently mutated genes, *NRAS*, *KRAS* and *BRAF* in the MAP kinase pathway are most frequently mutated, occurring in bone marrow plasma cells from approximately 50% of patients at diagnosis. Moreover, activating mutations in the MAP kinase pathway are of interest because they are potential therapeutic targets.²²⁻²⁵

In this study, we explored ctDNA as a biomarker of multiple myeloma and focused on mutations in recurrently mutated genes including *NRAS*, *KRAS* and *BRAF*. We measured the concentration of specific mutations in serum through several responses and relapses for up to 7 years in 20 patients and found a remarkable covariation with the concentration of M protein. However, in terminal aggressive disease, ctDNA appears to reflect the development of the disease better.

Methods

Study design and patient inclusion

We conducted a retrospective study measuring ctDNA in archived serum samples from patients with multiple myeloma. Mutations of interest were identified in a bone marrow biopsy or purified bone marrow plasma cells and subsequently measured in serum by mutation-specific digital droplet polymerase chain reaction (ddPCR). Patients were included based on the following criteria: (i) presence of one or more mutations in genes recurrently mutated in myeloma^{17,18} and (ii) availability of relevant serum or plasma samples. Twenty patients from two sources were included in this study: one previously published study of the *BRAF*^{V600E} mutation in myeloma and an on-going whole exome sequencing (WES) study.²⁶ A flowchart describing the patients' inclusion in detail is presented in the supplemental material (*Online Supplementary Figure S1*).

Clinical data were obtained from the patients' records and archived blood smears were evaluated for the presence of plasma cells. All patients had given written consent. The study was approved by the Regional Committee for Medical and Health Research Ethics (2016/821).

Details about the following experimental procedures are provided in the *Online Supplementary Methods*.

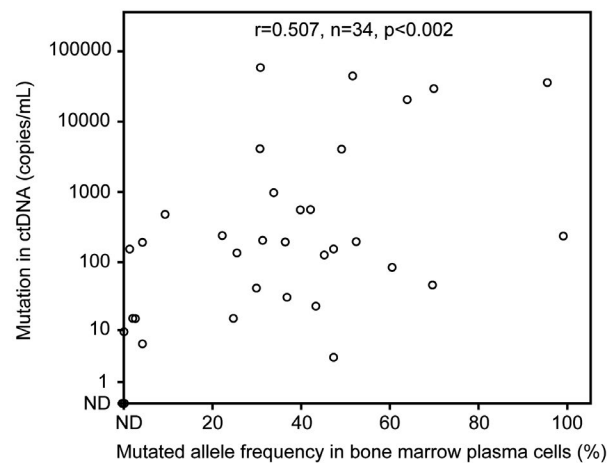


Figure 1. Correlation between mutation levels in bone marrow plasma cells and serum samples. Time-matched bone marrow and serum samples were obtained within 10 days of each other. Purified bone marrow plasma cells were analyzed by WES. In three cases in which WES was negative, positive results from more sensitive ddPCR of bone marrow plasma cells were reported instead. Serum samples were analyzed by ddPCR.

Detection of mutations in serum by digital droplet polymerase chain reaction

Serum (n=249) and citrate-plasma (n=2) samples were obtained from the Norwegian Multiple Myeloma Biobank. DNA was extracted from a median sample volume of 1.8 mL (range, 0.4-3 mL) using a QiaAmp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany). To detect mutations, we used the ddPCR system QX100/200 from Bio-Rad Laboratories (Hercules, CA, USA).²⁷ Detailed assay information is presented in *Online Supplementary Table S1* and raw data examples in *Online Supplementary Figure S2*. Patients' samples were considered to be mutation-positive if the mutant concentration in the sample was higher than the 95% confidence interval of the assay-specific false positive rate (*Online Supplementary Table S2*, *Online Supplementary Figure S3*). The estimated number of mutant copies required in a sample to be considered mutation positive ranged from 0.84 to 2.96 copies of mutated DNA (median 1.4). The quantity of mutated DNA in positive samples was reported in copies per mL of serum.

Whole exome sequencing

WES of purified plasma cells and matched germline controls was performed as previously described.²⁶ The target coverage of >100x was achieved for 85% of exonic regions. The limit of detection of WES was a mutated allele fraction of 2-4 % in the bone marrow sample.

Statistical analysis

Bivariate correlations were performed by the Spearman correlation rank test. The level of statistical significance with two-tailed *P*-values was *P*<0.05. Statistical analyses were carried out in SPSS v. 21 (IBM Corporation, Armonk, NY, USA).

Results

A summary of clinical and mutational data for each patient is given in Table 1.

Relation between tumor mutations in serum and bone marrow plasma cells

We started by determining whether mutations found in

Table 1. Summary of clinical data and mutations.

Patient	Mutation(s)	Sex	Age (years)	Survival (months)	N. of treatments	M-Protein	ISS-stage	Hb (g/L)	Ca-corr (mmol/L)	Creatinine (μ mol/L)	Bone disease
1	BRAF V600E	M	69	51	6	IgA kappa	-	-	-	-	-
2	NRAS Q61K	F	66	40	4	IgA kappa	3	8.7	2.53	105	Yes
3	KRAS Q61H	F	77	35*	2	IgG kappa	2	11.9	2.71	58	No
4	FAM46C S27Y, IRF4 K123R, KRAS A146P	F	54	52	6	IgG kappa	2	11.9	2.31	71	-
5	KRAS Q61R, TP53 Y236N	M	58	9	2	IgG kappa	1	15.3	2.42	54	-
6	BRAF V600E	F	81	64	2	IgG kappa	3	12.8	2.51	94	Yes
7	BRAF V600E	M	57	77	10	IgG lambda	-	11.9	-	107	No
8	BRAF V600E	F	68	104*	3	IgG lambda	1	14.2	2.26	60	Yes
9	KRAS Q22K	F	61	107	9	IgG lambda	2	10.9	2.36	61	Yes
10	BRAF V600E	M	54	79	6	Lambda	1	14.1	2.53	70	Yes
11	BRAF V600E	M	75	22	3	Lambda	3	7.8	3.53	248	No
12	NRAS G12D	F	48	58*	4	IgA kappa	-	10.9	-	82	Yes
13	NRAS Q61K	M	67	55*	2	IgA kappa	3	9.9	3.2	81	-
14	DIS3 H788R, NRAS Q61R	M	73	24	3	IgA kappa	1	14.5	2.31	93	Yes
15	NRAS G12A	M	68	23*	2	IgG kappa	1	14.1	2.23	30	-
16	NRAS Q61R	M	61	33*	2	IgA lambda	2	9.4	-	-	Yes
17	NRAS Q61K	M	50	32*	2	IgA kappa	2	12.3	2.76	97	No
18	KRAS Q22K	F	83	28*	3	IgA kappa	3	12.6	2.27	63	Yes
19	BRAF V600E, NRAS Q61K	F	69	46*	1	IgA lambda	2	10	2.44	79	Yes
20	BRAF V600E	M	64	42	2	Lambda	2	11.3	2.85	125	Yes

Clinical parameters are reported from the time of diagnosis of multiple myeloma. Survival is calculated from the date of diagnosis to the date of death or last observation. M, male; F, female; *, patient still alive; -, missing data; Ca-corr, albumin-corrected serum calcium; Hb, hemoglobin.

bone marrow plasma cells could be detected in time-matched serum samples by ddPCR, and found that this was the case for 17 of 18 patients (34 of 35 mutations). We examined the quantitative relationship between the concentrations of circulating mutated DNA and the allele fractions of the same mutations in bone marrow plasma cells. There was a moderate positive correlation between the two ($r=0.507$, $n=34$, $P<0.002$) (Figure 1). Thus, the concentration of a mutation in serum reflects the fraction of tumor cells harboring the same mutation.

Relation between levels of recurrent mutations and M protein in serum

Eleven patients had sequential serum samples available, spanning a median of 50 months (range, 8-90). In these patients, we monitored the concentration of mutated DNA over time in relation to tumor mass and treatment response as evaluated by M protein concentration. All 11 patients had a MAP kinase pathway mutation and two had at least one additional mutation (Table 1, patients 1-11). Most of these mutations were highly present in the bone marrow at diagnosis, with 75-100% mutation-positive plasma cells by immunohistochemistry or >50% mutated allele fraction by WES. Slightly lower MAP kinase mutated allele fractions of 34% and 26% were found at diagnosis in patients 2 and 5, respectively, and patient 11 had 25-50% *BRAF*^{V600E}-mutated cells by

immunohistochemistry. No diagnostic bone marrow samples were available from patients 9 and 10.

The concentrations of MAP kinase mutations in serum showed marked covariation with M protein levels. For example, patient 1 (Figure 2A) was monitored by M protein as well as circulating *BRAF*^{V600E} mutation during 51 months, from diagnosis through five relapses until death. Every change in disease activity, as reflected by the M protein level, was accompanied by similar changes in serum *BRAF*^{V600E} mutation levels. Similar observations were made in ten of the 11 patients with available sequential samples (Figures 2 and 3 and *Online Supplementary Figure S4*). The observed covariation in ten patients was confirmed by a formal correlation analysis of 210 time-matched measurements of M protein and circulating MAP kinase pathway mutation with correlation coefficients ranging from 0.63 to 0.96 (*Online Supplementary Table S3*). Only in patient 10 (*Online Supplementary Figure S4B*) was there no correlation. In this patient the *BRAF*^{V600E} mutation became undetectable after being present at a very low concentration (<10 copies/mL) at an early time point.

An important aspect of ctDNA analysis in myeloma is its sensitivity, compared to conventional methods, to detect low levels of disease. When looking at the ability to predict relapse, we found that serum mutation levels tended to increase before or at the same time as M protein in most cases in which the two methods could be compared

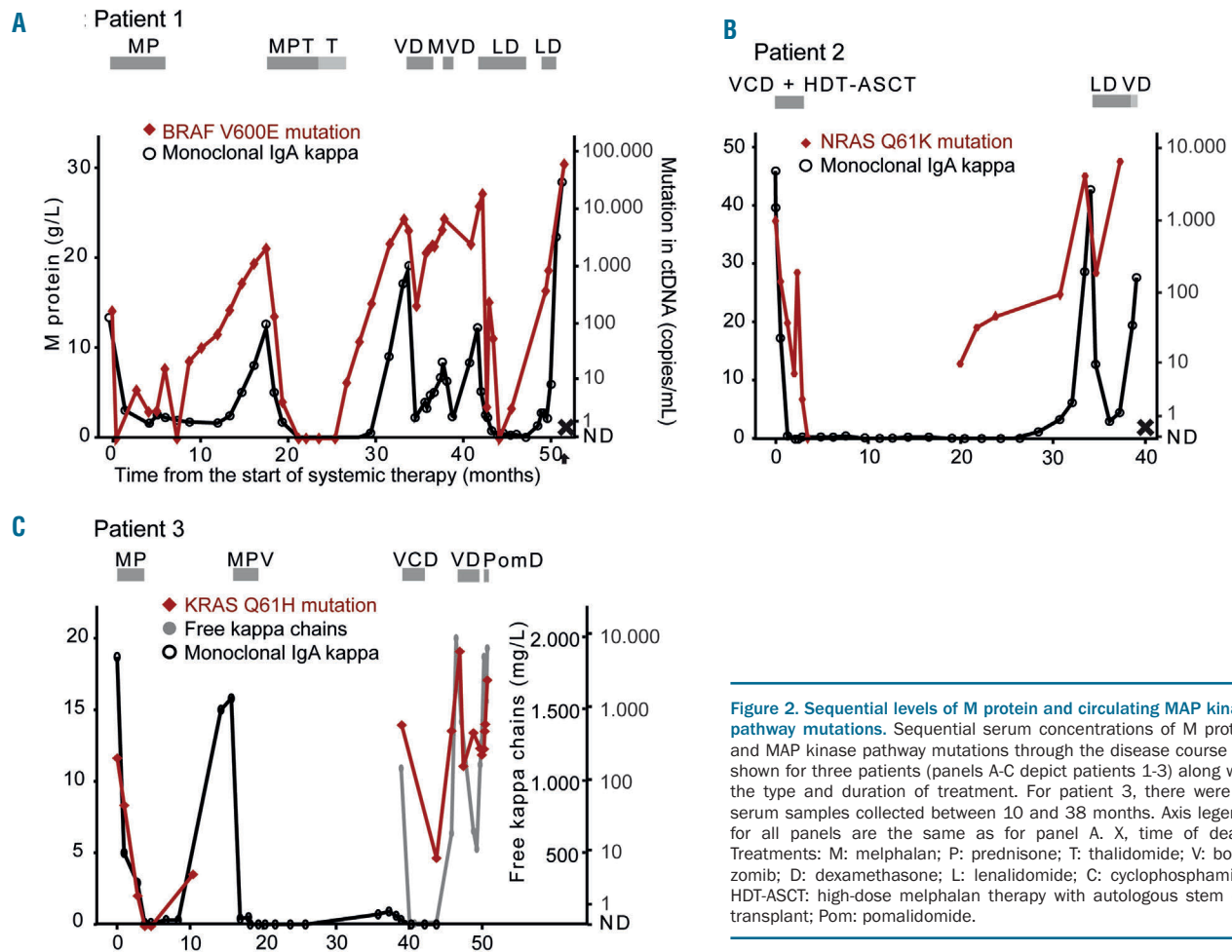


Figure 2. Sequential levels of M protein and circulating MAP kinase pathway mutations. Sequential serum concentrations of M protein and MAP kinase pathway mutations through the disease course are shown for three patients (panels A-C depict patients 1-3) along with the type and duration of treatment. For patient 3, there were no serum samples collected between 10 and 38 months. Axis legends for all panels are the same as for panel A. X, time of death. Treatments: M: melphalan; P: prednisone; T: thalidomide; V: bortezomib; D: dexamethasone; L: lenalidomide; C: cyclophosphamide; HDT-ASCT: high-dose melphalan therapy with autologous stem cell transplant; Pom: pomalidomide.

(Figures 2 and 3 and *Online Supplementary Figure S4*). Notably, a relapse from complete remission in patient 2 (Figure 2B) was detected by ctDNA 9 months before M protein became detectable. Relapses were also detected earlier by ctDNA in patient 1 (Figure 2A), although with somewhat shorter lead-times. On the other hand, in patient 11 (*Online Supplementary Figure S4F*) the second relapse was heralded by an increase in urine M protein 4 months before ctDNA became detectable. Furthermore, ctDNA often became undetectable during periods of remission even though low levels of M protein were still detectable, or the concentration of ctDNA would fluctuate around the limit of detection. In summary, ctDNA showed relapse earlier in two patients (3 and 9 months), later in one patient (4 months) and in three patients there was no difference.

In patient 3 (Figure 2C), we initially detected a *KRAS*^{Q61H} mutation in plasma cells by WES as well as by ddPCR of serum. Light chain escape occurred at the second relapse, 38 months after the start of treatment, when the tumor cell secretion converted from IgA- κ to κ chains only. Despite this change, we could monitor the disease by the serum concentration of *KRAS*^{Q61H} mutation.

In two patients, we monitored one or two recurrent mutations in addition to the MAP kinase pathway mutations (Figure 3A,B). Patient 4 (Figure 3A) had an *IRF4* mutation highly present in bone marrow plasma cells at

the last relapse. At diagnosis, this mutation was not detected in plasma cells by WES, but a few copies were found by ddPCR of plasma cells and serum. The concentration of the mutation in serum increased abruptly after initiation of therapy and covaried with M protein level for the rest of the disease course. Conversely, a *FAM46C* mutation present at a 60% allele fraction in plasma cells at diagnosis became undetectable in both serum and plasma cells during the disease course. In patient 5 (Figure 3B), the concentrations of M protein and *KRAS* and *TP53* mutations followed similar patterns in serum, despite a plasma cell allele fraction of only 4% for the *TP53* mutation at diagnosis.

Altogether, we monitored 14 mutated clones in 11 patients. Twelve of the mutations were detectable in serum at each relapse and covaried with M protein, whereas two mutations became undetectable during the disease course (*FAM46C*^{S279N} in patient 4 and *BRAF*^{V600E} in patient 10). These observations suggest that the serum concentration of recurrent mutations over long periods of time reflect the changes in total tumor mass in most myeloma patients.

Serum mutation levels in aggressive disease

In patients 1, 4, and 5 (Figure 2A, Figure 3 A,B) we noticed a marked increase in serum mutation levels in the terminal phase of the disease. At that time the patients

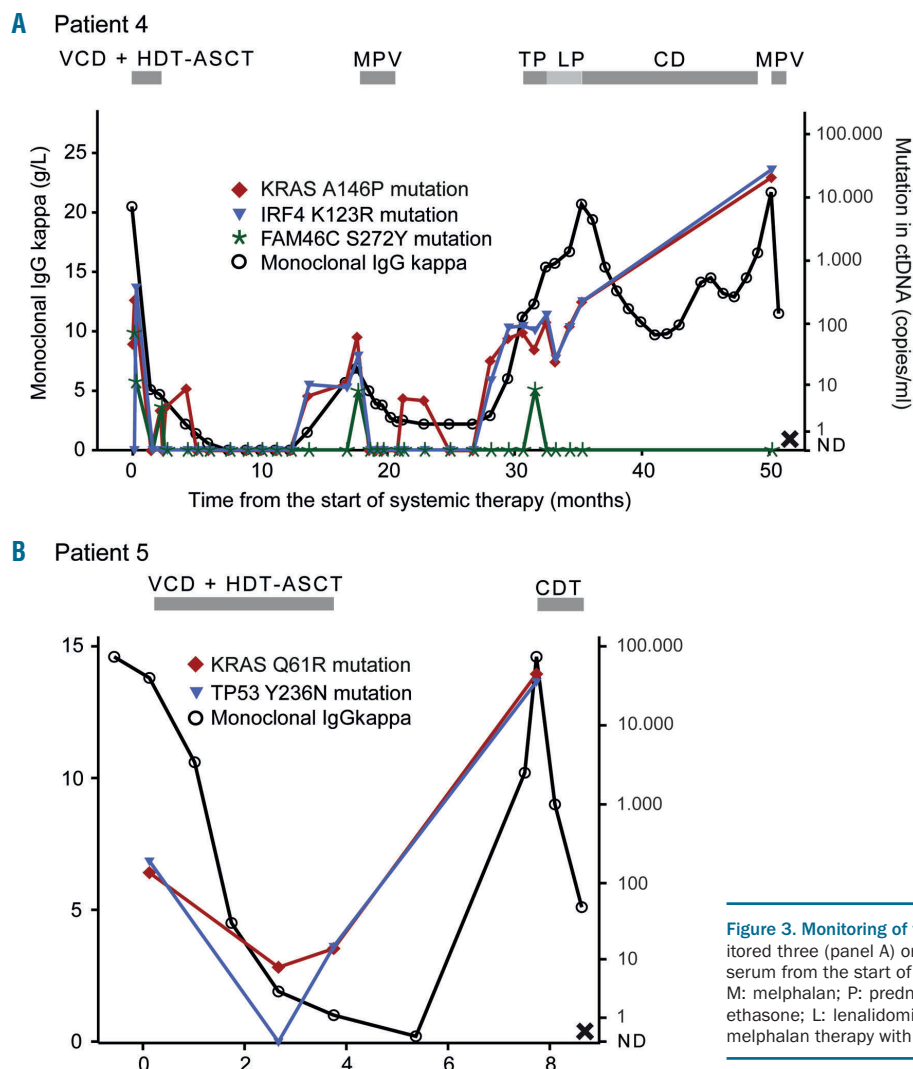


Figure 3. Monitoring of three or two mutations in serum. Here, we monitored three (panel A) or two (panel B) recurrent mutations by ddPCR of serum from the start of treatment until the terminal phase. Treatments: M: melphalan; P: prednisone; T: thalidomide; V: bortezomib; D: dexamethasone; L: lenalidomide; C: cyclophosphamide; HDT-ASCT: high-dose melphalan therapy with autologous stem cell transplant

had treatment refractory disease and remained alive only for a few weeks or months. To further analyze the dynamics of ctDNA over time, we compared the peak levels of mutations and M protein at each relapse (Figure 4). To facilitate the comparison between patients, we normalized the concentrations of M protein and the ctDNA as indicated in the legend to Figure 4. Only one mutation per patient is shown in Figure 4, however, in patients 4 and 5, mutations in *IRF4*, *TP53* and *KRAS* behaved in the same manner indicating that they were all characteristics of the same aggressive clone. The discrepancy between ctDNA and M protein in patients 1, 4 and 5 was particularly evident in the terminal phase when the ratio of ctDNA to M protein was up to 400-fold higher than at the start of treatment.

We analyzed several aspects of these patients which can contribute to the marked increase in serum concentrations of mutations. Patient 1 and 5 had plasma cells with immature morphology, whereas patients 1 and 4 had secondary plasma cell leukemia, with >20% plasma cells in blood. No plasma cells were found in blood from patient 5. In two of the patients there was >10% increase in mutated allele fraction in bone marrow plasma cells from the start of treatment to the time of terminal disease (34-49% in patient 2 and 26-52% in patient 5). Thus, several factors

may have contributed to the increased concentrations of ctDNA that were evident after transformation to a more aggressive disease.

Discussion

We studied the serum concentrations of recurrent mutations identified in bone marrow plasma cells from 20 patients with multiple myeloma. Our comprehensive series of samples covering the entire disease course from diagnosis to death of several patients provides a unique insight into the dynamics of ctDNA in relation to disease activity. The most striking findings were a marked covariation with the concentration of M protein, the gold standard biomarker to monitor tumor mass in multiple myeloma, and increasing concentrations of ctDNA relative to M protein as the disease became more aggressive.

To explain the increase of ctDNA, it is useful to discern between tumor mass and activity of the cells. The latter includes a number of functional aspects, such as proliferative rate and degree of adherence to the bone marrow environment. M protein is a typical tumor mass marker as long as the mechanisms of production and secretion of immunoglobulin are intact. As shown, ctDNA and M pro-

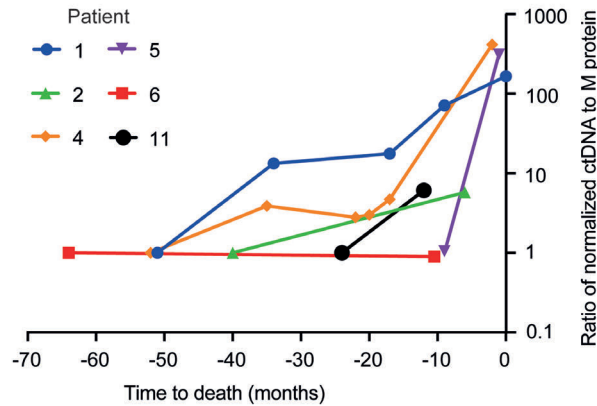


Figure 4. Ratio between serum levels of recurrent mutations and M protein during long-term follow-up. Diagnostic serum concentrations of M protein and MAP kinase mutations were normalized to one for each patient. Subsequent data are from the time points of peak M protein level at relapses, before a new treatment was started. The ratio of normalized mutation level to M protein was calculated for each data point. Patients were included in the figure if the diagnostic and at least one peak value of ctDNA and M protein were available. Patient 3 was excluded because of light chain escape, and patient 9 (Online Supplementary Figure S4A) was excluded because most peak values of ctDNA were too low to be confidently quantified.

tein seem to reflect tumor mass equivalently during long periods of the disease when the secretory mechanisms are operative and cellular functions relatively stable. This picture changes, however, when there is transformation to a more proliferative disease with high turnover of cells and perhaps a larger fraction of non-secretory cells. Furthermore, myeloma cells may be present in the circulation as shown in two of our patients. Although its clinical significance is unclear, ctDNA seems to reflect disease activity and progression differently from M protein.

Our serum samples were stored for up to 11 years before analysis. Despite reports of DNA degradation during protracted sample storage,²⁸ we found no statistically significant correlation between DNA yield and storage time, as shown in the methods section. Furthermore, it is recommended that ctDNA is analyzed in plasma rather than serum because of DNA released from leukocytes during sample preparation.²⁹ However, to our knowledge, serum and plasma have not been directly compared in a clinical setting, and previous studies have successfully used stored serum samples.^{30,31} The close covariation between ctDNA and M protein found in our study adds to the evidence that stored serum can provide meaningful results and is a valuable material for the study of ctDNA.

A weakness of this study was the low number of patients, limiting the generalizability of our results. Another weakness was the low and variable volume of serum available for analysis at each time-point, as reported in the methods section and elaborated in the Online Supplementary Methods. Because the ability of ddPCR to detect low levels of mutations is primarily limited by the sample volume and concentration of DNA, the sensitivity of our ctDNA measurements varied and was suboptimal in many samples. The potential to detect early relapse and minimal residual disease by ctDNA was, therefore, most likely under-estimated in our study.

Mithraprabhu *et al.* recently reported the detection and monitoring of ctDNA in myeloma patients.¹⁶ Their design

differed from ours as they sequenced DNA from plasma as well as bone marrow plasma cells, targeting recurrently mutated regions in the *NRAS*, *KRAS*, *BRAF* and *TP53* genes. Interestingly, they found 24% of mutations exclusively in plasma, consistent with the spatial heterogeneity of multiple myeloma previously demonstrated by multi-region DNA sequencing of bone marrow plasma cells.^{32,33} They also monitored specific mutations by ddPCR in three to six sequential samples from seven patients¹⁶ and our results are essentially in agreement with their observations.

There are also apparent discrepancies between the studies. We detected 97% of mutations in serum when they had been identified in a time-matched bone marrow sample, whereas the corresponding number was only 39% (38/97) in the study by Mithraprabhu *et al.*¹⁶ This may be explained by the high sensitivity of their procedure as the majority of mutations they detected in bone marrow plasma cells had a mutated allele fraction between 0.01 and 1%. In comparison, the limit of detection by WES of bone marrow plasma cells in our study was 2–4 % mutated allele fraction, which is in line with previous studies using WES.^{17,18}

There are several potential applications of ctDNA in multiple myeloma. The mechanisms by which M protein and ctDNA are released into the bloodstream appear to be independent of each other. Thus, monitoring the disease using ctDNA may be possible in situations in which M protein is not a reliable biomarker, such as in light chain escape and non-secretory or oligo-secretory disease.^{6,7,16} Furthermore, non-invasive detection of specific mutations may be useful to guide the use of targeted drugs such as BRAF or MEK inhibitors in patients with *BRAF*, *NRAS* or *KRAS* mutations.^{22–24}

In principle, any tumor-specific DNA sequence such as a somatic mutation or a translocation breakpoint could be monitored by ddPCR.^{12,34,35} Alternatively, targeted sequencing may be applied directly to plasma or serum DNA to detect several targets simultaneously.^{13,30,36} This approach has the potential to describe tumor clonal evolution over time and its relation to clinical phenomena such as drug resistance^{37,38} and may be preferred in many situations. The choice of method will depend on the purpose.

Altogether, this study provides detailed insight into the development of ctDNA levels over long periods of time in a limited number of patients. Circulating tumor DNA appears to be a multi-faceted biomarker of mutated cells, total tumor mass and transformation to a more aggressive disease in patients with multiple myeloma. However, several important questions remain unanswered, including the potential of ctDNA in minimal residual disease assessment and early detection of relapse.

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