High throughput flow cytometry-based drug sensitivity testing for melflufen in multiple myeloma

Master´s Thesis
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Abstract

Multiple myeloma (MM) is a hematological plasma cell malignancy in the bone marrow. Lately, increased knowledge of MM pathogenesis and advances in therapy have improved the survival of MM patients. However, due to the unique and complex genome of each patient, some patients are resistant to standard therapies while others achieve durable response but eventually experience relapse. Therefore, new strategies especially for relapsed and refractory and high-risk multiple myeloma (RRMM, HRMM) patients, who have poor response to current therapies, are required. Melflufen, a novel prodrug of the alkylating agent melphalan, has shown significantly decreased resistance effects and more selective cytotoxicity compared to melphalan *in vitro* and *in vivo*, but the molecular markers identifying the sensitive subgroups of MM patients have not yet been discovered. The aim of this study was to identify a melflufen-sensitive subgroup of MM patients by utilizing a high throughput flow cytometry-based drug sensitivity testing platform. For the assessment, mononuclear cells from bone marrow of MM patients were derived and melflufen sensitivity of different cell populations tested. As a result, malignant plasma cells were significantly more sensitive to melflufen than to melphalan. Importantly, all MM patient samples including RRMM and HRMM patients were sensitive to melflufen although to varying degrees suggesting these patients may benefit from melflufen treatment. Further integration of the results with additional molecular information may lead to discovery of new biomarkers and thus the prediction of melflufen responses can result in more effective disease management and save patients from ineffective therapies.

Keywords*:* multiple myeloma, melflufen, personalized medicine

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1 Introduction

1.1 Multiple myeloma

Multiple myeloma (MM) is a heterogenous and hematological plasma cell malignancy. Plasma cells (PCs) are type of blood cells derived from the lymphoid B-cell lineage during hematopoiesis which is defined as the formation of blood cell components (Jagannathan-Bogdan and Zon, 2013). These components originate from self-renewing hematopoietic stem cells, which are differentiated to either lymphoid progenitor cells, lymphoblasts, lymphocytes and plasma cells (Fig. 1), or to myeloid progenitor cells, granulocytes, erythrocytes, megakaryocytes and macrophages. During development, B cells mature in the lymph organs, where they undergo somatic hypermutation. They eventually return to the bone marrow (BM) as terminally differentiated PCs and produce and secrete antibodies essential for normal function of the immune system. However, in MM, malignant monoclonal PCs replace normal PCs in the BM, resulting in overabundance of clonal antibodies. Also, the number of normal blood cells and functional antibodies are decreased.

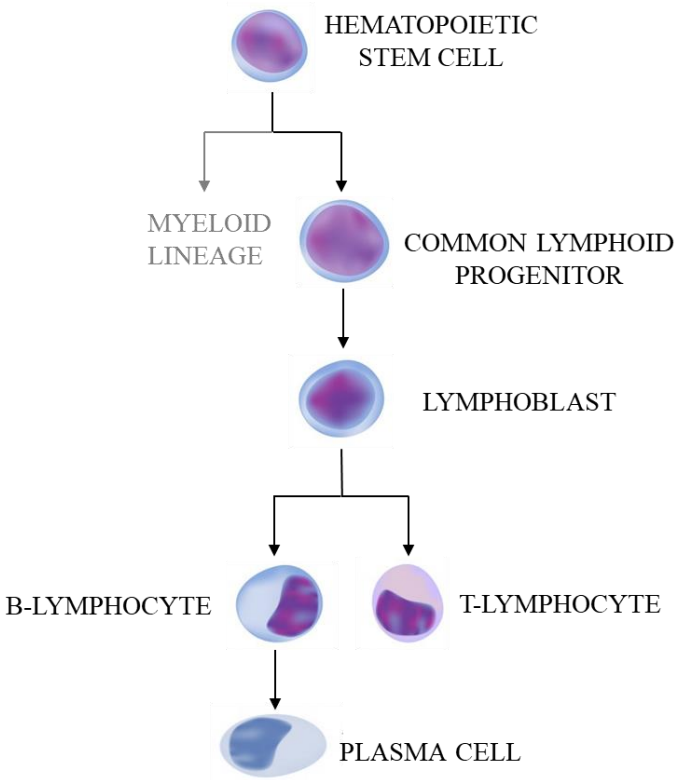

Figure 1. The plasma cells are originated from lymphoid B-cell linage. Blood cells are derived from hematopoietic stem cells either by lymphoid or myeloid lineage. During hematopoiesis of the lymphoid linage, hematopoietic stem cells proliferate to lymphoid progenitor cells, lymphoblasts, lymphocytes and finally to plasma cells.

1.1.1 Incidence, Disease Progression and Risk Factors

MM accounts for 1% of all cancers and 10–15% of hematologic malignancies, and is the second most common hematologic cancer worldwide (Rajkumar, 2018). In the United States, over 30 000 new cases and over 12 000 deaths of MM are reported each year (Siegel *et al.,* 2015), while the estimated 5-year prevalence is about 230 000 patients worldwide (Kazandjian, 2016). Furthermore, median age of patients diagnosed with MM is approximately 65 years, and the ratio between male and female patients is 1.4:1 indicating that the disease is slightly more common in males than females (Kyle *et al.,* 2003).

MM begins often as monoclonal gammopathy of undetermined significance (MGUS), which progresses to smoldering multiple myeloma (SMM) and finally develops to MM. MGUS is usually asymptomatic and has been present in the patients for more than 10 years prior to the diagnosis (Therneau *et al.,* 2012). It progresses to MM at a rate of 1% per year (Kyle *et al.,* 2002). In contrast, SMM patients usually do not have any symptoms, but they have paraprotein in the blood and clonal PCs in the BM (10–60%) without any organ or tissue damage. During the first five years after diagnosis, SMM progresses to MM at a rate of 10% per year, over the next five years 3% per year and thereafter 1.5% per year (Rajkumar, 2018). Lastly, MM can develop into plasma cell leukemia in which clonal PCs disseminate from the BM, are independent of the BM microenvironment and can survive in the blood (Gundesen *et al.,* 2019).

Although risk factors of MM are poorly understood, there are some factors that are observed to increase the risk of developing MM in addition to age and male sex. These are MGUS and SMM, a family history of MGUS, SMM, MM or other B-cell malignancy (Brigle and Rogers, 2017), and environmental or occupational exposure to ionizing radiation, asbestos, industrial chemicals, benzene and pesticides (Eriksson and Karlsson, 1992). Also, long-lasting exposure to antigens used as a treatment for example in chronic infections and rheumatoid arthritis can predispose to MM.

1.1.2 Clinical Features and Diagnosis

The imbalance of blood cells causes the common symptoms of MM such as a weakened immune system and continuous infections, anemia and tiredness, as well as abnormal bruising and bleeding (Kyle and Rajkumar, 2008). Also, skeletal fractures and bone pain are quite common accounting for 58% of cases at diagnosis (Brigle and Rogers, 2017),

which result in calcium release into the bloodstream and therefore hypercalcemia (Fairfield *et al.,* 2016). Additionally, fatigue, weight loss and renal dysfunction are often observed. However, when MM is diagnosed, nonspecific symptoms for an extended period of time are usually presented, which challenges diagnosis.

The criteria for MM diagnosis are recommended by the International Myeloma Working Group (IMWG) and World Health Organization (WHO) (Rajkumar *et al.,* 2014). The diagnosis requires either plasmacytoma proved by biopsy or examination of clonal PCs on BM, in addition to one of more myeloma defining events (MDE). MDE consists of CRAB (hyper**c**alcemia, **r**enal failure, **a**nemia, lytic **b**one lesions) and three specific biomarkers: 1) clonal BM PCs ≥60%; 2) serum free light chain ratio ≥100; and 3) at least 5mm diameter focal lesion(s) on magnetic resonance imaging (MRI).

1.1.3 Molecular Classification, Genetic Complexity and Risk Stratification

Antibodies, also known as immunoglobulins, consist of two immunoglobulin heavy (IgH) and two light chains (IgL). In MM, the most common abnormal chains are IgG and IgA heavy chains. There is also possibility that only light chains are produced, however in these cases, the prognosis of patients is poorer and the frequency of additional disorders such as renal failure and amyloidosis is higher compared to patients with IgG and IgA subtypes (Zhang *et al.* 2014). In addition, the differences of IgL subtypes (kappa and lambda) and their influence on MM outcome and progression have not yet been studied in more detail (Zhang *et al.* 2014).

The genome of the myeloma cell has multiple structural alterations and mutations in several oncogenes and tumor suppressor genes and thus displays genomic instability. Primary cytogenetic abnormalities can be divided into two subgroups based on fluorescent in situ hybridization (FISH) of BM and the karyotype of the patients (Bergsagel and Chesi, 2013). 40–50% of patients have hyperdiploid karyotype which is characterized by odd-numbered chromosomes including trisomies in chromosomes 3, 5, 7, 9, 11, 15, 19 and 21. In contrast, hypodiploid karyotype is characterized by frequent translocations in the IgH and the loss of chromosomes 13, 14, 16, and 22. Importantly, hyperdiploid karyotype has a better prognosis than hypodiploid karyotype.

Secondary cytogenetic abnormalities occur during the MM progression and often cause the invasion of myeloma cells from the BM. These changes include *RAS* mutations, *MYC* translocations and aberrations such as del(17p) which is the locus of *TP53* gene, gain(1q)

locus of *MCL1*, *CKS1B*, *ANP32E* and *BCL9* genes, and del(13q) the locus of *RB1*, *DIS3*, *mir15a* and *mir16.1* genes, which all are related to cancer development and progression (Castaneda and Baz, 2019). Since chromosomal translocations are already observed in MGUS and SMM stages, there is evidence that aberrant DNA repair is involved in disease progression (Castaneda and Baz, 2019). Additionally, at the later stages of MM, signaling pathways are disturbed, the BM microenvironment becomes more favorable to malignant PCs and begins to nurture and support the proliferation of these cells, and epigenetic changes, such as DNA methylation and micro-RNA changes, are observed (Rajkumar, 2018). The primary and secondary genomic events in MM are presented in Table 1.

Table 1. The primary and secondary genomic events in multiple myeloma (MM) with the frequency and prognostic value. Adapted from "*Multiple Myeloma Genomics - A Concise Review"* by Castaneda and Baz, 2019.

Genomic event	Abnormality	Frequency in MM (%)	Prognostic value
Primary events			
Trisomies	Odd-numbered chromosomes	~45	Favorable (trisomy 21 may have worse outcome)
Translocations	t(11;14)	15	Neutral
	t(4;14)	15	Adverse
	t(6;14)	2	Neutral
	t(14;16)	5	Adverse
	t(14;20)	1	Adverse
Secondary events			
Chromosome gains	1q	40	Adverse
	8q	15	Neutral
	11q	15	Neutral
Chromosome losses	1p	30	Adverse
	12p	15	Adverse
	14q	10	Not determined
	16q	30	Neutral
	17p	10	Adverse
	13q	40	Neutral
Translocations	t(8;14), t(8;11)	15	Adverse

The International Staging System (ISS) divides MM into three stages based on serum β2-macroglobulin (β2-M) and albumin measurements. Since the staging system does not take into account karyotype information, which is the most important prognostic factor in MM, the Revised International Staging System (RISS) was created by the IMWG to combine elements of both tumor burden and disease biology (Rajkumar *et al.,* 2014). In stage 1 (low-risk), serum albumin is more than 3.5 gm/dl, β2-M is less than 3.5 mg/l, there is no high-risk cytogenetics, and serum lactate dehydrogenase level is normal. In contrast, in

the stage 3 (high-risk), serum β2-M is more than 5.5 mg/l and it has high-risk cytogenetics – t(4;14), t(14;16), or del(17p) – or elevated serum lactate dehydrogenase level. Stage 2 (standard-risk) does not fit in neither stage 1 nor 3. Although any cytogenetic abnormality is considered as high-risk MM (HRMM), some of the abnormalities are considered as poor-risk markers. For example, the 8-year survival rate of patients with del(17p) has been identified as 52%, t(4;14) translocation 33%, and gain(1q) abnormality 36% indicating the worst prognosis among the cytogenetic aberrations (Majumder *et al.,* 2017).

1.1.4 Current Treatment

The pipeline for treating MM is one of the most diverse in oncology. Treatment options for MM include alkylators (melphalan, doxorubicin), steroids (dexamethasone, prednisone), immunomodulators (thalidomide, lenalidomide, pomalidomide), proteasome inhibitors (bortezomib, carfilzomib), histone deacetylase (HDAC) inhibitors (panobinostat), monoclonal antibodies (daratumumab, elotuzumab) and nuclear export inhibitors (selinexor), which are used individually or in combination. The choice of treatment depends on the presence or absence of cytogenetic features and the progression stage (initial treatment, maintenance treatment, relapse treatment). Moreover, one of the main problems of MM treatment is adverse effects such as secondary leukemia caused by chemotherapy of used drugs. Hence, only MM with symptoms is treated.

If a patient with low or standard-risk cytogenetic aberrations is eligible, initial treatment is usually started with stem cell transplant in combination with multi-drug therapy such as bortezomib-containing regimens or lenalidomide with dexamethasone (Mateos and San Miguel, 2017). For most patients, lenalidomide is the standard drug for maintenance therapy after the transplant. For high-risk patients, carfilzomib-lenalidomide-dexamethasone combination is recommended as initial therapy and proteasome inhibitor-based regimens as maintenance therapy.

Unfortunately, stem cell transplantation is an intensive treatment method meaning that it is valid for only a small number of patients such as young patients. Therefore, older patients are often treated with less intensive methods. Initial therapy is preferred to be bortezomib-lenalidomide-dexamethasone, followed by lenalidomide maintenance in low and standard-risk patients, and bortezomib maintenance in high-risk patients. In frail elderly patients, initial therapy is often started with lenalidomide-dexamethasone and

followed until progression. If patient is not eligible for lenalidomide treatment, melphalan-based regimens are also recommended (Rajkumar, 2018).

Although some patients respond well to treatment and reach stable disease stage, eventually almost all patients will develop resistance to therapy and experience relapse. The duration of remission decreases with each regimen (Kumar *et al.,* 2004). Many factors, such as the timing of the relapse, response to prior therapy, aggressiveness of the relapse, and performance status (TRAP) affects the choice of therapy. A triplet regimen, of which at least two drugs are new and patient is not refractory to, especially proteasome inhibitors and monoclonal antibodies, are highly used (Rajkumar, 2018). When treating relapsed and refractory MM (RRMM) patients, the focus is in palliative treatment and providing as good quality of life (QoL) as possible (Sonneveld and Broijl, 2016).

1.2 Melphalan and melflufen

Melphalan is a classical alkylating chemotherapeutic drug developed in 1953 for various malignancies such as ovarian and breast cancers, lymphomas, leukemia and MM (Bayraktar *et al.,* 2013). It has increased the lifespan of patients for a several years (Trippoli *et al.,* 1998) and it has been used in clinics mainly as combination chemotherapy (Wickström *et al.,* 2008). Therefore, melphalan has proved its important role in MM therapy. However, especially with higher doses, it has cytotoxic and drug-resistant effects (Chauhan *et al.,* 2013), and thus some novel biological drugs have replaced it. Hence, the melphalan prodrug melphalan flufenamide ethyl ester (melflufen, previously denoted as J1) (Fig. 2), was developed by Oncopeptides AB to achieve more targeted efficacy and hence more specific responses and less adverse effects.

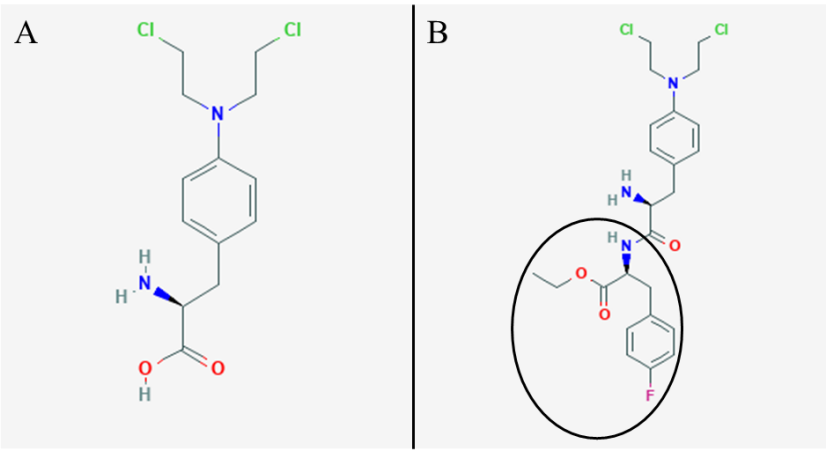

Figure 2. The chemical structure of melphalan and its prodrug melflufen. The difference between structures of (A) melphalan and (B) melflufen is circled. Structures are from PubChem, accessed Aug 16, 2019, from https://pubchem.ncbi.nlm.nih.gov/.

Melflufen, a lipophilic peptide-conjugated alkylator, has a novel mechanism of action in MM (Fig. 3). Specific aminopeptidases, which are important for normal cellular function, are highly expressed in several malignant cells and are associated with tumor cell invasion, differentiation and proliferation (Wickström *et al.,* 2011). Melflufen is potentiated by these aminopeptidases and due to its high lipophilicity (logP 4.04) it is able to rapidly penetrate membranes of malignant PCs (Wickström *et al.,* 2017). Immediate enzymatic cleavage of the peptide bond of melflufen by specific peptidases causes high intracellular concentrations of melphalan inducing extensive DNA damage and eventually apoptosis (Wickström *et al.,* 2017).

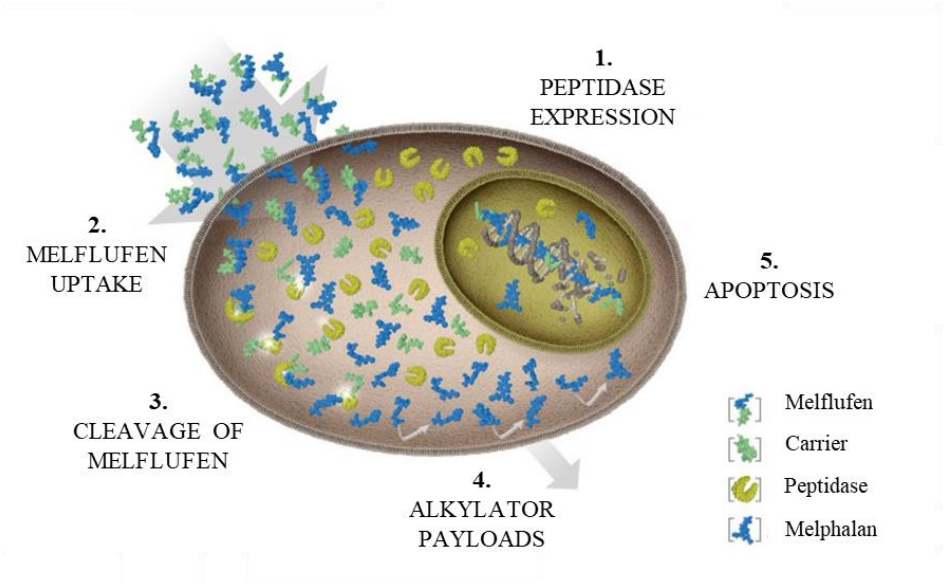

Figure 3. The mechanism of action of melflufen. 1. Specific peptidases are expressed in MM cells. 2. Due to its high lipophilicity, melflufen is rapidly taken up by malignant plasma cells. 3. Inside the cells, melflufen is immediately cleaved by the specific peptidases. 4. The melphalan payloads are entrapped. 5. Melflufen induces DNA damage leading to apoptosis of malignant plasma cells. Adapted from "Mechanism of Action", accessed Aug 29, 2019, from https://www.oncopeptides.se/en/mechanism-of-action/

The potential use of melflufen has been studied for several malignancies including hematological cancers, such as lymphoma (Delforoush *et al.,* 2016) and leukemia (Strese et *al.,* 2017), as well as solid malignancies (Berglund *et al.,* 2015), such as urothelial carcinoma (Viktorsson *et al.,* 2016) and ovarian cancer (Carlier *et al.,* 2016). It has been shown that despite identical alkylating capacity, melflufen has significantly higher activity, more selective cytotoxicity and decreased resistance effects *in vitro* compared to melphalan (Ray *et al.,* 2016; Wickström *et al.,* 2010; Wickström *et al.,* 2017). Furthermore, melflufen has 67-fold lower IC50 values than melphalan in hematological malignancies *ex vivo* (Wickström *et al.,* 2008).

In addition, melflufen has been tested *in vivo* in rodents with conventional xenograft models and hollow fiber models (Chauhan *et al*., 2013; Chesi *et al.,* 2012; Gullbo *et al.,* 2004). For example, Chauhan *et al.* investigated a significant delay in tumor growth and prolongation of survival in SCID mice xenografted with myeloma cells treated with melflufen (Chauhan *et al.,* 2013). Despite an expected short half-life in the blood circulation (1 h), melflufen does not exhibit decreased activity when compared to melphalan (Gullbo *et al.,* 2003). Recently, melflufen has also shown promising results for late-stage MM and hence it is currently in phase III clinical trials for RRMM ("NIH Clinical Trial OCEAN", study identifier NCT03151811).

1.3 Precision and personalized medicine

A single drug can be either therapeutically effective, toxic or non-responsive among a large group of patients. Therefore, a precision and personalized medicine approach – to get the right drugs to the right patients at the right time – has been utilized especially in the field of oncology. For example, trastuzumab for HER2 positive breast cancer, panitumumab for KRAS positive colorectal cancer and niraparib for BRCA1 and 2 positive ovarian cancer have been developed and approved by United States Food and Drug Administration (FDA) (Saadeh, Bright and Rustem, 2019). Moreover, individualized medicine approaches are also seen among novel RRMM therapies including panobinostat targeting HDACs (Maiso *et al.,* 2006) and venetoclax targeting BCL-2 (Kumar *et al.,* 2017).

Precision and personalized medicine is based on genomic characterisation of normal and cancer genomes in individual patients and guidance of targeted treatments. For robust utilization, patients should be stratified based on these cytogenetic markers for better prediction of drug responses and disease progression. Consequently, MM patients should be stratified based on individual cytogenetic groups rather than heterogeneous risk categories (Rajkumar, 2018), and be identified prior the initial therapy for predicting treatment responses in each patient and for achieving the maximal response and benefit. It can also minimize adverse events and costs of treatments. However, one of the main challenges of personalized medicine is the dependence of available biomarkers which are needed for the prediction of safety and efficacy of novel drugs.

1.4 High throughput flow cytometry-based drug sensitivity testing

Flow cytometry is a technique used for identifying different cell populations from cell suspension. Microscopic particles such as cells and chromosomes flow individually through laser light beam where their scattering and possible fluorescence emitting can be detected. The scattering can be measured in two different angles: 1) forward scatter (FSC) which is comparable to cell size, and 2) side scatter (SSC) which is relative to the granularity or internal complexity of the cells. Additionally, fluorescence dyes, usually fluorophores conjugated into antibodies, can be utilized at different wavelengths. Cells are usually labelled with several different fluorophores for detecting different cell populations.

Immunophenotyping is the clinical application of flow cytometry used for defining differentiation linage and stage of cells based on the antigens expressed on the surface of the cells. In MM, immunophenotyping can be used for 1) differential diagnosis from other hematological diseases and lymphomas, 2) predicting the development of MGUS and SMM into MM, 3) defining different subtypes, and 4) defining the remission stage when monitoring the treatment responses (Jelinek *et al.,* 2017). By using immunophenotyping and cell surface molecules (cluster of differentiation, CD), malignant PCs can be separated from the other cell populations by using CD138 and CD38 markers. The most widely used CD markers for separating healthy and malignant PCs are CD19, CD20, CD27, CD28, CD33, CD45, CD54, CD56, CD81 CD117, CD200 and CD307 (Flores-Montero *et al.,* 2016; Jelinek *et al.,* 2017). For example, 90% of malignant PCs are CD19-, 99% CD45-/CD45low and 70% CD56+ (Kuehl and Bergsagel, 2012; Pérez-Persona *et al*., 2007). By contrast, healthy PCs usually express CD19 and CD45 markers while lacking CD56. However, there is an ongoing debate regarding the expressed markers of malignant PCs, and therefore what are the proper CD markers for identifying malignant PCs from healthy ones.

To utilize this cell population separation, a high throughput, multiparametric, flow cytometry-based drug sensitivity and resistance testing (DSRT) platform has been set up and performed previously (Javarappa *et al.,* 2018; Kuusanmäki *et al.,* 2019; Majumder *et al.,* 2019). It is based on detecting the responses of drugs in specific cell populations including malignant cells and other healthy cell populations. The platform can be used for validating the most effective therapy options for individual patients by identifying molecular signaling pathways and genetic markers of drug responses (Saarela *et al.,*

2014). Importantly, inefficient therapies can be avoided, new combinational therapy possibilities provided, and drug sensitivities linked to the predictive biomarkers.

1.5 The aim of the study

Despite the increased knowledge of the MM genome and improved survival of MM patients due to advances in therapy, there are still unmet medical needs with the treatment of MM as mentioned above. First, due to the unique and complex genome of each patient, some patients are resistant to standard therapies while others initially achieve long, durable response. Second, almost every patient will experience relapsed stage and eventually become resistant to standard therapies. Third, adverse effects, such as development of secondary leukemia remain a major problem. Therefore, new strategies and personalized medicine approaches for MM treatment, especially for HRMM and RRMM patients, who have poor responses to few available treatment options and who have the worst prognosis among the MM patients, are required. Thus, the first aim of this study was to show that melflufen is more effective drug than melphalan *ex vivo* using a multiparametric, flow cytometry-based DSRT platform. In addition, other drugs were also tested but the results were not included in this thesis. The second aim was to identify melflufen-sensitive subgroup(s) of MM patients. Furthermore, the overall goal is to unveil MM specific mutations or gene expression patterns by integrating our results with molecular profiling for new biomarker discovery in the future. The workflow of the study is presented in Figure 4.

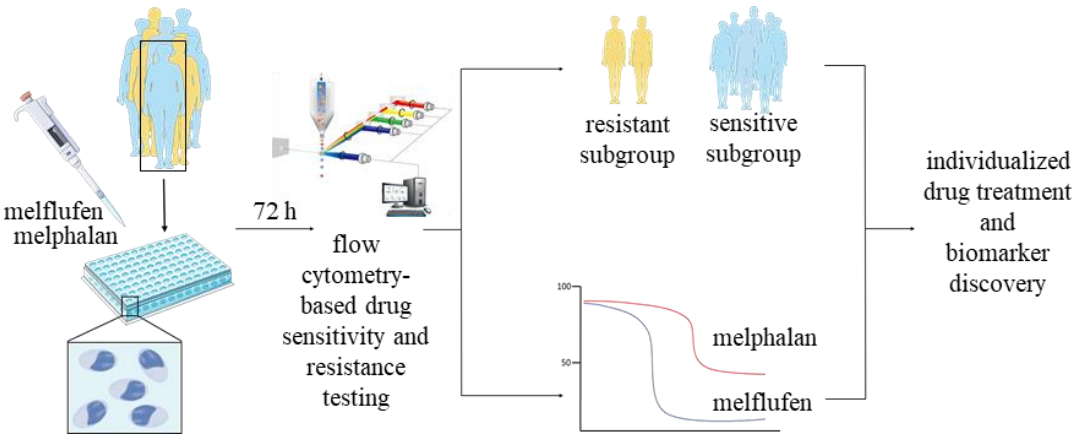

Figure 4. The workflow of the study. Briefly, the sensitivity of mononuclear cells isolated from bone marrow of individual MM patients were tested to melflufen and melphalan in seven concentrations. On day three, fluorescently labelled antibodies specific for different cell surface and viability markers were added to the cells for detecting plasma cells and other healthy cell populations, and read with a high-throughput flow cytometer. After data analysis, the objective was to observe whether melflufen is more effective than melphalan and stratify patients into melflufen-sensitive subgroup(s) based on clinical characteristics and cytogenetic factors. Furthermore, the goal is to integrate the results with molecular profiling for new biomarker discovery and individualized drug treatment in the future.

2 Results

2.1 Selected samples and clinical characteristics of patients

On day three, 16 chosen samples had reasonable cell viability (mean 50.70%, SD 0.14%) and number of CD138+/CD38+ (malignant PCs) cells in control dimethyl sulfoxide (DMSO) wells (mean 1236.3 cells, SD 1713.0 cells) (Appendix 1), which is 10.7% (SD 13.8%) from all live cells. The 16 samples came from 15 MM patients from which two samples were from the same patient at diagnosis and relapse stages.

Clinical characteristics of the patients are presented in Table 2 (for detailed characteristics of each patient, see Appendix 2). The average age of the patients at diagnosis was 64 years (SD 11.8 years), 7 (47%) were younger than 65 years and 8 (53%) older than 65 years. 7 (44%) were from newly diagnosed and 9 (56%) RRMM patients, and 6 (40%) were female and 9 (60%) male. 4 (25%) patients had not been previously treated, while 7 (44%) and 5 (31%) had received 1-2 or more than 3 lines of treatment, respectively. Based on ISS, 2 (13%) patients were scored with 1, 5 (33%) with 2 and 4 (27%) with 3. 3 (20%) patients had IgA and 6 (40%) IgG heavy chain, while 9 (60%) had kappa and 3 (20%) lambda light chain. 3 (20%) patients had only light chain without heavy chains. 8 (53%) of the patients had high-risk cytogenetic markers del(17p) and/or t(4;14). The majority of patients, 11 (73%) and 13 (87%), also had common MM cytogenetic aberrations del(13q) and gain(1q), respectively. ISS, IgH and IgL data was not available (NA) for all the patients (for 4 (27%), 6 (40%) and 3 (20%) patients, respectively).

Table 2. Clinical characteristics of patients and drug sensitivity scores (DSSs) after melflufen treatment. Patients were stratified based on age at diagnosis, disease status, gender, treatment line, international staging system (ISS), immunoglobulin heavy and light chains, and presence of del(13q), gain(1q) and high-risk (del(17p) and/or t(4;14)) aberrations. RRMM=relapsed and refractory multiple myeloma, NA=not available, IgL=immunoglobulin light chain.

Characteristic	Distribution of patients	n (%)	Mean DSS (SD or Q1, Q3)	*p*
Age at diagnosis (years)	<65	7 (46.7)	38.19 (6.07)	0.6387
	≥65	8 (53.3)	36.32 (8.73)	
Disease status	Diagnosis	7 (43.75)	34.34 (8.08)	0.1987
	RRMM	9 (56.25)	39.31 (6.67)	
Gender	Female	6 (40)	37.23 (8.03)	0.9700
	Male	9 (60)	37.08 (7.62)	

Treatment line	0 1-2 ≥3	4 (25) 7 (43.75) 5 (31.25)	34.25 (31.10, 37.30) 35.60 (30.60, 45.30) 44.00 (42.70, 44.00)	0.4113
ISS	1 2 3 NA	2 (13.3) 5 (33.3) 4 (26.7) 4 (26.7)	38.25 (33.80, 42.70) 38.75 (26.85, 44.65) 31.20 (27.90, 38.85)	0.8606
Immunoglobulin heavy chain	IgA IgG NA	3 (20) 6 (40) 6 (40)	35.90 (10.87) 37.62 (4.84)	0.7380
Immunoglobulin light chain	Kappa Lambda NA	9 (60) 3 (20) 3 (20)	34.64 (7.62) 41.53 (3.21)	0.1644
Presence of immunoglobulins	IgA IgG IgL only NA	3 (20) 6 (40) 3 (20) 3 (20)	35.90 (10.87) 37.62 (4.84) 33.90 (SD 8.75	0. 8016
del(13q)	Yes No	11 (73.3) 4 (26.7)	36.12 (7.78) 40.20 (6.54)	0.3638
gain(1q)	Yes No	13 (86.7) 2 (13.3)	38.84 (6.88) 29.77 (6.17)	0.3638
High-risk cytogenetic markers	Both del(17p) alone t(4;14) alone None	1 (6.7) 3 (20) 4 (26.6) 7 (46.7)	8 (53.3) 26.85 (23.30, 30.40) 42.70 (33.80, 45.30) 37.30 (33.65, 42.65) 39.60 (31.80, 44.00)	0.2319

2.2 Drug responses in malignant plasma cells

Dose responses of melflufen and melphalan in malignant PCs among all 16 samples (Fig. 5A) and in each sample individually (Fig. 5B) showed that melflufen was more potent drug than melphalan. Mean drug sensitivity score (DSS, for definition see section 4.4.3) of melflufen was 37.14 (SD 7.51) and of melphalan 15.47 (SD 4.76) in malignant PCs (Appendix 3), and DSSs of melflufen were significantly higher compared to DSSs of

melphalan (p<0.0001) (Fig. 5C). Also, Pearson correlation showed that responses of melflufen and melphalan were not significantly associated (r=0.22, p=0.41) (Fig. 5D). Since all samples had high DSS and thus seemed to be sensitive to melflufen, the responses in malignant PCs were stratified into more (<50nM) and less (>50nM) sensitive subgroups (Appendix 4A) rather than sensitive and resistant groups. However, when DSSs of melflufen in these subgroups were compared, there was no statistically significant difference (p=0.1599) (Appendix 4B).

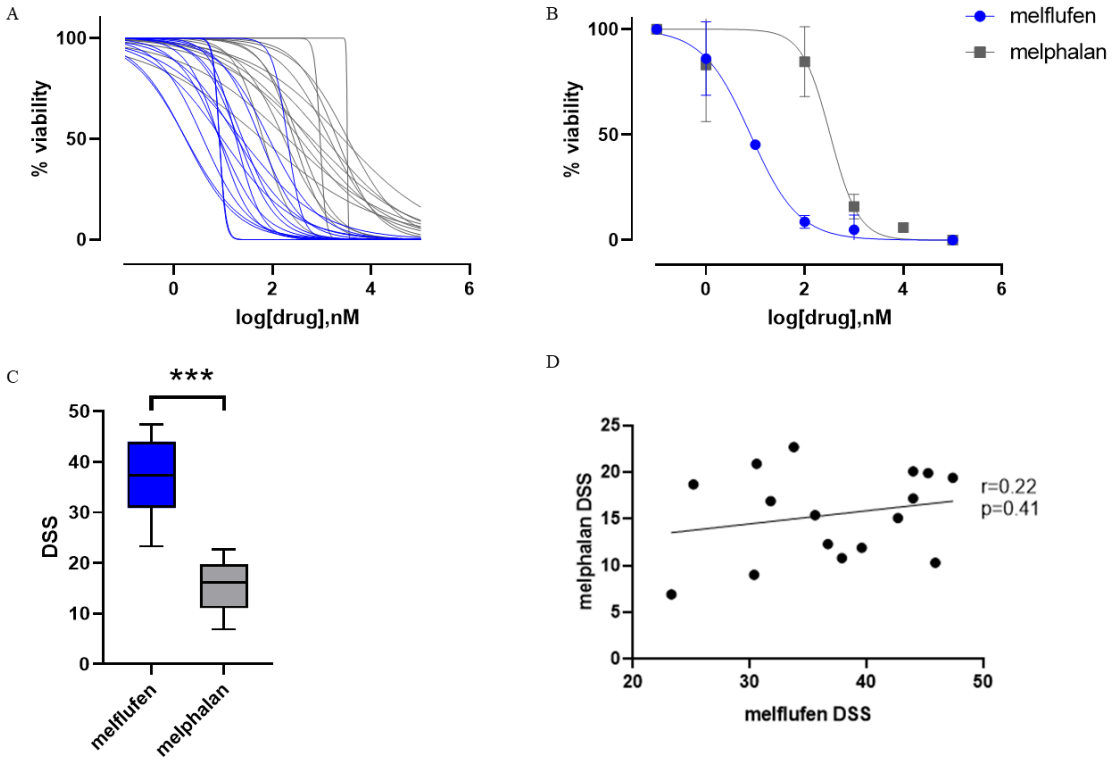

Figure 5. The responses of melflufen and melphalan in malignant plasma cells. The dose-response curves of melflufen and melphalan (A) from all 16 MM patients, and (B) from individual patients showed that melflufen was more potent drug than melphalan. (C) When drug sensitivity scores (DSSs) of drugs in malignant plasma cells were compared, DSSs of melflufen were significantly higher than DSSs of melphalan (p<0.0001). (D) Pearson correlation showed no significant association between DSS of melflufen and melphalan (r=0.22, p=0.41). Blue=melflufen, grey=melphalan. DSS values are presented as means in Fig. 5C.

2.3 Identification of melflufen-subgroups

Although all samples seemed to be sensitive to melflufen, to assess whether clinical characteristics of MM patients affected the responses of melflufen, patients were stratified into different subgroups (Table 2). First, to observe whether disease status and the number of received treatment lines had influence on melflufen responses, patients were stratified into diagnosis and relapsed groups, and 0, 1-2 and ≥3 treatment line groups. Mean DSS of melflufen was 34.34 (SD 8.08) for diagnosed patients and 39.31 (SD 6.67) for relapsed patients (Table 2). Median DSS was 34.25 (31.10, 37.30) for patients who had not

received any treatment, 35.60 (30.60, 45.30) for patients with one or two treatment lines and 44.00 (33.00, 45.70) for patients with three or more treatment lines (Table 2). Although samples from relapsed patients (Fig. 6A) and patients who had received three or more treatment lines (Fig. 6B) tended to be more sensitive compared to newly diagnosed patients and to patients with less than three lines of treatments, statistically significant differences were not observed (p=0.1987 and 0.4113, respectively) (Table 2).

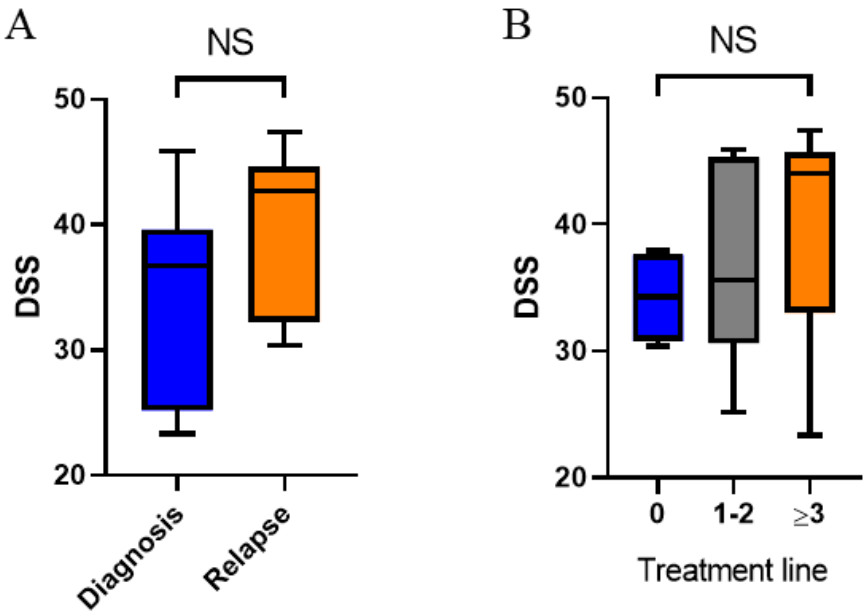

Figure 6. The responses of melflufen in malignant plasma cells among newly diagnosed and relapsed patients, and patients with 0, 1-2, and 3 or more lines of treatment. (A) Samples from relapsed patients seemed to be more sensitive to melflufen compared to newly diagnosed patients but drug sensitivity scores (DSSs) were not significantly higher (p=0.1987). (B) Samples from patients who have received three or more treatment lines tended to be more sensitive to melflufen than patients with less than three treatment lines but DSSs were not significantly higher (p=0.4113). DSS values are presented as means in Fig. 6A and as medians in Fig. 6B. NS=not significant.

Second, to observe whether the aggressiveness of the disease and high-risk cytogenetics affected the melflufen responses, patients were stratified based on ISS status (1-3) and the presence of t(4;14), del(17p) and gain(1q) aberrations (Table 2). Median DSS was 31.20 (27.90, 38.85) for patients with ISS score 3, 38.75 (26.85, 44.65) for patients with score 2, and 38.25 (33.80, 42.70) with score 1. In contrast, mean DSS of melflufen was 38.84 (SD 6.88) for patients with gain(1q) aberration and 29.77 (SD 6.17) for patients without the aberration. Median DSS was 26.85 (23.30, 30.40) for patients with both t(4;14) and del(17p) mutations, 42.70 (33.80, 45.30) for patients with only del(17p) mutation, 37.30 (33.65, 42.65) for patients with only t(4;14) mutation, and 39.60 (31.80, 44.00) for patients without either of these mutations (Table 2).

Patients with the highest ISS score seemed to be less sensitive to melflufen compared to patients with ISS score 1 or 2 (Fig. 7A), while patients with t(4;14), del(17p) and gain(1q) seemed to be more sensitive to melflufen than patients without these alterations (Fig. 7B and 7C). However, if patients had both t(4;14) and del(17p) alterations, they seemed to be even less sensitive to melflufen than wildtype patients (Fig. 7C). However, no statistically significant differences were observed between ISS scores (p=0.8606), gain(1q) aberration and wildtype (p=0.3638), and between other high-risk cytogenetic aberration subgroups (p=0.2319) (Fig. 7, Table 2).

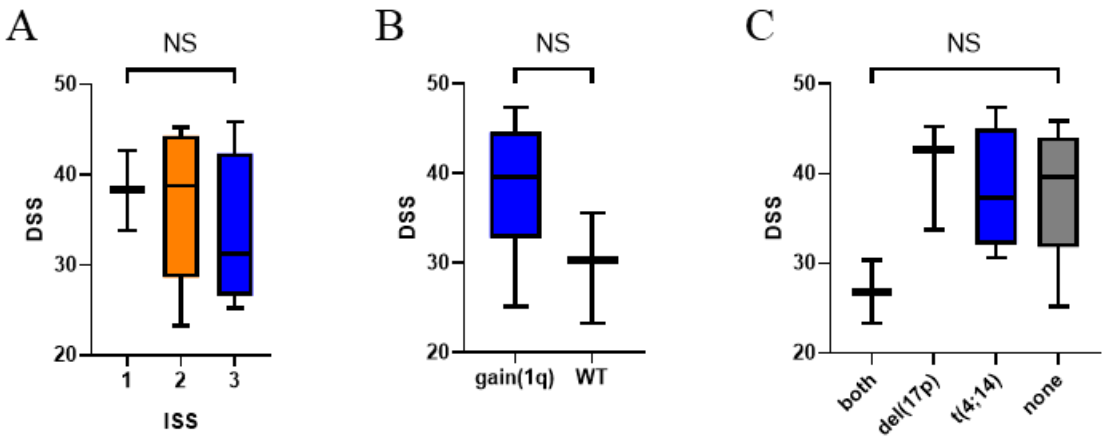

Figure 7. The responses of melflufen in malignant plasma cells among patients with different ISS scores, and patients with gain(1q), del(17p) and/or t(4;14) or none of these alterations. (A) Samples from patients with the highest ISS score seemed to be less sensitive to melflufen compared to patients with ISS score 1 or 2, but drug sensitivity scores (DSSs) were not significantly lower in high ISS score patients (p=0.8606). (B) Samples from patients with gain(1q) and (C) other high-risk cytogenetic factors, t(4;14) and del(17p), seemed to be more sensitive to melflufen than patients without these alterations. However, patients with both t(4;14) and del(17p) seemed to be less sensitive to melflufen than wildtype patients. Yet, significant differences were not observed between gain(1q) and wildtype (p=0.3638) and between other high-risk cytogenetic subgroups (p=0.2319). DSS values are presented as medians in Fig. 7A and C, and as means in Fig. 7B. NS=not significant, WT=wild type.

Furthermore, patients were stratified based on age at diagnosis (<65 years and ≥65 years old), gender, IgL (kappa and lambda), IgH (IgA and IgG), presence of immunoglobulins (IgG, IgA or light chain only) and presence of del(13q) aberration (Table 2). Mean DSS was 38.19 (SD 6.07) for patients younger than 65 years and 36.32 (SD 8.73) for patients 65 years old or older, and 37.23 (SD 8.03) for females and 37.08 (SD 7.62) for males. Mean DSS was 34.64 (SD 7.62) for patients with kappa and 41.53 (SD 3.21) for patients with lambda IgL, 35.90 (SD 10.87) for patients with IgA and 37.62 (SD 4.84) for patients with IgG IgH, and 33.90 (SD 8.75) for patients with only light chain. Furthermore, mean DSS was 36.12 (SD 7.78) for patients with del(13q) and 40.20 (SD 6.54) for patients without the aberration. Although all patients seemed to be sensitive to melflufen, significant differences between subgroups of age, gender, IgL, IgH, presence of

immunoglobulins and presence of del(13q) aberration were not detected (p=0.6387, 0.9700, 0.1644, 0.7380, 0.8016 and 0.3638, respectively) (Appendix 5).

2.4 Drug responses in lymphocytes

To observe the cytotoxic effects of melflufen and melphalan in healthy cell populations, lymphocytes were used as internal controls. Mean DSS of melphalan was 15.51 (SD 3.51) and of melflufen 40.27 (SD 7.22) in lymphocytes (Appendix 3). DSSs of melphalan were similar in malignant PCs and lymphocytes (p=0.9460) (Fig. 8A), whereas DSSs of melflufen were significantly higher in lymphocytes compared to malignant PCs (p=0.0002) (Fig. 8B).

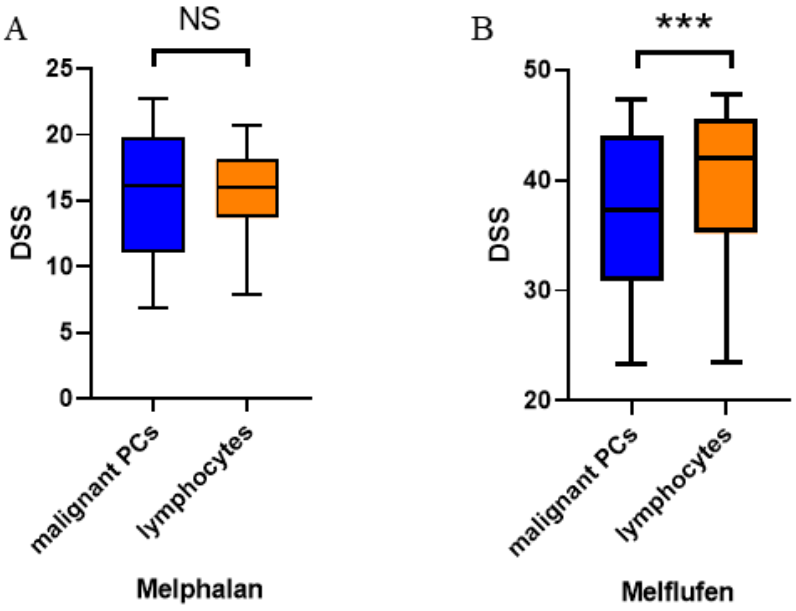

Figure 8. The responses of melphalan and melflufen in lymphocytes. (A) The drug sensitivity scores (DSSs) of melphalan were similar in lymphocytes and malignant plasma cells (PCs) (p=0.9460). (B) DSSs of melflufen were significantly higher in lymphocytes compared to malignant PCs (p=0.0002). DSS values are presented as means in Fig. 8A and as medians in Fig. 8B. NS=not significant.

3 Discussion

3.1 Melflufen sensitivity

Despite the evolving treatment landscape and advances of ongoing research, MM remains incurable. Although some patients achieve long and durable remission, almost all patients will relapse and thus there is an unmet medical need especially regarding treatment of RRMM and HRMM patients. In this study, we tested the sensitivity of malignant PCs derived from MM patients to melflufen, a novel alkylating anticancer agent, and

compared the results to melphalan. Further patient stratification allowed us to detect whether there is a specific melflufen-sensitive subgroup(s) of MM patients. When comparing DSSs of melflufen and melphalan in malignant PCs, our results showed that malignant PCs were more sensitive to melflufen than melphalan (p<0.0001) (Fig. 5A, B and C). In addition, there was almost no correlation between melflufen and melphalan responses (0.22) and the relationship was lacking significance (p=0.41) (Fig. 5D), indicating that the responses of melflufen are not associated with the responses of melphalan. These results indicated that melflufen might be more effective than melphalan already at reduced doses and if patient is not eligible for melphalan, melflufen treatment might be beneficial.

To observe how RRMM and HRMM patient samples responded to melflufen, drug response profiles were compared based on subgroups of disease status and treatment line as well as ISS score and high-risk (gain(1q), del(17p) and t(4;14)) cytogenetic markers. By comparing DSSs of melflufen in these different subgroups, we evaluated that all samples from MM patients including RRMM and HRMM patients seemed to be sensitive to melflufen although to varying degrees. Samples from relapsed patients and patients with three or more treatment lines tended to be more sensitive to melflufen than samples from newly diagnosed patients and patients with 0-2 treatment lines (Fig. 6), although no significant differences were observed. However, our results show that RRMM patient samples responded well to melflufen treatment.

Furthermore, samples from patients with the highest ISS score seemed to be less sensitive to melflufen compared to patients with lower scores (Fig. 7A), while samples from HRMM patients seemed to be more sensitive to melflufen than patients without high-risk alterations (Fig. 7B and 7C). However, patients with both t(4;14) and del(17p) alterations seemed to be less sensitive to melflufen compared to patient samples without these alterations (Fig. 7C), although the significance was lacking. Since samples from patients with the highest ISS score and both t(4;14) and del(17p) aberrations did not respond as effectively as patient samples with lower ISS score and each mutation alone, it should be investigated if patients with individual aberrations benefit from melflufen treatment more than patients with several aberrations. Nevertheless, samples from HRMM patient also responded to melflufen treatment.

Although intravenous administration of melflufen affects several cell populations present in the body, the response characterisation was limited only in hematopoietic cell types.

To observe the cytotoxicity of melflufen, the responses in lymphocytes were detected. When comparing DSSs of melflufen in malignant PCs and lymphocytes, lymphocytes were even more sensitive to melflufen than malignant PCs (p=0.0002) (Fig. 8B). This suggest that melflufen might have some adverse cytotoxic effects. In fact, it has already been observed in phase 1-2 clinical studies that the most common severe adverse events of melflufen are neutropenia and thrombocytopenia both in solid tumors (Berglund *et al.,* 2015) and in RRMM (Richardson *et al.,* 2020). Importantly, adverse events observed in trials were reversible and clinically manageable in all patients. Nevertheless, cytotoxicity should be studied in more detail in the future. Also, drug responses should be studied in other cell populations and healthy BM cells for providing a deeper understanding of malignant and non-malignant responses and adverse effects. With this approach, dose levels with maximal efficacy against malignant PCs and minimal effects towards healthy cell populations can be identified. Also, the cycle length of melflufen administration can be increased to provide additional time for hematological recovery allowing patients to stay on treatment longer (Richardson *et al.,* 2020).

It is also noteworthy that these specific patients do not respond well to current treatments, so it should be studied whether adverse effects of melflufen are milder compared to the symptoms and onset of RRMM. There is an ongoing debate on whether MM should be treated aggressively with multi-drug combinations aiming at complete response or whether MM should be treated in a way that the disease is in control which emphasizes the overall survival (OS) and QoL of patients (Rajkumar, 2008). Especially, the predicted prognosis for RRMM and HRMM patients is currently extremely poor compared to low and standard-risk patients. When using risk stratification model of IMWG (see section 1.1.3), median OS among low-risk patients is often more than ten years, among standard-risk patients approximately seven years, and among high-risk patients approximately two years (Hanbali *et al.,* 2017; Iriuchishima *et al.,* 2015). Moreover, median OS among RRMM patients who are refractory to lenalidomide and bortezomib is only nine months (Kumar *et al.* 2012).

3.2 Challenges and Future Perspective

Interpretation of this study was limited by the small sample size. One of the main challenges of the assay used in the study was poor viability and low number of malignant PCs of the cryopreserved patient samples. Our study was dependent on patient-derived samples since they provide a more predictive model to clinical efficacy compared to

cancer cell lines. Although *in vitro* and *in vivo* studies based on cell line models may provide an important understanding of the pathogenesis of MM and drug responses, these models cannot mimic the molecular complexity of MM genome. For example, clinical drug sensitivity in 57–83%, and drug resistance in 90% accuracy have been predicted from i*n vitro* results (Volm and Efferth, 2015), while *ex vivo* drug sensitivity has been proven to have clear relationship with clinical outcome (Majumder *et al.,* 2017; Snijder *et al*., 2017). However, frozen cells are challenging to use since freezing-thawing cycle of PCs is quite radical resulting in weak survival of the cells and loss of CD138 expression (Dorwal, Thakur and Rawat, 2014; Kawano, *et al.,* 2012). Therefore, some fresh patient-derived MNCs may be tested for increased viability and the number of malignant PCs.

Since multiple signaling pathways are activated and clonal heterogenicity is presence in MM, the use of combination therapy which can target various molecules simultaneously, might be justified (Nijhof *et al.,* 2018). Consequently, when using combination, therapeutic efficacy can be achieved at reduced doses of melflufen and thus toxicity and adverse effects can be minimized. In fact, previous studies show that melflufen has had synergistic effects with bortezomib (Wickström *et al.,* 2008) and induced antagonistic effects with docetaxel (Wikström *et al.,* 2007) *in vitro*. Moreover, the ongoing clinical trial which combines melflufen with dexamethasone supports the use of combination therapy ("NIH Clinical Trial OCEAN"). Hence, different drug combinations with melflufen should be studied *ex vivo* as well.

Lastly, there are only few drugs for clinically validated cancer mutations available showing how poor the understanding of relationship between complex genome and cellular phenotype still is (Friedman *et al.,* 2015). Also, regardless many benefits of DSRT, it still lacks standardization needed for more robust and accurate results. Recently, a new approach called individualized system medicine (ISM) has been developed for acute myeloid leukemia (AML) (Pemovska *et al.,* 2013). It is based on optimizing the safest and most effective treatment for each patient as well as trying to understand the mechanism of drug resistance by utilizing *ex vivo* DSRT, clinical implementation of predicted effective treatments, and studies of samples from treated patients. Therefore, ISM approach implementation for other hematological malignancies including MM may improve the understanding of biology of disease and drug resistance (Majumder, 2018; Majumder *et al.,* 2017). Additionally, DSRT standardization may lead to better utilization of the platform also in clinical use. Furthermore, DSRT platform should be developed further so for example BM microenvironment effects on drug responses can be

considered. Although the used cell culture medium was from the HS-5 human BM stromal cell line mimicking the environment of BM, cell adhesion mediated drug responses or hypoxia for example could not be taken into account (Majumder *et al.,* 2017).

3.3 Conclusions and summary

The reason why some patients relapse and what causes drug resistance remains still unknown. Possible explanations are for instance the impact of cytogenetic and epigenetic alterations, the role of deregulated signaling pathways and the BM microenvironment as well as MM cancer stem cells (Abdi *et al.,* 2013). During disease progression, the genomic complexity of MM is increasing due to cytogenetic alterations both by the number and structure of chromosomes, which are key challenges of MM therapies. Since the pipeline for treating MM is one of the most diverse in oncology and more and more drugs are getting marketing authorization for MM, determining the best treatment option for individual patients becomes more difficult. In addition, newer, usually more expensive drugs and many lines of treatment lead to high costs of MM therapies, and the costs are expected to rise even more due to aging of the population and extended patient survival (Roy *et al.,* 2015). Hence, DSRT platform and *ex vivo* testing can therefore be clinically important approaches and guide which treatments are the most effective for individual patients. The significance of the study relies on the linking of data from *ex vivo* drug responses to complex molecular profiles for biomarker discovery, personalized medicine development and ultimately improved clinical outcome. Moreover, the results from the study are directly translatable to patients and can guide the timing and treatment options.

In this study, we were able to observe more sensitive melflufen responses in malignant PCs from MM patients compared to melphalan *ex vivo*. Although statistically significant melflufen-sensitive subgroups were not observed using standard clinical criteria and features, this study showed that all samples from MM patients including RRMM and HRMM patients were sensitive to melflufen suggesting these patients may benefit from melflufen treatment and thus our results are clinically relevant and valuable. In the future, melflufen sensitivity studies may be repeated with drug combinations, cell co-cultures and healthy BM samples as well as the mechanism of action of melflufen, especially the association of esterases and peptidases to melflufen sensitivity, may be studied. Further integration of our drug response results with additional molecular information and clinical profiles may lead to discovery of MM specific mutations or gene expression patterns and

identification of novel predictive and prognostic biomarkers. Taken together, the prediction of melflufen responses and a precision medicine approach can lead to more effective disease management, increase QoL of the patients, save patients from extremely expensive and ineffective therapies, and reduce the economic burden of MM.

4 Materials and methods

4.1 Patient material and sample processing

BM aspirates were obtained from MM patients (n=51) after signing a written Informed Consent Form (ICF) in accordance with the Declaration of Helsinki and the study was approved by The Coordinating Ethics Board of Helsinki University Hospital Comprehensive Cancer Center (study permits 303/13/03/01/2011 approved on 5.11.2012 and 239/13/03/00/2010 approved on 12.10.2010). Clinical data have been collected, handled and stored appropriately and each patient has been pseudonymized in a way that patients cannot be identified.

Samples were obtained from the Finnish Hematology Clinical Biobank and Registry (FHRB, www.fhrb.fi) and selected especially from RRMM and HRMM patients. Briefly, samples were processed by isolating MNCs from BM aspirates using Ficoll-Paque density gradient centrifugation (Ficoll Paque Premium; GE Healthcare, Chalfont St Giles, Buckinghamshire, UK), viably cryopreserved and stored in liquid nitrogen.

4.2 Reagents, antibodies and controls

Conditioned medium (CM; 25% conditioned medium from the HS-5 human BM stromal cell line in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin (10 000 units/ml and 10 000 µg/ml, respectively)) was used for culturing the cells. Cells were counted with Trypan Blue stain 0.4% (#T10282; Invitrogen, Waltham, MA, USA), cell counting chamber slides (#100078809; Invitrogen) and Countess automated cell counter (Invitrogen). Melflufen (Recipharm AB, Stockholm, Sweden) and melphalan (Sigma-Aldrich, St. Louise, MO, USA) were dissolved in DMSO (#D4540, Sigma-Aldrich), stored in -80°C as 10 mM stock, and thawed in 37°C water bath just prior to use. Antibody dilution mix (AbM, Table 3) diluted in staining buffer (SB; 5% FBS in 1 x Dulbecco's phosphate-buffered saline (DPBS)) and PE Annexin V Apoptosis Detection kit with 7-AAD (#559763; BD Bioscience, Eysins, Switzerland) diluted in mQ were used for MNCs staining. To

compensate spectral overlap of fluorescence from several fluorophores, the compensation was performed with UltraComp eBeads Compensation Beads (#01-2222; Invitrogen) according to their protocol (50 ul/test well).

Table 3. Information of antibodies and fluorophores of the antibody dilution mix used for staining of mononuclear cells on day three. Antibodies were diluted to staining buffer.

Marker	Fluorophore	Concentration (ng/ul)	Clone	Source	Manufacturer	Catalogue#	Lot
CD19	BV421	0.2	HIB19	Mouse	BD Biosciences	562440	8270584
CD138	BV605	1.33	MI15	Mouse	BD Biosciences	563294	9073759
CD38	BV786	2.0	HIT2	Mouse	BD Biosciences	563964	8304886
CD45	FITC	0.5	HI30	Mouse	BD Biosciences	561865	8134922
CD56	PE-Cy7	1.0	B159	Mouse	BD Biosciences	557747	8340788
CD319	APC	1.33	162.1	Mouse	Biolegend	331810	B260180

4.3 High-throughput drug sensitivity and resistance testing

CM and the frozen cell vials were warmed and thawed in +37°C water bath. The cells were transferred carefully into a falcon tube with CM, and CM was added 1:10 slowly on the cells. Cells were centrifuged at 400 x g for 6 min and the supernatant was removed. The cell pellet was re-suspended in CM and 50 ul/ml of RQ1 RNase-free DNaseI (#M6101; Promega) was added. The cells were incubated at humidified environment at +37°C and 5% CO_2 for 1h. The cells were then diluted to 2 M cells/ml in CM, transferred to a non-treated 6-well plate and incubated overnight at +37°C and 5% CO_2.

On the next day, the cells were mixed gently with a pipette, filtered through a 70 µm cell strainer (#22363548; Fisher Scientific, Pittsburg, Pennsylvania, USA) and counted. The cells were centrifuged at 400 x g for 6 min and the supernatant was removed. The cell pellet was re-suspended in CM in 2 M cells/ml. First, 50 µl of CM was added to control wells (0.2% DMSO) of a V-bottom 96-well plate and the plate was shaken with the plate shaker for a couple of minutes (750 rpm). Then 100 000 cells/well were dispensed to control and drug wells (Fig. 9). Melflufen and melphalan were added directly on top of the cells in seven concentrations made fresh in CM ranging from 0.1–100 000 nM as duplicates (Fig. 9). The final volume of each well was 100 µl. The plate was shaken with the plate shaker for a couple of minutes (450 rpm) and incubated at +37°C and 5% CO_2 for 72 h. For the day 0 control, which was performed to check the viability and number of malignant PCs, two wells of an empty 96-well plate were plated with 100 000 cells/well and centrifuged at 500 x g for 6 min. The supernatant was removed by turning the plate upside down. 25 µl of AbM was added to the staining well and 25 µl of SB to the

unstaining well. The plate was incubated at RT for 30 min in the dark. 100 µl of SB was added to wells for washing and the plate was centrifuged at 500 x g for 6 min. Supernatants were removed and 25 µl of Annexin V Apoptosis Detection solution was added to each well. The plate was shaken with the plateshaker for a couple of minutes (450 rpm). The plate was read with a high-throughput flow cytometer (iQue Screener Plus, Sartorius, Albuquerque, NM, USA). For the day 3 DSRT analysis, the same staining protocol was repeated for treated cells after 72 h incubation.

		Melflufen		**Melphalan**				**Melflufen**		**Melphalan**		
	1	2	3	4	5	6	7	8	9	10	11	12
A		0,1		0,1				0,1		0,1		
B		1		1				1		1		
C		10		10				10		10		
D	DMSO	DMSO		DMSO	DMSO	DMSO	DMSO	DMSO		DMSO	DMSO	DMSO
E		100		100				100		100		
F		1000		1000				1000		1000		
G		10000		10000				10000		10000		
H		100000		100000				100000		100000		

Figure 9. Layout of 96-well drug plate. Melflufen and melphalan concentrations were ranging from 0.1–100 000 nM as duplicates. DMSO was used as negative control. In addition to melflufen and melphalan, other drugs were also tested in the same plate but the results were not included in this thesis.

4.4 Data analysis

4.4.1 Sample selection criteria

Out of 51 tested samples, 16 were chosen for further data analysis based on reasonable cell viability (viability >25%) and constant number of CD138+/CD38+ (malignant PCs) cells in control DMSO wells (>50 cells) for data normalization (Appendix 1). Moreover, samples should have had enough malignant PCs in the drug wells for creating robust dose-response. Also, samples were not chosen for data analysis if there were problems with antibody staining or flow cytometer run (Appendix 1).

4.4.2 Gating and dose-response curves

DSRT readouts were analysed with ForeCyt software (Sartorius) and the counts of different cell populations including malignant PCs and lymphocytes were observed by utilising specific antibodies (Table 3). Gating strategy is presented in Figure 10. All cells were detected with FSC and SSC, single cells were detected from all cells with FSC height (FSC-H) and FSC area (FSC-A). Live, apoptotic and dead cells were separated using annexin V and 7-AAD. Malignant PCs were separated from other live cell populations with anti-CD138 and anti-CD38 antibodies (Flores-Montero *et al.,* 2016;

Jelinek *et al.,* 2017) whereas lymphocytes were separated with anti-CD45 antibody and low SSC (Akanni and Palini, 2006).

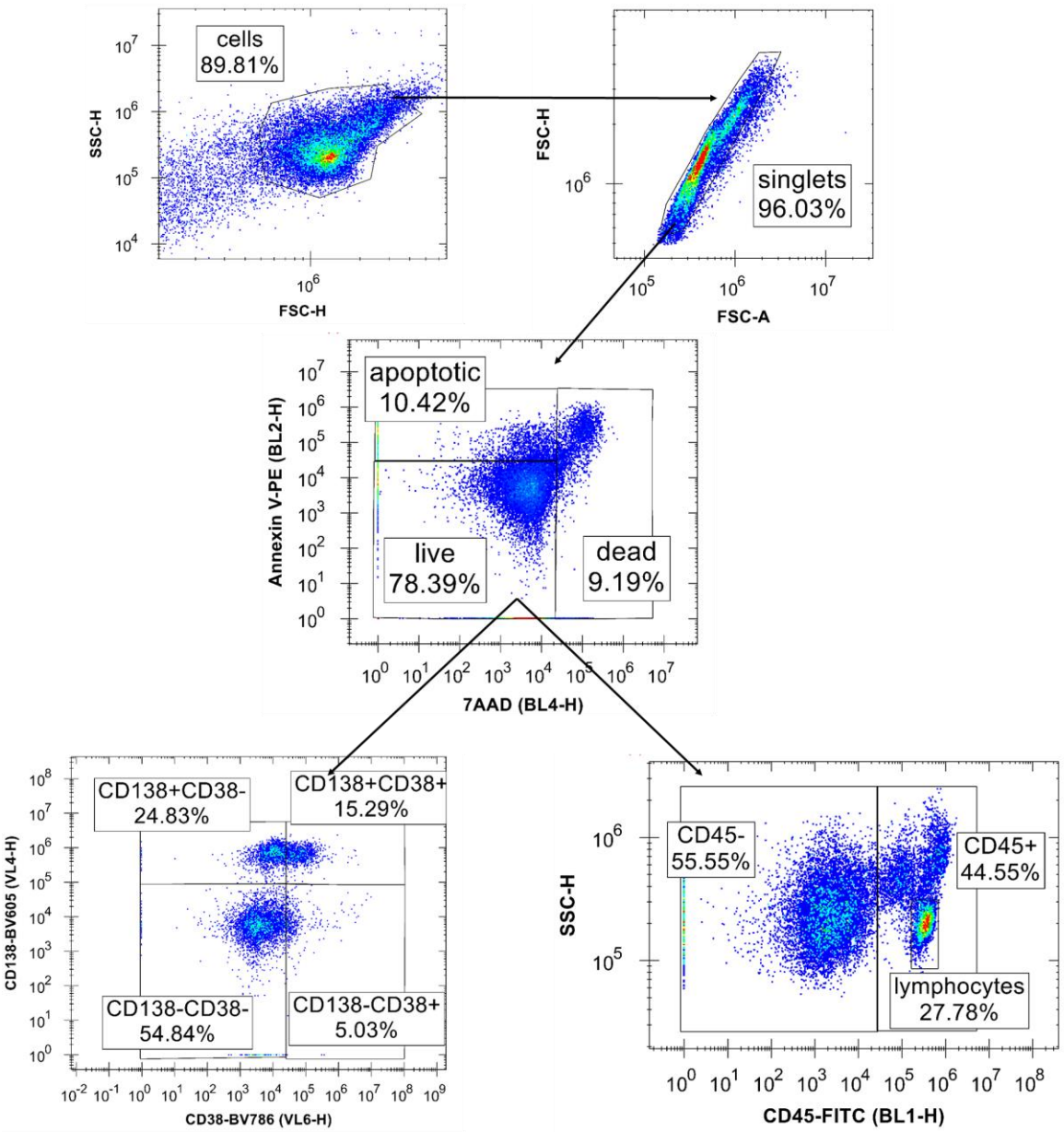

Figure 10. Gating strategy. All cells were detected with FSC and SSC, single cells were detected from all cells with FSC height (FSC-H) and FSC area (FSC-A). Live, apoptotic and dead cells were separated with annexin V and 7-AAD. Malignant PCs were separated from other live cell populations utilizing anti-CD138 and anti-CD38 antibodies whereas lymphocytes were separated utilizing anti-CD45 antibody and low SSC. BL4, BL2, VL6, VL4 and BL1 are channels of flow cytometer.

GraphPad Software (GraphPad Prism 8.4.2, San Diego, CA, USA) was used for nonlinear regression analysis and for generating logistic dose-response curves to melflufen and melphalan. The data was normalized to means of the highest and lowest concentrations of drug duplications of each sample.

4.4.3 Drug sensitivity score

Drug sensitivity score (DSS), an approach used for creating a single metric from multiparametric dose-response relationships which takes into account IC50, slope maximal and minimal response as well as minimal activity (Yadav, B. *et al.*, 2015), to each patient was obtained from FIMM's main data repository (TheDB). It has been shown that two samples can have equal single model parameters, for example IC50 values, while having different activity patterns (Yadav, B. *et al.*, 2015). Therefore, in some cases IC50 is not informative enough to detect the differences in response patterns of patient samples, and thus DSS is used in this study to identify selective drug response patterns. The higher DSS associates with smaller IC50 value and reflects higher sensitivity of the sample.

4.4.4 Statistical analysis

Statistical analysis were done with GraphPad Prism. Normal distribution assumption for DSSs was checked visually using normal quantile plot and with Shapiro-Wilk test. Mean and standard deviation (SD) are reported for normally distributed variables, and median with 25% and 75% quartiles (Q1, Q3) to nonparametric variables. Differences were considered statistically significant when associated with a p-value of <0.05, and highly significant with <0.001.

The difference of melflufen and melphalan responses in malignant PCs was obtained by comparing DSSs with paired two-sample t-test. PC specific drug sensitivity was obtained by comparing DSSs of melflufen with Wilcoxon signed rank test and DSSs of melphalan with paired two-sample t-test in malignant PCs and lymphocytes. Correlation of melflufen and melphalan responses (DSS) of malignant PCs was analysed with Pearson correlation.

Since all samples seemed to be sensitive to melflufen, to identify melflufen-sensitive subgroup, the responses of melflufen in malignant PCs were stratified into more (IC50 <50nM) and less (IC50 >50nM) sensitive subgroups, rather than sensitive and resistant groups, based on IC50 values. Further, to assess whether clinical characteristics (Table 2) influence the responses of melflufen in malignant PCs, MM patients were stratified into different subgroups based on disease status (diagnosis, relapse), age at diagnosis (<65 years and ≥65 years old), gender (females and males), IgL (kappa and lambda), IgH (IgA and IgG), presence of immunoglobulins (IgG, IgA and light chain only), ISS score (1-3), lines of treatment (0, 1-2, ≥3) and the presence of del(13q), gain(1q), del(17p) and t(4;14)

aberrations. DSSs of melflufen in sensitivity, disease status, age, gender, IgL, IgH as well as del(13p) and gain(1q) aberration subgroups were compared with unpaired t-test. DSSs of melflufen in ISS, treatment lines and genetic aberrations (del(17p) and/or t(4;14), or neither of them) subgroups were compared with Kruskal-Wallis test. DSSs of melflufen in immunoglobulin subgroups were compared with one-way ANOVA test.

5 Acknowledgements

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6 Abbreviations list

β2-M	β2-Macroglobulin
BM	Bone Marrow
CD	Cluster of Differentiation
CM	Conditioned Medium
DSRT	Drug Sensitivity and Resistance Testing
DSS	Drug Sensitivity Score
FSC	Forward Scatter
HRMM	High-Risk Multiple Myeloma
IgH	Immunoglobulin Heavy chain
IgL	Immunoglobulin Light chain
IMWG	International Myeloma Working Group
ISS	International Staging System
MGUS	Monoclonal Gammopathy of Undetermined Significance
MM	Multiple Myeloma
MNC	Mononuclear Cell
NA	Not Available
OS	Overall Survival
PC	Plasma Cell
QoL	Quality of Life
RRMM	Relapsed and Refractory Multiple Myeloma
SB	Staining Buffer
SMM	Smoldering Myeloma
SSC	Side Scatter

7 References

Abdi, J., G. Chen, and H. Chang. 2013. Drug resistance in multiple myeloma: latest findings and new concepts on molecular mechanisms. Oncotarget. 4:2186–2207. doi: 10.18632/oncotarget.1497.

Akanni, E.O., and A. Palini. 2006. Immunophenotyping of Peripheral Blood and Bone Marrow Cells by Flow Cytometry. EJIFCC, 17:17–21.

Bayraktar, U.D., Q. Bashir, M. Qazilbash, R.E. Champlin, and S.O. Ciurea. 2013. Fifty years of melphalan use in hematopoietic stem cell transplantation. Biol. Blood Marrow Transplant.19:344–356. doi: 10.1016/j.bbmt.2012.08.011.

Berglund, Å., A. Ullén, A. Lisyanskaya, S. Orlov, H. Hagberg, B. Tholander, R. Lewensohn, P. Nygren, J. Spira, J. Harmenberg, M. Jerling, C. Alvfors, M. Ringbom, E. Nordström, K. Söderlind, and J. Gullbo. 2015. First-in-human, phase I/IIa clinical study of the peptidase potentiated alkylator melflufen administered every three weeks to patients with advanced solid tumor malignancies. Invest. New Drugs. 33:1232-1241. doi:10.1007/s10637-015-0299-2.

Bergsagel, P.L., and M. Chesi. 2013. V. Molecular classification and risk stratification of myeloma. Hematol. Oncol. 31:38-41. doi:10.1002/hon.2065.

Brigle, K., and B. Rogers. 2017. Pathobiology and Diagnosis of Multiple Myeloma. Semin. Oncol. Nurs. 33(3):225-236. doi: 10.1016/j.soncn.2017.05.012.

Carlier, C., S. Strese, K. Viktorsson, E. Velander, P. Nygren, M. Uustalu, T. Juntti, R. Lewensohn, R. Larsson, J. Spira, E. De Vlieghere, W.P. Ceelen, and J. Gullbo. 2016. Preclinical activity of melflufen (J1) in ovarian cancer. Oncotarget. 7:59322-59335. doi: 10.18632/oncotarget.11163.

Castaneda, O., and R. Baz. 2019. Multiple Myeloma Genomics - A Concise Review. Acta Med. Acad. 48:57-67. doi: 10.5644/ama2006-124.242.

Chauhan, D., A. Ray, K. Viktorsson, J. Spira, C. Paba-Prada, N. Munshi, P. Richardson, R. Lewensohn, and K.C. Anderson. 2013. *In Vitro* and *In Vivo* Antitumor Activity of a Novel Alkylating Agent, Melphalan-Flufenamide, against Multiple Myeloma Cells. Clin. Cancer Res. 19:3019-3031. doi:10.1158/1078-0432.CCR-12-3752.

Chesi, M., G.M. Matthews, V.M. Garbitt, S.E. Palmer, J. Shortt, M. Lefebure, A.K. Stewart, R.W. Johnstone, and P.L. Bergsagel. 2012. Drug response in a genetically engineered mouse model of multiple myeloma is predictive of clinical efficacy. Blood. 120:376-385. doi:10.1182/blood-2012-02-412783.

Delforoush, M., S. Strese, M. Wickström, R. Larsson, G. Enblad, and J. Gullbo. 2016. *In vitro* and *in vivo* activity of melflufen (J1) in lymphoma. BMC Cancer. 16:263. doi: 10.1186/s12885-016-2299-9.

Dorwal, P., R. Thakur, and S. Rawat. 2014. CD138 expression in plasma cells is volatile and time-lag dependent. The Egyptian Journal of Haematology, 39:258-259. doi:10.4103/1110-1067.153978

Eriksson, M., and M. Karlsson. 1992. Occupational and other environmental factors and multiple myeloma: a population based case-control study. Br. J. Ind. Med. 49:95-103. doi: 10.1136/oem.49.2.95.

Fairfield, H., C. Falank, L. Avery, and M.R. Reagan. 2016. Multiple myeloma in the marrow: pathogenesis and treatments. Ann. N. Y. Acad. Sci. 1364:32-51. doi: 10.1111/nyas.13038.

Flores-Montero, J., R. de Tute, B. Paiva, J.J. Perez, S. Böttcher, H. Wind, L. Sanoja, N. Puig, Q. Lecrevisse, M.B. Vidriales, van Dongen, Jacques J.M., and A. Orfao. 2016. Immunophenotype of normal vs. myeloma plasma cells: Toward antibody panel specifications for MRD detection in multiple myeloma. Cytometry B. Clin. Cytom. 90:61-72. doi: 10.1002/cyto.b.21265.

Gullbo, J., E. Lindhagen, S. Bashir-Hassan, M. Tullberg, H. Ehrsson, R. Lewensohn, P. Nygren, M. De La Torre, K. Luthman, and R. Larsson. 2004. Antitumor efficacy and acute toxicity of the novel dipeptide melphalanyl-p-L-fluorophenylalanine ethyl ester (J1) *in vivo*. Invest. New Drugs. 22:411-420. doi:10.1023/B:DRUG.0000036683.10945.bb.

Gullbo, J., M. Wickström, M. Tullberg, H. Ehrsson, R. Lewensohn, P. Nygren, K. Luthman, and R. Larsson. 2003. Activity of hydrolytic enzymes in tumour cells is a determinant for anti-tumour efficacy of the melphalan containing prodrug J1. J. Drug Target. 11:355-363. doi: 10.1080/10611860310001647140.

Gundesen, M.T., T. Lund, H. Moeller, and N. Abildgaard. 2019. Plasma Cell Leukemia: Definition, Presentation, and Treatment. Curr. Oncol. Rep. 21:8. doi: 10.1007/s11912-019-0754-x.

Hanbali, A., M. Hassanein, W. Rasheed, M. Aljurf, and F. Alsharif. 2017. The Evolution of Prognostic Factors in Multiple Myeloma. Adv. Hematol. 2017:4812637. doi: 10.1155/2017/4812637

Iriuchishima, H., T. Saitoh, H. Handa, A. Isoda, M. Matsumoto, M. Sawamura, A. Iwasaki, C. Ushie, H. Hattori, Y. Sasaki, T. Mitsui, A. Yokohama, N. Tsukamoto, H. Murakami, and Y. Nojima. 2015. A new staging system to predict prognosis of patients with multiple myeloma in an era of novel therapeutic agents. Eur. J. Haematol. 94:145-151. doi: 10.1111/ejh.12407

Jagannathan-Bogdan, M. and L.I. Zon. 2013. Hematopoiesis. Development .140:2463-2467. doi: 10.1242/dev.083147

Javarappa, K.K., D. Tsallos, and C.A. Heckman. 2018. A Multiplexed Screening Assay to Evaluate Chemotherapy-Induced Myelosuppression Using Healthy Peripheral Blood and Bone Marrow. SLAS Discov. 23:687-696. doi: 10.1177/2472555218777968.

Jelinek, T., R. Bezdekova, M. Zatopkova, L. Burgos, M. Simicek, T. Sevcikova, B. Paiva, and R. Hajek. 2017. Current applications of multiparameter flow cytometry in plasma cell disorders. Blood Cancer J. 7:e617. doi: 10.1038/bcj.2017.90.

Kawano, Y., S. Fujiwara, N. Wada, M. Izaki, H. Yuki, Y. Okuno, K. Iyama, H. Yamasaki, A. Sakai, H. Mitsuya, and H. Hata. 2012. Multiple myeloma cells expressing

low levels of CD138 have an immature phenotype and reduced sensitivity to lenalidomide. Int. J. Oncol. 41:876-884. doi: 10.3892/ijo.2012.1545

Kazandjian, D. 2016. Multiple myeloma epidemiology and survival: A unique malignancy. Semin. Oncol. 43:676-681. doi: 10.1053/j.seminoncol.2016.11.004.

Kuehl, W.M., and P.L. Bergsagel. 2012. Molecular pathogenesis of multiple myeloma and its premalignant precursor. J. Clin. Invest. 122:3456-3463. doi: 10.1172/JCI61188

Kumar, S.K., J.H. Lee, J.J. Lahuerta, G. Morgan, P.G. Richardson, J. Crowley, J. Haessler, J. Feather, A. Hoering, P. Moreau, X. LeLeu, C. Hulin, S.K. Klein, P. Sonneveld, D. Siegel, J. Bladé, H. Goldschmidt, S. Jagannath, J.S. Miguel, R. Orlowski, A. Palumbo, O. Sezer, S.V. Rajkumar, B.G.M. Durie, International Myeloma Working Group. 2012. Risk of progression and survival in multiple myeloma relapsing after therapy with IMiDs and bortezomib: a multicenter international myeloma working group study. Leukemia, 26:149-157. doi: 10.1038/leu.2011.196.

Kumar, S.K., T.M. Therneau, M.A. Gertz, M.Q. Lacy, A. Dispenzieri, S.V. Rajkumar, R. Fonseca, T.E. Witzig, J.A. Lust, D.R. Larson, R.A. Kyle, and P.R. Greipp, 2004. Clinical course of patients with relapsed multiple myeloma. Mayo Clin. Proc. 79:867-874. doi: 10.4065/79.7.867.

Kumar, S., J.L. Kaufman, C. Gasparetto, J. Mikhael, R. Vij, B. Pegourie, L. Benboubker, T. Facon, M. Amiot, P. Moreau, E.A. Punnoose, S. Alzate, M. Dunbar, T. Xu, S.K. Agarwal, S.H. Enschede, J.D. Leverson, J.A. Ross, P.C. Maciag, M. Verdugo, and C. Touzeau. 2017. Efficacy of venetoclax as targeted therapy for relapsed/refractory t(11;14) multiple myeloma. Blood. 130 (22): 2401–2409. doi: 10.1182/blood-2017-06-788786.

Kuusanmäki, H., A. Leppä, P. Pölönen, M. Kontro, O. Dufva, D. Deb, B. Yadav, O. Brück, A. Kumar, H. Everaus, B.T. Gjertsen, M. Heinäniemi, K. Porkka, S. Mustjoki, and C.A. Heckman. 2019. Phenotype-based drug screening reveals association between venetoclax response and differentiation stage in acute myeloid leukemia. Haematologica. doi: 10.3324/haematol.2018.214882.

Kyle, R.A., and S.V. Rajkumar. 2008. Multiple myeloma. Blood. 111:2962-2972. doi: 10.1182/blood-2007-10-078022.

Kyle, R.A., M.A. Gertz, T.E. Witzig, J.A. Lust, M.Q. Lacy, A. Dispenzieri, R. Fonseca, S.V. Rajkumar, J.R. Offord, D.R. Larson, M.E. Plevak, T.M. Therneau, and P.R. Greipp. 2003. Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin. Proc. 78:21-33. doi: 10.4065/78.1.21.

Kyle, R.A., T.M. Therneau, S.V. Rajkumar, J.R. Offord, D.R. Larson, M.F. Plevak, and L.J. Melton. 2002. A long-term study of prognosis in monoclonal gammopathy of undetermined significance. N. Engl. J. Med. 346:564-569. doi: 10.1056/NEJMoa01133202.

Maiso, P., X. Carvajal-Vergara, E.M. Ocio, R. López-Pérez, G. Mateo, N. Gutiérrez, P. Atadja, A. Pandiella, and J.F. San Miguel. 2006. The histone deacetylase inhibitor LBH589 is a potent antimyeloma agent that overcomes drug resistance. Cancer Res. 66:5781-5789. doi: 10.1158/0008-5472.CAN-05-4186.

Majumder, M.M., R. Silvennoinen, P. Anttila, D. Tamborero, S. Eldfors, B. Yadav, R. Karjalainen, H. Kuusanmäki, J. Lievonen, A. Parsons, M. Suvela, E. Jantunen, K. Porkka, and C.A. Heckman. 2017. Identification of precision treatment strategies for relapsed/refractory multiple myeloma by functional drug sensitivity testing. Oncotarget, 8:56338-56350. doi: 10.18632/oncotarget.17630.

Majumder, M.M., A. Leppä, M. Hellesøy, P. Dowling, A. Malyutina, R. Kopperud, D. Bazou, E. Andersson, A. Parsons, J. Tang, O. Kallioniemi, S. Mustjoki, P. O'Gorman, K. Wennerberg, K. Porkka, B.T. Gjertsen, and C.A. Heckman. 2019. Multi-parametric single cell evaluation defines distinct drug responses in healthy hematological cells that are retained in corresponding malignant cell types. Haematologica. doi: 10.3324/haematol.2019.217414.

Majumder, M.M. 2018. Improving Precision in Therapies for Hematological Malignancies. Doctoral Dissertation, University of Helsinki, Helsinki, Finland

Mateos, M., and J.F. San Miguel. 2017. Management of multiple myeloma in the newly diagnosed patient. Hematology Am. Soc. Hematol. Educ. Program. 2017:498-507. doi: 10.1182/asheducation-2017.1.498.

NIH Clinical Trial. A Study of Melphalan Flufenamide (Melflufen)-Dex Or Pomalidomide-Dex For RRMM Patients Refractory to Lenalidomide. Available at: https://clinicaltrials.gov/ct2/show/NCT03151811?term=melflufen&cond=Multiple+Myeloma&draw=2&rank=3 [Accessed 25 May 2020].

Nijhof, I.S., N. van de Donk, S. Zweegman, and H.M. Lokhorst. 2018. Current and New Therapeutic Strategies for Relapsed and Refractory Multiple Myeloma: An Update. Drugs, 78:19-37. doi: 10.1007/s40265-017-0841-y.

Pemovska, T., M. Kontro, B. Yadav, H. Edgren, S. Eldfors, A. Szwajda, H. Almusa, M.M. Bespalov, P. Ellonen, E. Elonen, B.T. Gjertsen, R. Karjalainen, E. Kulesskiy, S. Lagström, A. Lehto, M. Lepistö, T. Lundán, M.M. Majumder, J.M.L. Marti, P. Mattila, A. Murumägi, S. Mustjoki, A. Palva, A. Parsons, T. Pirttinen, M.E. Rämet, M. Suvela, L. Turunen, I. Västrik, M. Wolf, J. Knowles, T. Aittokallio, C.A. Heckman, K. Porkka, O. Kallioniemi, and K. Wennerberg. 2013. Individualized systems medicine strategy to tailor treatments for patients with chemorefractory acute myeloid leukemia. Cancer Discov. 3:1416-1429. doi: 10.1158/2159-8290.CD-13-0350.

Pérez-Persona, E., M. Vidriales, G. Mateo, R. García-Sanz, M. Mateos, A.G. de Coca, J. Galende, G. Martín-Nuñez, J.M. Alonso, N. de Las Heras, J.M. Hernández, A. Martín, C. López-Berges, A. Orfao, and J.F. San Miguel. 2007. New criteria to identify risk of progression in monoclonal gammopathy of uncertain significance and smoldering multiple myeloma based on multiparameter flow cytometry analysis of bone marrow plasma cells. Blood. 110:2586-2592. doi: 10.1182/blood-2007-05-088443.

Rajkumar S.V. 2008. Treatment of myeloma: cure vs control. Mayo Clin. Proc. 83: 1142-1145. doi: 10.4065/83.10.1142

Rajkumar, S.V. 2018. Multiple myeloma: 2018 update on diagnosis, risk-stratification, and management. Am. J. Hematol. 93:1091-1110. doi: 10.1002/ajh.25117.

Rajkumar, S.V., M.A. Dimopoulos, A. Palumbo, J. Blade, G. Merlini, M. Mateos, S. Kumar, J. Hillengass, E. Kastritis, P. Richardson, O. Landgren, B. Paiva, A.

Dispenzieri, B. Weiss, X. LeLeu, S. Zweegman, S. Lonial, L. Rosinol, E. Zamagni, S. Jagannath, O. Sezer, S.Y. Kristinsson, J. Caers, S.Z. Usmani, J.J. Lahuerta, H.E. Johnsen, M. Beksac, M. Cavo, H. Goldschmidt, E. Terpos, R.A. Kyle, K.C. Anderson, B.G.M. Durie, and J.F.S. Miguel. 2014. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. Lancet Oncol. 15:538. doi: 10.1016/S1470-2045(14)70442-5.

Ray, A., D. Ravillah, D.S. Das, Y. Song, E. Nordström, J. Gullbo, P.G. Richardson, D. Chauhan, and K.C. Anderson. 2016. A novel alkylating agent Melflufen induces irreversible DNA damage and cytotoxicity in multiple myeloma cells. Br. J. Haematol. 174:397-409. doi: 10.1111/bjh.14065.

Richardson, P.G., S. Bringhen, P. Voorhees, T. Plesner, U.H. Mellqvist, B. Reeves, C. Paba-Prada, H. Zubair, C. Byrne, D. Chauhan, K. Anderson, E. Nordström, J. Harmenberg, A. Palumbo, and P. Sonneveld. 2020. Melflufen plus dexamethasone in relapsed and refractory multiple myeloma (O-12-M1): a multicentre, international, open-label, phase 1-2 study. Lancet. Haematol. 7:e395–e407. doi: 10.1016/S2352-3026(20)30044-2.

Roy, A., J.K. Kish, L. Bloudek, D.S. Siegel, S. Jagannath, D. Globe, E.T. Kuriakose, and K. Migliaccio-Walle. 2015. Estimating the Costs of Therapy in Patients with Relapsed and/or Refractory Multiple Myeloma: A Model Framework. Am. Health. Drug Benefits. 8:204–215.

Saadeh, C., D. Bright, and D. Rustem. 2019. Precision Medicine in Oncology Pharmacy Practice. Acta Med. Acad. 48:90-104. doi: 10.5644/ama2006-124.246.

Saarela, J., Kulesskiy, E., Laamanen, K., Pemovska, T., Pietiäinen, V., Potdar, S., von Schantz-Fant, C., Turunen, L., Östling, P. and Wennerberg, K., 2014. A personalised medicine drug sensitivity and resistance testing platform and utilisation of acoustic droplet ejection at the Institute for Molecular Medicine Finland. Synergy, 1:78.

Siegel, R.L., K.D. Miller, and A. Jemal. 2015. Cancer statistics, 2015. CA. Cancer J. Clin. 65:5-29. doi: 10.3322/caac.21254.

Snijder, B., G.I. Vladimer, N. Krall, K. Miura, A.S. Schmolke, C. Kornauth, O. Lopez de la Fuente, H.S. Choi, E. van der Kouwe, S. Gültekin, L. Kazianka, J.W. Bigenzahn, G. Hoermann, N. Prutsch, O. Merkel, A. Ringler, M. Sabler, G. Jeryczynski, M.E. Mayerhoefer, I. Simonitsch-Klupp, K. Ocko, F. Felberbauer, L. Müllauer, G.W. Prager, B. Korkmaz, L. Kenner, W.R. Sperr, R. Kralovics, H. Gisslinger, P. Valent, S. Kubicek, U. Jäger, P.B. Staber, and G. Superti-Furga. 2017. Image-based *ex-vivo* drug screening for patients with aggressive haematological malignancies: interim results from a single-arm, open-label, pilot study. Lancet. Haematol. 4:e595-e606. doi: 10.1016/S2352-3026(17)30208-9.

Sonneveld, P., and A. Broijl. 2016. Treatment of relapsed and refractory multiple myeloma. Haematologica. 101:396-406. doi: 10.3324/haematol.2015.129189.

Strese, S., S.B. Hassan, E. Velander, C. Haglund, M. Höglund, R. Larsson, and J. Gullbo. 2017. *In vitro* and *in vivo* anti-leukemic activity of the peptidase-potentiated alkylator melflufen in acute myeloid leukemia. Oncotarget. 8:6341-6352. doi: 10.18632/oncotarget.13856.

Therneau, T.M., R.A. Kyle, L.J. Melton, D.R. Larson, J.T. Benson, C.L. Colby, A. Dispenzieri, S. Kumar, J.A. Katzmann, J.R. Cerhan, and S.V. Rajkumar. 2012. Incidence of Monoclonal Gammopathy of Undetermined Significance and Estimation of Duration Before First Clinical Recognition. Mayo Clin. Proc. 87:1071-1079. doi: 10.1016/j.mayocp.2012.06.014.

Trippoli, S., A. Messori, P. Becagli, R. Alterini, and E. Tendi. 1998. Treatments for newly diagnosed multiple myeloma: analysis of survival data and cost-effectiveness evaluation. Oncol. Rep. 5:1475-1482. doi: 10.3892/or.5.6.1475.

Viktorsson, K., C. Shah, T. Juntti, P. Hååg, K. Zielinska-Chomej, A. Sierakowiak, K. Holmsten, J. Tu, J. Spira, L. Kanter, R. Lewensohn, and A. Ullén. 2016. Melphalan-flufenamide is cytotoxic and potentiates treatment with chemotherapy and the Src inhibitor dasatinib in urothelial carcinoma. Mol Oncol. 10:719-734. doi: 10.1016/j.molonc.2015.12.013.

Volm, M., and T. Efferth. 2015. Prediction of Cancer Drug Resistance and Implications for Personalized Medicine. Front Oncol. 5:282. doi: 10.3389/fonc.2015.00282

Wickström, M., C. Haglund, H. Lindman, P. Nygren, R. Larsson, and J. Gullbo. 2008. The novel alkylating prodrug J1: diagnosis directed activity profile *ex vivo* and combination analyses *in vitro.* Invest. New Drugs. 26:195-204. doi: 10.1007/s10637-007-9092-1.

Wickström, M., K. Viktorsson, L. Lundholm, R. Aesoy, H. Nygren, L. Sooman, M. Fryknäs, L.K. Vogel, R. Lewensohn, R. Larsson, and J. Gullbo. 2010. The alkylating prodrug J1 can be activated by aminopeptidase N, leading to a possible target directed release of melphalan. Biochem. Pharmacol. 79:1281-1290. doi: 10.1016/j.bcp.2009.12.022.

Wickström, M., P. Nygren, R. Larsson, J. Harmenberg, J. Lindberg, P. Sjöberg, M. Jerling, F. Lehmann, P. Richardson, K. Anderson, D. Chauhan, and J. Gullbo. 2017. Melflufen - a peptidase-potentiated alkylating agent in clinical trials. Oncotarget. 8:66641-66655. doi: 10.18632/oncotarget.18420.

Wickström, M., R. Larsson, P. Nygren, and J. Gullbo. 2011. Aminopeptidase N (CD13) as a target for cancer chemotherapy. Cancer Sci. 102:501-508. doi: 10.1111/j.1349-7006.2010.01826.x.

Yadav, B., T. Pemovska, A. Szwajda, E. Kulesskiy, M. Kontro, R. Karjalainen, M.M Majumder, D. Malani, A. Murumägi, J. Knowles, K. Porkka, C.A. Heckman, O. Kallioniemi, K. Wennerberg, and T. Aittokallio. 2014. Quantitative scoring of differential drug sensitivity for individually optimized anticancer therapies. Sci. Rep. 4:5193. doi: 10.1038/srep05193.

Zhang, J.J., W.J. Sun, Z.X. Huang, S.L. Chen, Y.P. Zhong, Y. Hu, N. An, M. Shen, and X. Li. 2014. Light chain multiple myeloma, clinic features, responses to therapy and survival in a long-term study. World J. Surg. Oncol. 12:234-237. doi: 10.1186/1477-7819-12-234.

8 Appendices

Appendix 1. A list of tested samples and selection criteria for data analysis. Out of 51 tested samples, 16 were chosen for further data analysis based on reasonable cell viability (>25%) and number of malignant plasma cells (PCs) in DMSO wells (>50 cells). Moreover, samples should have had enough malignant PCs in the drug wells for creating the robust dose-response curves. Also, samples were not chosen for data analysis if there were problems with antibody staining or flow cytometer run. NA= not available. DG=diagnosis, RRMM=relapsed and refractory multiple myeloma.

Sample ID	Flow-based viability on day 3 (%)	Average number of PCs in DMSO well on day 3	Comments
MM01	31.8	138.4	Chosen for data analysis
MM02	35.9	1708	
MM03	37.8	360.8	
MM04_DG	49.8	6912.6	
MM04 RRMM	50.4	53.6	
MM06	68.4	850.8	
MM07	63.4	2092.9	
MM08	47.8	257.4	
MM09	54.1	559.3	
MM10	29.9	558.3	
MM11	57.0	1777.4	
MM12	40.1	2430.7	
MM13	50.6	153.2	
MM14	61.5	70.3	
MM15	79.0	220.4	
MM16	53.7	1636	
mean	**50.7**	**1236.26**	
MM17	38.5	38.5	Low number of PCs
MM18	50.7	9	
MM19	26.6	27.3	
MM20	25.4	2.6	
MM21	45.2	3.1	
MM22	38.6	32.1	
MM23	15.5	127.6	Poor viability
MM24	8.4	625.4	
MM25	12.1	32.7	Low number of PCs and poor viability
MM26	11.4	15.1	
MM27	13.9	6.1	
MM28	0.0	11	
MM29	13.4	7.9	
MM30	21.0	2	
MM31	13.9	19.9	
MM32	NA	NA	Low number of PCs and poor viability already on day 0, and thus DSRT was not run on day 3 for treated cells
MM33	NA	NA	
MM34	NA	NA	
MM35	NA	NA	
MM36	NA	NA	
MM37	NA	NA	
MM38	73.3	110.3	Variable number of cells in drug wells causing problems for determining dose response
MM39	37.5	324.3	
MM40	68.2	83	
MM41	35.5	345.9	
MM42	18.0	NA	

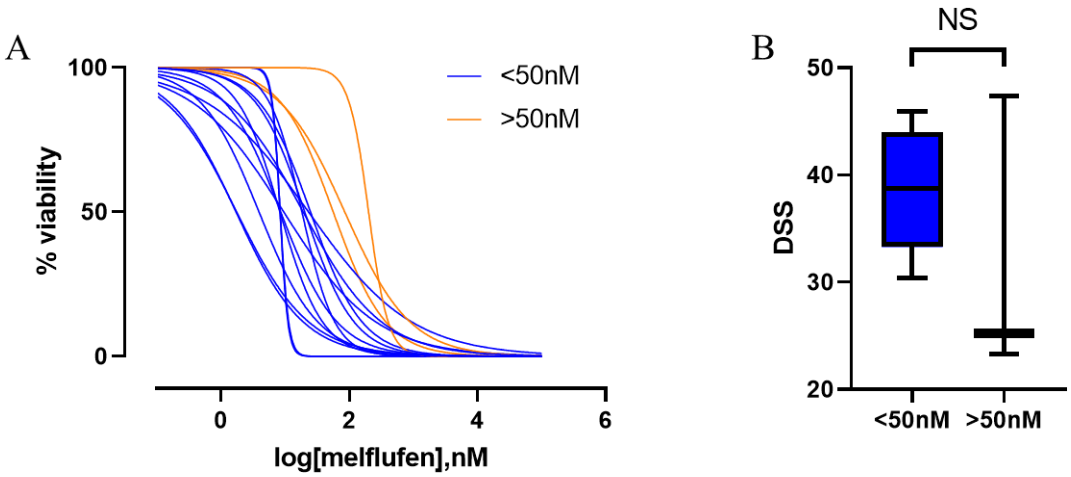
MM43	53.0	NA	Variable number of cells in DMSO wells leading to problems with normalization
MM44	27.4	NA	Problems with antibody staining
MM45	33.9	NA	
MM46	35.9	176.4	
MM47	55.0	1430.5	
MM48	NA	NA	Clog during flow run
MM49	NA	NA	
MM50	41.2	218.3	
MM51	53.2	134.3	

Appendix 2. Detailed clinical characteristics of each patient. R=RRMM=relapsed and refractory multiple myeloma, D=DG=diagnosis, F=female M=male, ISS=international staging system, IgH=immunoglobulin heavy chain, IgL=immunoglobulin light chain, NA=not available.

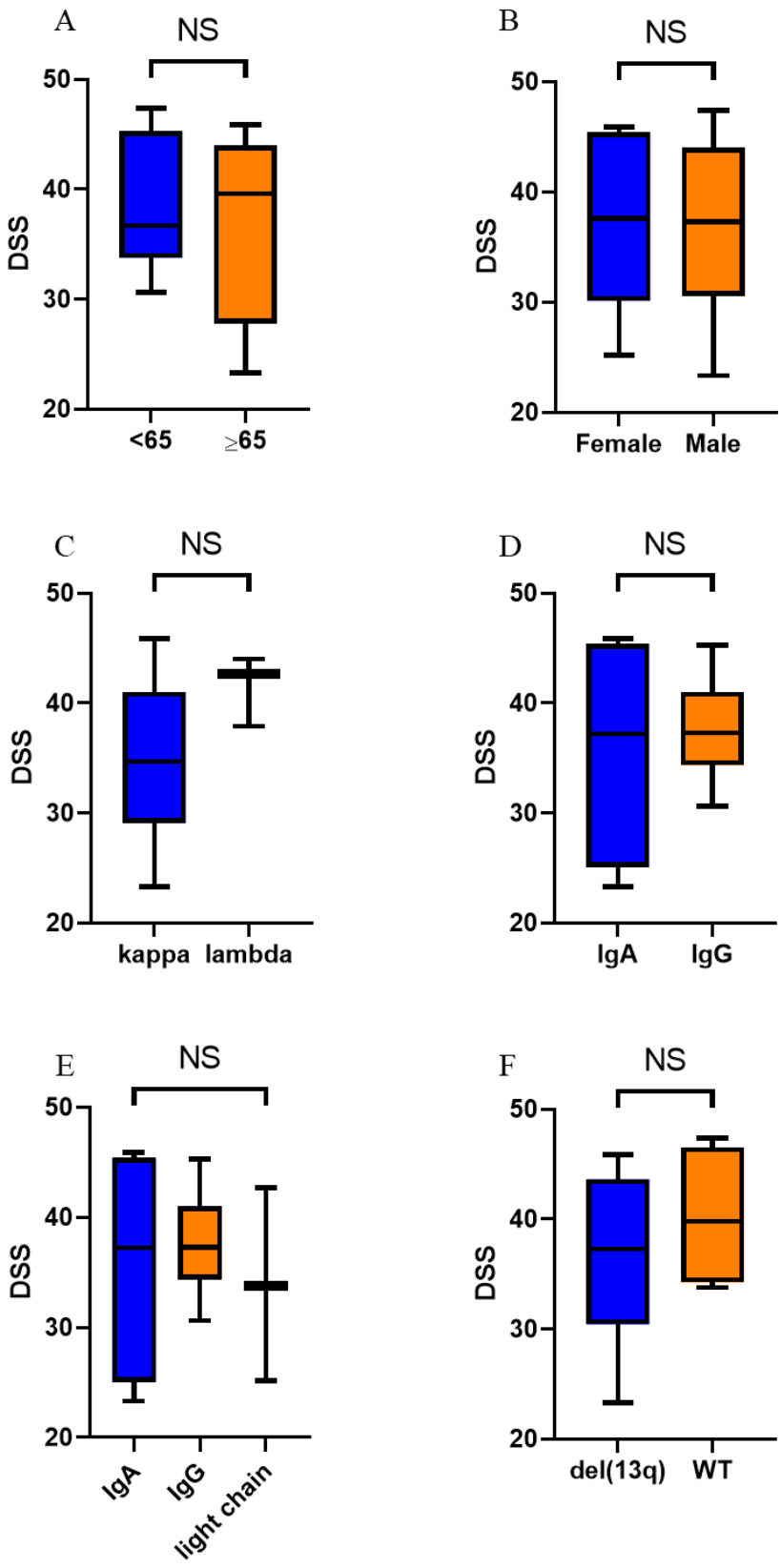
Sample ID	Age at diagnosis	Disease status	Gender	Treatment line	ISS	IgH	IgL	del (13q)	gain (1q)	High-risk cytogenetics
MM01	61	R	M	1	1	NA	kappa	NA	x	del17p
MM02	51	R	M	1	3	IgG	kappa	x	x	t(4;14)
MM03	74	R	M	4	2	NA	NA	x	x	none
MM04 DG	77	D	M	0	2	IgA	kappa	x	NA	both
MM04 RRMM	77	R	M	5	2	IgA	kappa	x	NA	both
MM06	58	R	F	1	2	IgG	kappa	x	x	del17p
MM07	77	R	M	5	1	NA	lambda	x	x	del17p
MM08	55	R	F	2	NA	IgG	kappa	x	NA	none
MM09	73	R	M	3	NA	IgA	lambda	x	x	none
MM10	37	R	M	9	NA	NA	NA	x	x	t(4;14)
MM11	69	D	F	1	3	NA	kappa	x	x	none
MM12	70	D	F	0	3	NA	NA	x	x	none
MM13	77	D	F	2	3	IgA	kappa	x	x	none
MM14	71	D	F	1	2	IgG	kappa	x	x	none
MM15	58	D	M	0	NA	IgG	kappa	x	x	t(4;14)
MM16	55	D	M	0	2	IgG	lambda	x	x	t(4;14)

Appendix 3. Drug sensitivity scores (DSSs) of melflufen and melphalan in malignant plasma cells (PCs) and lymphocytes. *=statistically significant difference.

Drug	Cell population	DSS (SD)	*p*
Melflufen	Malignant PCs	37.14 (7.51)	<0.0001*
Melphalan		15.47 (4.76)	
Melflufen	Malignant PCs	37.14 (7.51)	0.0002*
	Lymphocytes	40.27 (7.22)	
Melphalan	Malignant PCs	15.47 (4.76)	0.9460
	Lymphocytes	15.51 (3.51)	



Appendix 4. The stratified responses of melflufen by IC50 values in malignant plasma cells. (A) The responses of melflufen in malignant plasma cells from MM patient samples were stratified based on IC50 values into more (<50nM, blue) and less (>50nM, orange) sensitive groups. (B) When drug sensitivity scores (DSSs) of melflufen between these sensitivity groups were compared, significant difference was not detected (*p*=0.1599). NS=not significant.



Appendix 5. The responses of melflufen in malignant plasma cells among patients stratified by age at diagnosis, gender, immunoglobulin light and heavy chains and presence of del(13q) aberration. MM patients were stratified based on (A) age at diagnosis (<65 years and ≥65 years old), (B) gender (females and males), (C) immunoglobulin light (kappa and lambda) and (D) heavy chains (IgA and IgG), (E) presence of immunoglobulins (IgG, IgA or light chain only), and (F) presence of del(13q) aberration. Although all patients seemed to be sensitive to melflufen, significant differences between subgroups were not detected (p=0.6387, 0.9700, 0.1644, 0.7380, 0.8016 and 0.3638, respectively). DSS=drug sensitivity score, NS=not significant, WT=wild type.