

**Charles University  
Faculty of Medicine in Hradec Králové**

**Doctoral Degree Programme  
Internal Medicine**

**Unfavourable Biological Prognostic Factors  
in Multiple Myeloma**

**Nepříznivé biologické prognostické faktory  
u mnohočetného myelomu**

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Hradec Králové, 2019

## **Author's Declaration**

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### **Declaration:**

I declare hereby that this dissertation thesis is my own original work and that I indicated by references all used information sources. I also agree with depositing my dissertation in the Medical Library of the Charles University, Faculty of Medicine in Hradec Králové and with making use of it for study and educational purpose provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

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**Hradec Králové, 2019**

**Utkarsh Painuly, M.D.**

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## 0.0 KEY

ActRIIA – Activin A receptor type IIA  
ADCC – Antibody-dependent cellular cytotoxicity  
AKI – Acute kidney injury  
ANXA2 – Annexin A2  
ASC – Antigen secreting cell  
ASCT – Autologous stem cell transplantation  
ASO-PCR – Allele specific polymerase chain reaction  
ATRA – All-trans retinoic acid  
BCL – B-cell lymphoma  
BFGF – Basic fibroblast growth factor  
BM – Bone marrow  
BMA – Bone marrow aspirate  
BMSC – Bone marrow stromal cell  
BP – Bendamustine, Prednisolone  
BTK – Burton's tyrosine kinase  
CAM-DR – Cell adhesion mediated drug resistance  
CARD11 – Caspase recruitment domain-containing protein 11  
CBF – Core binding factor  
CCL-3 – Chemokine (C-C motif) ligand 3(CCL3)  
CD – Cluster of differentiation  
CDKN2A – Cyclin dependent kinase inhibitor 2A  
Cereblon – Cerebral protein with ion protease  
cIAP – Cellular inhibitor of apoptosis protein  
CKD – Chronic kidney disease  
CKD-EPI – Chronic kidney disease epidemiology collaboration  
COT- Combination therapy  
CR – Complete response  
Cr – Creatinine  
CrCl – Creatinine clearance  
CSR – Class switch recombination  
CTD – Cyclophosphamide, thalidomide and dexamethasone

CXCR – Chemokine receptor  
CXCR – Ligand receptor CXC-family  
CYLD – Cylindromatosis  
DaraRd – Daratumumab, lenalidomide, dexamethasone,  
DaraVD – Daratumumab, bortezomib, dexamethasone  
DaraVD – Daratumumab, bortezomib, dexamethasone  
DEP – Dishevelled, EGL-10 and pleckstrin  
DEPTOR – DEP domain containing TOR interacting protein  
DH – Diversity domain  
Dkk – Dickkopf-1  
DSS – Durie salmon staging  
DVL – Dishevelled [Dsh] homolog  
DVT – Deep vein thrombosis  
EBI – Erythroblastic islands  
ECM – Extracellular matrix  
eGFR – Estimated glomerular filtration rate  
EloRd – Elotuzumab, lenalidomide, dexamethasone  
EloVD – Elotuzumab, bortezomib, dexamethasone  
EMD – Extramedullary disease  
EPO – Erythropoietin  
ERK – Extracellular signal-regulated kinase  
ESA – Erythrocyte stimulating agent  
FBXO – f box only protein  
FCB – Follicular B cell  
FGFR3 – Fibroblast growth factor receptor  
FISH – Fluorescence in-situ hybridization  
FL – Fas ligand  
GATA – Erythroid transcription factor  
GC – Germinal centre  
GCSF – Growth colony stimulation factor  
GDP – Guanosine diphosphate  
GSK3 – Glycogen synthase kinase-3  
GTP – Guanosine triphosphate  
GWAS – Genome wide association studies

HDAC – Histone deacetylase inhibitor  
HDT – High dose therapy  
HGF – Hepatocyte growth factor  
HIF – Hypoxia induced factor  
HOMOX1 – Haem oxygenase 1  
HSP – Heat shock protein  
Hyper-CVAD- Cyclophosphamide, vincristine, doxorubicin, dexamethasone  
ICAM – Intracellular adhesion molecule  
iCR – Immunophenotypic complete response  
IF – Immunofixation  
iFISH – Interphase fluorescence hybridization  
Ig – Immunoglobulin  
IGF – Insulin like growth factor  
IHC – Immunohistochemistry  
IKBIP – Inhibitor of nuclear factor kappa-B kinase  
IL- Interleukin  
IL6R – Interleukin 6 receptor  
IMiD – Immunomodulatory  
IMWG – International Myeloma Work Group Diagnostic criteria  
INF – Interferon  
IRS – Insulin receptor substrate  
ISS – International staging system  
IXaRd – Ixazomib, lenalidomide, dexamethasone  
JH – Joining domain  
JNK – c-Jun N-terminal kinase  
JNK – Jun N-terminal kinase  
KD – Carfilzomib dexamethasone  
Kd – Carfilzomib, dexamethasone  
KRD – Carfilzomib lenalidomide dexamethasone  
KRd – Carfilzomib, lenalidomide, dexamethasone  
LCDD – Light chain deposition disease  
LDH – Lactate dehydrogenase  
LFE – Lymphoid enhancer factor  
LGALS1 – Galectin-1

LTBR – lymphotoxin beta receptor  
mAb – Monoclonal antibody  
MAF – Musculoaponeurotic fibrosarcoma  
MAPK – Mitogen activated protein kinases  
mCR – Molecular complete response  
MDE – Myeloma defining event  
MDSC – Myeloma derived suppressor cell  
MGUS – Monoclonal gammopathy of undetermined significance  
MHC – Major histocompatibility  
MIP – Macrophage inflammatory protein  
miRNA – MicroRNA  
MM – Multiple myeloma  
MM – Multiple Myeloma  
MMPC – Multiple myeloma plasma cells  
MMSET – Multiple myeloma SET domain  
MP – Melphalan, Prednisolone  
MPF – Multiparametric flowcytometry  
MPT – Melphalan, prednisolone, thalidomide  
MRD – Minimal residual disease  
MRDR – Modification of diet in renal disease  
mTOR – Mammalian target of rapamycin/mechanistic target of rapamycin  
MTORC – Mammalian target of rapamycin complex  
MVD – Micro-vessel density  
MZB – Marginal zone B cell  
NF-kB – Nuclear factor kappa light chain enhancer of B cell  
NGF – Next generation flowcytometry  
NGS – Next generation sequencing  
NHBCL – Non Hodgkin b-cell lymphoma  
NIK – NF-kappa-B-inducing kinase  
NK -Natural killer  
NKG2D – Natural killer group 2D  
NOD/SCID – Non-obese diabetic/severe combined immunodeficiency  
OB – Osteoblast  
OC – Osteoclast

OP – Osteoprotegerin  
OPN – Osteopontin  
OS – Overall survival  
PAD – Bortezomib, doxorubicin, dexamethasone  
PanoVD – Panobinostat, bortezomib, dexamethasone  
PARP – Poly-ADP ribose polymerase  
PC – Plasma cells  
PCL – Plasma cell leukaemia  
PD – Progressive disease  
PFS – Progression free survival  
PI – Proteasome inhibitor  
PI3K – Phosphatidylinositol 3-kinase  
PKC – Protein kinase C  
PLC $\gamma$  - Phospholipase C gamma  
PSGL – P-selectin glycoprotein ligand  
PSMB4 – Proteasome subunit beta type-4  
PSMD3 – 26S proteasome non-ATPase regulatory subunit 3  
PTN – Pleiotrophin  
RAC – Ras-related C3 botulinum toxin substrate 1  
RAG – Recombinant activating genes  
RANK – Receptor activator of nuclear factor  $\kappa$  B  
RANK – Receptor activator of nuclear factor  $\kappa$ B  
RANKL – Receptor activator of nuclear factor  $\kappa$ B ligand  
RB – Retinoblastoma  
RD – Revlimid, dexamethasone  
RIFLE – Risk, injury, failure, loss and end stage kidney disease criteria  
RIPK – Receptor-interacting serine/threonine-protein kinase  
RNA – Ribonucleic acid  
RTK – Receptor tyrosine kinase  
RTK – Receptor tyrosine kinase  
SCID – Severe combined immunodeficiency  
sCR – Stringent complete response  
sFLC – Serum free light chain



SLAMF7 – Signalling lymphocytic activation molecule F7  
SMAD – Mothers against decapentalegic homolog  
sMICA – shedding of MHC class I chain related protein A  
SMM – Smouldering MM  
SMM – Smouldering multiple myeloma  
sMZL – Splenic marginal zone lymphoma  
SNP – Single nucleotide polymorphisms  
SPEP – Serum protein electrophoresis  
STAT – Signal transducer and activator of transcription  
TAC1 – Transmembrane activator and calcium modulator and cyclophilin ligand interactor  
TAM – Tumour associated macrophages  
TCF – T cell factor  
TGF – Tissue growth factor  
TGF – Transforming growth factor  
Th – T-helper  
TIAM – T-cell lymphoma invasion and metastasis-inducing protein  
TLR – Toll like receptor  
TLR – Toll like receptor  
TNF – Tumour necrosis factor  
TNFR – Tumour necrosis factor receptor  
TRAF- TNF receptor associated factor  
TRAIL – Tumour necrosis factor-related apoptosis-inducing ligand  
Treg – T- regulatory  
TT3 – Total therapy three  
TTP – Time to progression  
UBE2Q1 – Ubiquitin-conjugating enzyme E2 Q1  
UPR – Unfolded protein response  
VAD- Vincristine, doxorubicin, dexamethasone  
VCAM – Vascular cell adhesion molecule  
VCD – Bortezomib, cyclophosphamide, dexamethasone  
VD – Bortezomib, dexamethasone  
VEGF – Vascular endothelial growth factor  
VGPR – Very good partial response

VH – Variability domain

VLA - Very late antigen

VMCP- Vincristine, cyclophosphamide, melphalan, prednisolone

VMP – Bortezomib, melphalan, prednisolone

VRD – Bortezomib, lenalidomide, dexamethasone

VTD – Bortezomib, thalidomide, dexamethasone

WNT – Wingless and integration pathway

XIAP – X-linked inhibitor of apoptosis

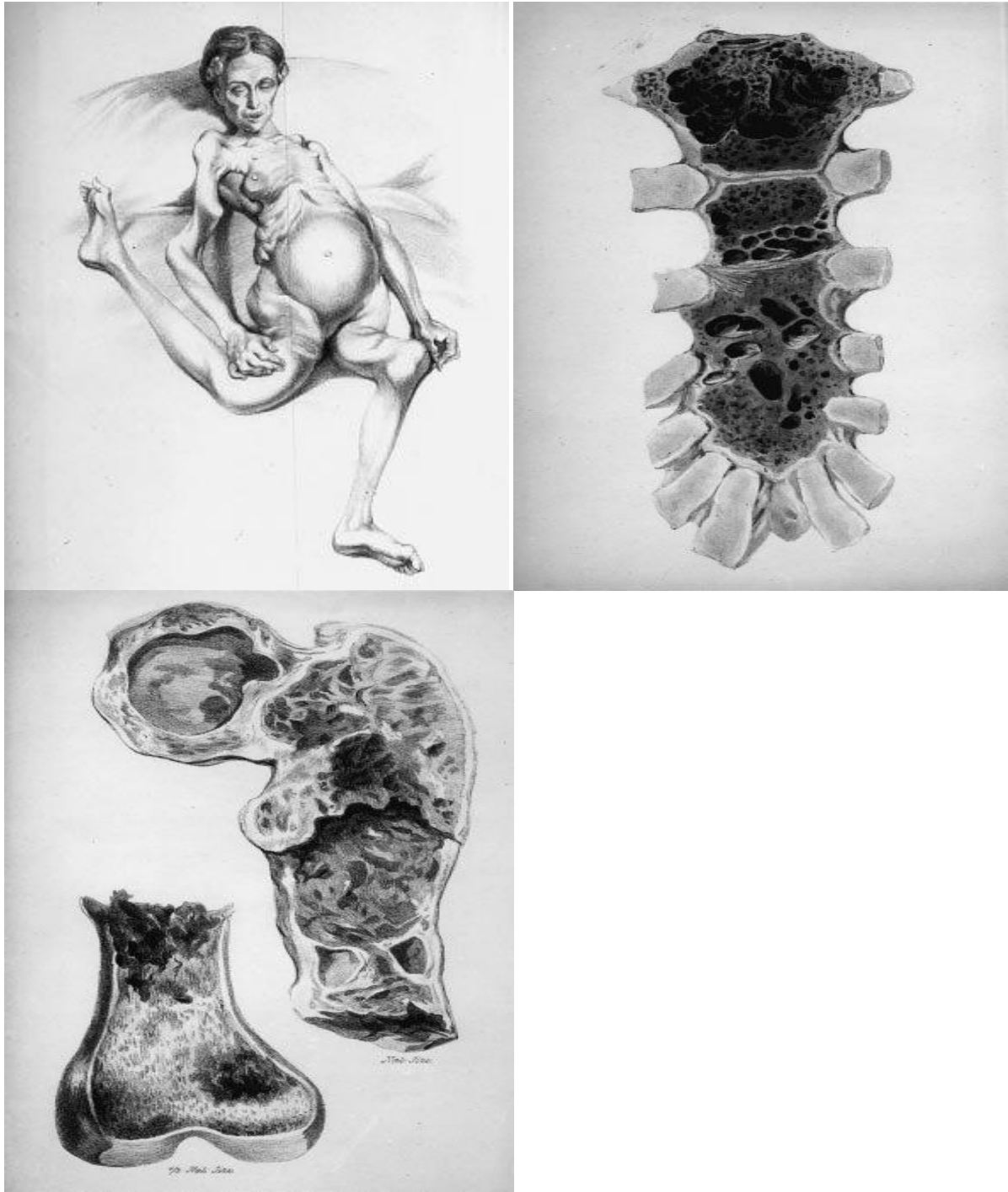
## 1.0 A brief history of Multiple Myeloma (MM)

### 1.1 History of MM:

The understanding of biology and subsequent determination of appropriate treatment has seen a paradigm shift in Multiple Myeloma. The first recorded case is well noted during the 19<sup>th</sup> century by Dr Solly where post-mortem examination of a 39-year-old female Sarah Newbury revealed multiple bone fractures, bone deformities and replacement of marrow with grumous material which did not resemble or contain pus. Dr Solly and Mr Birkett of Guy's Hospital further noted that the cells in this grumous material were nucleated with oval outlines and one or occasional two bright central nucleoli.



**Fig.1: An early drawing of plasma cells(PC): Dalrymple, John. On the Microscopical Character of Mollities Ossium. 1846 [2]**



**Fig.2: Sarah Newbury with a) bone deformities and fractures. b) and c) bony destruction of sternum and femur from Solly 1884 [3]**

While a similar case of fragile and brittle bones was being reviewed, by Dr McIntyre, a general practitioner at Harley Street, London of Mr McBean; a reputed 44 year old grocer experienced polyuria with nocturnal accidents which stiffened his linens with no urethral discharge otherwise. Mr McBean passed away three years later and autopsy

revealed fragile bones, with bone marrow of a gelatinous consistency. Presenting with his symptoms, Dr McIntyre also noted increased specific gravity of urine, predominantly attributed to viscous albumin like substance which stiffened the body linen of the subject.

Dr Henry Bence Jones, a renowned chemical pathologist at St. George's Hospital in London, who analysed the urine sample, exerted this substance to be a hydrated deuterioxide of albumen with a high index of suspicion as part of a more extensive disease process. Mr John Dalrymple, a surgeon at the Royal Ophthalmic Hospital Moorfields, London examined Mr Mcbean's diseased bone tissue and microscopically noted the presence of oval cells which were 1.5-2 times larger than red cells, with two nuclei each with a nucleolus. This was assumedly plasma cell(PC) description; however, the term itself was coined in Waldeyer in 1875 although the described cells were likely tissue mast cells.

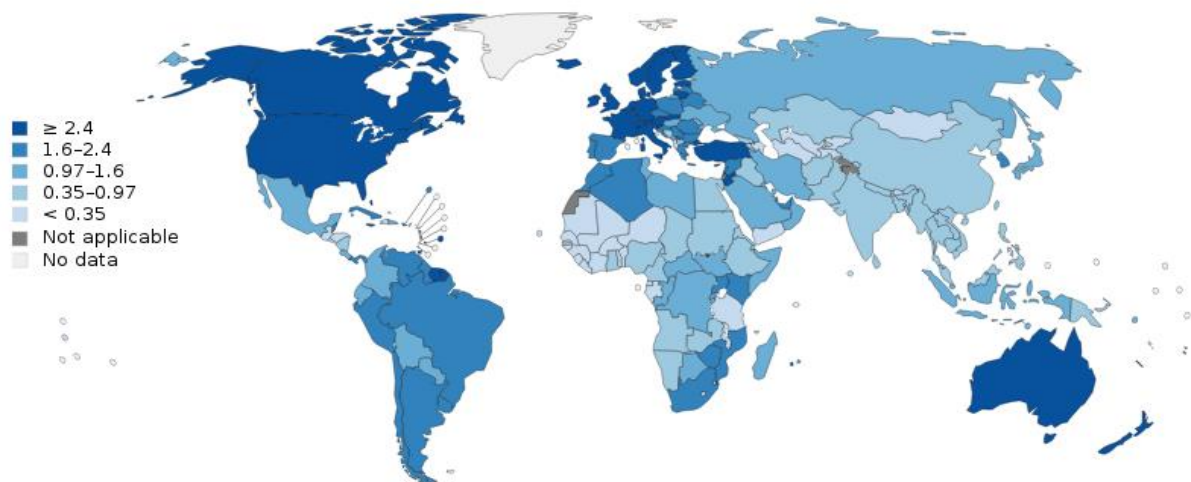
It was in the 1890s when an accurate description of PC with a typical microscopic appearance of an eccentrically positioned nucleus, a perinuclear pale area 'hof' and irregular cytoplasm was characterised. Bence Jones proteins were increasingly identified as abnormal related to the disease process, independent of dietary intake of proteins in early 20<sup>th</sup>-century being of two different types (1922 Bayne Jones and Williams). It was Korngold and Lipari (1956) who identified the presence of urine free light chains with kappa and lambda light chains attributed to them for their work. Edelman and Gally demonstrated the identical amino acid composition of light chains prepared from Immunoglobulin and the Bence Jones protein from the same patient.

## 1.2 Introduction

MM is a PC proliferation disorder characterised by a monoclonal population of terminally differentiated mature B cells which presents with disease-related features including anaemia, kidney insufficiency, bone lesions and hypercalcemia.

### 1.2.1 Epidemiology

The global incidence of MM is about 139 per 1000 cases with about 98 per 1000 deaths worldwide is also attributable to MM[4]. In the countries with high, middle and low socio-demographic index it ranks 21<sup>st</sup>, 27<sup>th</sup> and 24<sup>th</sup> most common cancer in both sexes[5].



**Fig3: Estimated age-standardized rates (World) of incident cases in both sexes with MM, worldwide in 2012(<http://gco.iarc.fr/today>). Data source: GLOBOCAN 2012[6]**

It is the 14<sup>th</sup> most common malignancy and the second most common hematological malignancy that accounts for 1 to 2% of all cancers in the United States and has an annual incidence of approximately 4-5 per 100,000 in the developed world and the mortality of 4.1/100,000/year[7, 8]. It is more common in the population over 60 years

of age with about 10 % population under 50 and 2 % under 40 years at diagnosis. The median age at diagnosis is 69, and the median age at death is 75. The estimated new cases in the United States were 30,770 in 2018 with approximately 12,700 deaths secondary to the disease accounting for 2.1 percent of all cancer-related mortality. Encouragingly the death rates were falling by 0.5% each year between 2006-2015 with 5-year relative survival percent at 53 in 2015 compared to 26.3 % in 1975[7]. The prevalence of MM IN US was estimated at 124,733 in 2015[7].

5,540 (two percent) of all newly diagnosed cancer cases in the United Kingdom were of MM in 2015 with almost 45% of new cases diagnosed in people over 75 years of age. Increased disease incidence rates are noted between 1992-1995 and 2013 to 2015 by thirty-two percent with a more considerable increase in the male population. An estimated 11% increase in the UK between 2014 and 2035 is projected for MM incidence. Encouragingly overall survival has seen a fourfold increase in the UK in the last 40 years with thirty-three percent surviving 10 or more years[9].

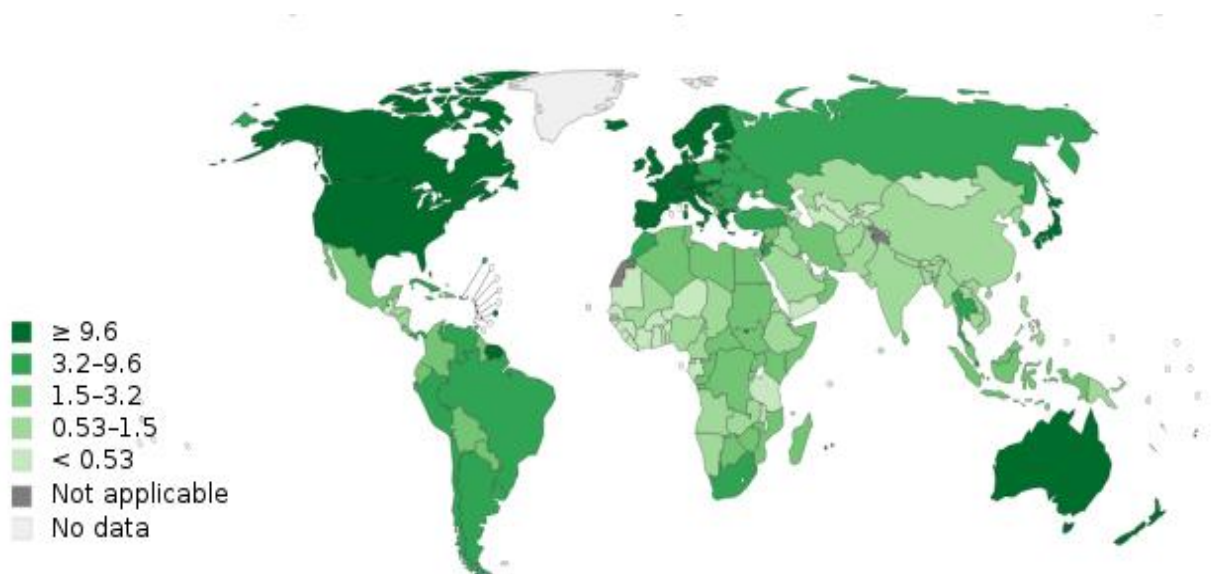
Asian countries were initially noted to have a relatively lower incidence of MM. However, newer studies indicate a rise in incidence and disease-related mortality. Taiwan noted a fourfold increase in the incidence of MM [10]while Korea reported a tenfold increase in 2012[11] making it possibly the second most common hematological malignancy in the country[11]. The higher disease incidence and prevalence is likely indicative of improved health care and access to advanced diagnostics.

In Latin America, while the incidence of MM appears similar to the overall incidence, decreasing incidence trends were reported in Ecuador (particularly for women) and Costa Rica[12]. Sub-Saharan Africa potentially under-reports its true incidence of MM in population given limited and skewed cancer registries available from these countries[13]. There is a higher incidence of MGUS in African Americans compared to the white population suggestive of possible similar results for the black population in Africa[14]

The incidence of MM is slightly higher in men when compared to women (1.4:1). MM is the most common hematological malignancy in the Black population along with earlier age of onset and 2-3 times higher incidence of the disease when compared to

the white population[14].

The prevalence of MM has also significantly increased. Reports from Sweden and Denmark show approximately a threefold increased disease prevalence between the 1980s until post immunomodulatory era (Ref 6, 46). This could partly be due to increased patient survival and further autologous haematopoietic stem cell transplantations in eligible patients.



**Fig4: Estimated number of prevalent cases (5-year), both sexes, multiple myeloma, worldwide in 2012(<http://gco.iarc.fr/today>). Data source: GLOBOCAN 2012[6]**



## 1.3 Aetiology

There is increasing evidence supporting a hereditary form of MM. Familial cases of MM are well documented[15-17]. Studies have demonstrated familial clustering of monoclonal gammopathy of undetermined significance (MGUS) particularly in first degree relatives with increased prevalence with increasing age to 12% for related probands between 60-69 years of age[17, 18]. Several case reports have highlighted upon the increased risk of disease transmission particularly in first degree relatives, in African American/Black families and male relatives within these family[19]. Genome-wide association studies(GWAS) have identified several loci single nucleotide polymorphisms(SNP) which could potentiate the development of MM by enhancing transcription of proto-oncogenes such as MYC which are well known to be dysregulated in MM[20].

While initially speculated, no significant increased risk of MM was reported amongst survivors of the atomic bomb[21]. Increased risk of developing MM has also been associated with a high body mass index(BMI)[22]. The increased relative risk of 1.38 of MM is noted in farmers, but no significant occupational exposures, infections, pesticides or solvents could show to be causal[23]. Exposure to hair dyes has also been not shown to increase the risk of MM[24].

## 1.4 B cell maturation and differentiation

Once produced in the bone marrow, pro-B cells undergo first molecular differentiation with heavy chain immunoglobulin(Ig) gene(IGH) rearrangements. Stochastic DNA deletions first cause 1 of the 27 DH(diversity domain) segment to 1 of the 6 JH (Joining domain) segments. Pro-B cells which successfully undergo the first differentiation further differentiate by combining the formed DH-JH with the VH(variability domain) segment. Recombination activating genes(RAG) precisely regulate these rearrangements by pattern recognition of specific DNA segments within JH, DH and VH segments.

Formed Pre- B cells next, undergo rearrangements in the genes for the light chains Kappa( $\kappa$ ) and Lambda( $\lambda$ )(IGL $\kappa$  and IGL $\lambda$ ) with IGL $\lambda$  gene rearrangement occurs when a pre-B cell fails to undergo IGL $\kappa$  gene rearrangement correctly. Hence these Mature B cells express IgM $\kappa$  or IgM and exit the marrow after undergoing negative selection where surface IgM is mandatory to pass the checkpoint. Majority of mature B cells migrate to the spleen and lymph nodes where successive negative selections ensure the survival of specific mature B cells. These mature B cells are termed as Follicular B cells(FC B cells). A subset of mature B cells migrates to the marginal zone(MZ B cells) of the splenic sinus'. This convenient location facilitates an encounter with various bloodborne pathogens and antigens possible.

The smallest subset of remaining B cells termed as the B1 cells localises to the peritoneal and pleural mucosal regions monitoring these physiologically pathogen enriched sites. The MZ and B1 cells are inherently adept at responding to T cell independent antigens. They produce short-lived antibodies with fixed antigen affinity and form an essential early response to a foreign antigen; however, FC cells preferentially respond to antigens causing simultaneous CD4+ T helper cell activation.

Antigen exposure via dendritic or T helper cells activates the FC cells and forms the germinal centres(GC) - the site of somatic hypermutation. These selective mutations permit selective survival of FC B cells which produce high-affinity Ig's to the culprit antigen. This process leads another step in the development of antigen secreting

cells(ASC) called the B lymphoblasts which then undergo Ig class switch recombination(CSR) expressing different Ig's; IgA, IgE and IgD becoming mature B cells in the process. These mature B cells are crucial for the formation of PC or memory B cell differentiating to produce ASC's on repeat antigen exposure.

## **1.5 Pathogenesis**

### **1.5.1 Origin of clonal plasma cells(PC)**

Hamburger et al. first demonstrated the clonogenic MM patient PC's by successfully growing colonies in vitro on soft agar plates with an efficiency ranging from 0.1 to 0.001[25]. Since then, the origin of MM plasma cell(MMPC) disease has been much studied. Different studies have identified several possible origins of clonotypic MM cells. These clonal cells persist despite treatment and can be transplanted with use of growth colony stimulation factor(GCSF) mobilization into secondary hosts[26].

#### **1.5.1.1 Clonal B cell origin**

Identical Ig gene sequences throughout the course of MM is indicative that the disease originated likely after B cell somatically mutated and formed ASC's. Identification of B cells harbouring Ig gene sequence and identical idiotype to the individual myeloma clone raised the possibility of a post-germinal B cell origin of MM [27, 28]. Further work by Matsui et al. by injecting CD19+ clonotypic B cell population from the peripheral blood sample of MM patients into Non-obese diabetic/severe combined immunodeficiency(NOD/SCID) mice and producing a mature CD138+ monoclonal plasma cell population in the mice indicated a B memory cell type precursor of the disease. Further, the engrafted CD19+ B cells from mice could be secondarily engrafted and produce the disease phenotype in the secondary recipient[29]. This monoclonal CD19+ Memory B cell type phenotype is resident to bone marrow(BM) niche and relatively resistant to most commonly used anti-myeloma therapy[27, 30, 31] while showing sensitivity to Rituximab (anti CD20).

The population of circulating clonotypic B cells increases with disease relapse in MM patients and can persist despite achieving a significant absence of PC frequency in the BM post bortezomib and autologous stem cell transplantation(ASCT)[32]. Further studies have indicated with changes in the cellular environment such as increased hypoxia can reduce CD138 expression/ or instead selectively promote CD20, CXCR4 expressing stem cell-like phenotype. These cells with increased stem cell-like transcription factors sensitized to Bortezomib treatment post ATRA treatment[33]

### **1.5.1.2 Clonal PC origin**

While work with clonotypic B cell in myeloma genesis shows promise, Chen et al. challenged the notion of clonal B cell origin of MM by showing that a minor proportion of circulating B cells in MM patients is clonally related to MMPC[34]. FISH-based assessment of bone marrow aspirates showed no significant association between the chromosomal aneuploidy in MM patients when comparing the B cell population and the monoclonal PC[35]. With the possibility of a B cell stem cell-like clonal origin of MM disease, It is hard to explain why CD20 depleted BM did not yield higher proportion and deeper response rates in MM patients treated with Rituximab[36]

Yaccobi et al. successfully engrafted human MMPC's in mice models enriched with foetal bones. The engrafted cells produced human Ig between 2-9 weeks of the transplantation and with hypercalcemia, osteoclast activation and selective foetal bone reabsorption[37]. Rawston et al. further identified CD138+/CD38+/VLA5- as markers of circulating and self-replenishing plasma cells using stringent flow based gating strategies[38].

Hosen et al. successfully demonstrated CD38 ++, CD138-/+ PC is capable of producing MM in severe combined immunodeficiency(SCID) mice models, with a more rapid disease progression in CD138- cell population. Importantly these BM cells were deficient in B cells when secondarily transplanted to SCID-rab recipient to generate the disease[39]. These results are similar to those of Kim et al. who used more immunocompromised host mice with xenografted human foetal bone grafts to populate

patient's fully differentiated CD138+ CD38+ cells causing MM disease in the host mice which was transferable to secondary hosts[40].

Given the possible heterogeneity of cells indicates a heterogeneous PC population with several subclones. The clonal population sensitive to chemotherapy can be tidally taken over by the resistant subclone. However, several factors can be implicated in disease progression and resistance to chemotherapy including tumour promoting bone marrow microenvironment, activated signalling pathways, genetic aberrations and MM cell plasticity as noted above.

## 1.6 Signalling pathways

MM cells depend on direct and indirect interaction with surrounding milieu micro-environment to maintain sustained growth, resistance to treatment and continued survival. These interactions cause dysregulated activation of signalling pathways within the MM cells which produce growth factors and cytokines for autocrine and paracrine influence on self and surrounding bone microenvironment. Mutations causing activation of some of these signalling pathways are prominently noted in active disease but not in MGUS or SMM stages. Currently used chemo-therapeutics target the signalling pathways implicated in MM.

### 1.6.1 NF- $\kappa$ B pathway

This family of five transcription factors ( RelA/p65), RelB, c-Rel, p50 and p52 widely regulate innate and adaptive immune responses. In a non-stimulated state, cells retain NF- $\kappa$ B in the cytoplasm bound to I $\kappa$ B or Inhibitory(I $\kappa$ B). Stimulus triggering either the classical(canonical) or alternative(non-canonical) pathways causes either proteasomal degradation of I $\kappa$ B(canonical pathway) and nuclear translocation of NF- $\kappa$ B subunit or involves selective processing of p100(precursor of p50 and p52) and subsequent nuclear translocation of p52/RelB complex and gene activation respectively[41].

Activated NF- $\kappa$ B has been noted in 40% MM cell lines and between 17-20% primary patient tumours[42-44]. Transcriptional activation of genes including MAPK31, MAP3K14, RIPK4, TLR4, TRAF2, TRAF3, CYLD, cIAP1/2, NIK, LTBR, CD40, TACI, BRTC, CARD11, IKBIP, NFKB1, IKBRB and TNFRSF1A which affect dysregulation in classical and alternative pathways is reported in MM[44]. Selective activation of regulators involved in proliferation( such as LTBR, CD40, NIK) while switching off the inhibitory regulators(cIAP1/2, TRAF 2/3) abnormally activates NF- $\kappa$ B in MM[43, 44].

Current therapeutics notably IMiDs(immunomodulatory drugs including thalidomide and lenalidomide) and a proteasome inhibitor(PI) such as Bortezomib affect NF- $\kappa$ B signalling. IMiDs are suggested to inhibit proinflammatory cytokines such as IL-6 which significantly upregulates pro-survival and proliferative signals in MM plasma

cells. IL-6 is one of the cytokines induced by activated classical NF- $\kappa$ B[45]. Bortezomib inhibits alternative NF- $\kappa$ B pathway however activates the classical pathway[46]. Hence, a significant anti-MM activity likely needs dual inhibition of classical and alternative NF- $\kappa$ B pathways[47].

### **1.6.2 RAS/RAF/MEK/ERK**

MAPK(Mitogen-activated protein kinase) belongs to serine/threonine kinases with four central kinases- extracellular signal-regulated kinase(ERK), ERK5, p38MAPK (p38) and c-Jun N-terminal kinase(JNK)[48]. On activation by receptor tyrosine kinase (RTK)/G protein-coupled receptor inactive GDP bound RAS gets activated to GTP-Ras complex which phosphorylates its effectors including RAF/MEK/ERK and PI3K/Akt and TIAM1/RAC1 pathways[49]. These pathways are essential in regulating cellular differentiation, cytoskeletal dynamic changes and flux of cellular proteins. Dysregulation of these pathways is implicated in tumour survival and progression[49].

Ras is arguably the most mutated oncogene with mutations in K-ras present in about 50% in lung and 90% in pancreatic cancers respectively[49]. Three human RAS oncogenes include H-ras, N-ras and K-ras of which K-ras and N-ras are highly mutated in MM. Recent study using whole genome exome sequencing identified mutated K-ras(21%), N-ras(19%) and BRAF(7%) in about 50% MM patients[50]. RAS mutations were previously reported in 7% of MGUS patients with increasing frequency of occurrence to 45 % of patients with relapsed MM[51].

Ras mutations are mutually exclusive and potentiate clonal heterogeneity[50, 52]. Higher frequency of such mutations at relapse likely suggests aggressive disease with likely increased drug resistance. N-ras mutations are associated with increased resistance to Bortezomib with reduced response rates(7% vs 53% in patients with mutant vs wild-type N-ras,  $p=0.00116$ ) with shorter progression free survival(PFS) [53]. Raab et al. demonstrated treatment response to BRAF inhibitor vemurafenib in a BRAF<sup>V600E</sup> mutated relapsed refractory MM patient. The response was sustained until

de-novo NRAS mutation caused selective disease progression [54]. This highlights the importance of targeting therapy in MM patients.

### **1.6.3 PI3/Akt/mTOR pathway**

Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB or Akt)/mammalian target of rapamycin(mTOR) signalling is dysregulated in several malignancies and impacts cellular division, motility growth and survival[55]. Mutated oncogenic drivers such as PIK2CA and Akt1 are frequently altered in various cancers and are found in a high grade of malignancies[56, 57]. Activating mutation causing self-activation of the pathway has not been found in PI3 and Akt pathway as demonstrated by Ismail et al. who found one point mutation in PIK3CA gene in MM patients with equal presence of the same in healthy individuals underscoring the importance of the mutation[58]. It implies either cross-activation amongst various signalling pathways or surplus stimuli from the microenvironment such as IL-6, VEGF upregulates the pathway in MM.

Insulin-like growth factor(IGF) causes phosphorylation of insulin receptor substrate (IRS) which activates Akt. Two important downstream isoforms of mammalian target of rapamycin(mTOR - evolutionarily conserved serine /threonine kinases) complexes, MTORC1 and MTORC2 are implicated in protein synthesis, inhibition of autophagy, glucose metabolism, cellular survival and proliferation. MTORC1 is essential for protein translation and cell growth while MTORC2 is involved in glucose metabolism, survival and proliferation. Negative feedback mechanisms are controlling the balance between the mTOR complexes and of the PI3K/Akt activation. These feedback mechanisms are noted to be altered in MM[55, 59].

Fernandez et al. showed F box only protein(FBXO9) a constituent of ubiquitin-protein ligase complex which is overexpressed in hyperdiploid MM, causes proteasomal degradation of MTORC1 leading to MTORC2 driven Akt auto-activation during starvation. If MM microenvironment is reportedly hypoxic, this could have significant implications for activation of MTORC2 driven Akt in MM[60].



Peterson et al. identified MTOR interacting protein DEP domain containing TOR interacting protein (DEPTOR) mRNA anomalously increased in MMPC's (28%) 4 folds compared to normal PC's. This increase is mainly clustered in the non-hyperdiploid subgroup of MM patients harbouring translocations for Cyclin D1, CyclinD3, c-MAF or MAFB. DEPTOR was identified to inhibit negative feedback loop regulating PI3K activation by MTORC1 hence leading to activation of Akt[61].

#### **1.6.4 Signalling pathway crosstalk**

Signalling pathways in MM are not mutually exclusive and can also overlap in activating signalling making treatment challenging and likely contributing to treatment failure. Further, given receptor homology, signalling molecule could simultaneously activate multiple signalling pathways. IL-6 has been shown to activate the PI3/Akt kinase and Ras/MEK/ERK pathway[62]. ERK inhibition has been shown to upregulate PI3K activating the PI3/Akt kinase pathway in MM[63]. Vice versa, selective inhibition of activated Akt has been shown to upregulate phosphorylated Erk in MM cell lines. Synergism was seen with simultaneous inhibition of Akt using Pan-Akt inhibitor with MEK inhibitor in MM cell lines which were resistant to Akt inhibition and exhibited upregulated MEK/ERK pathway likely the cause of resistance[64]. Inhibition of another well-known pathway the Janus kinase (Jak)/ signal transducer and activator of transcription proteins (STAT)– Jak2/Stat3 which responds to IL-6 signalling inducing resistance to apoptosis in MM, has been shown to cause upregulation phosphorylated-ERK and phosphorylated-Akt on inhibition using TG101209, a Jak2 inhibitor[65]. Several other mechanisms of cross-activation between the pathways have been reported. It would strongly underpin combination chemotherapy approach in the treatment of MM.

## 1.7 Chromosomal abnormalities

Evaluation of cytogenetics in MM has significant implications for disease progression, presentation, response to treatment and overall survival(OS). The use of FISH has superseded metaphase karyotype analysis. A more significant number of specific probes targeting the interface chromosomes yields increased detection of specific abnormalities which were cryptic to karyotypic chromosomal analysis; carried significant disease prognostication.

Broadly, molecular cytogenetic aberrations are classified as primary and secondary chromosomal abnormalities. This classification considers presence of primary chromosomal abnormalities in MGUS disease stage. Usually, primary chromosomal abnormalities are either trisomies or translocations. Trisomies usually involve odd number chromosomes including 3, 5, 7, 9, 11, 13, 15 forming an aneuploid/ hyperdiploid karyotype. Primary chromosomal translocations involve locus for heavy chain immunoglobulin IGH On chromosome 14 along with associated chromosome partners which most frequently are located on chromosome 4, 6,11,16 and 20[66]. Other less frequent Primary chromosomal abnormalities involve IgH translocations involve unusual partner chromosomes and simultaneous trisomes with IgH translocations[66].

<b>FISH abnormality</b>	<b>Approximate frequency (%)</b>	<b>Chromosome affected</b>
<b><i>Trisomy(No IgH abnormality)</i></b>	42	Odd number chromosomes
<b><i>IgH translocations without trisomies</i></b>	3015	
t(11;14)	6	<i>CCND1(Cyclin D1)</i>
t(4;14)	4	<i>FGFR3 and MMSET</i>
t(14;16)	<1	<i>C-MAF</i>
t(14;20)	5	<i>MAFB</i>
Other IgH translocations with uncommon partner chromosomes		
<b><i>IgH translocations with trisomies</i></b>	15	
t(11;14)	3	<i>CCND1(Cyclin D1)</i>

t(4;14)	4	<i>FGFR3 and MMSET</i>
t(14;16)	1	<i>C-MAF</i>
t(6;14)	<1	<i>CCND3(Cyclin D3)</i>
Other IgH translocations with uncommon partner chromosomes	7	
<b><i>Monosomy 14 without IgH translocations or trisomies</i></b>	4.5	
<b><i>Other cytogenetic abnormalities in the absence of IgH translocations or trisomy(ies) or monosomy 14</i></b>	5.5	
<b><i>Normal</i></b>	3	

**Table 1: Cytogenetic abnormalities in MM[67, 68]**

Primary chromosomal abnormalities have distinct characteristics. These events are observed at the MGUS stage and are observed to be present in the majority of clonal PC population. These events are none-overlapping; only one translocation involving chromosome 14 would be present in the driving clone of the disease; however secondary chromosomal abnormality could be simultaneously present.

Secondary chromosomal abnormalities are deemed to be a feature of progressive disease and are usually reflective of an adverse prognostic outcome. These include Deletion 13q/ monosomy 13, deletion 17p/ monosomy 17 And deletion 1p or gain/ amplification of chromosome 1q. The secondary chromosomal abnormalities are understood to be a sub-clonal population defining event leading disease progression and eventual treatment failure.

### **1.7.1 Chromosome numerical abnormalities (trisomies, hyperdiploidy and hypodiploidy)**

Chromosomal aberrations are seen almost 90% of MM patients[69]. These are complex structural and numerical abnormalities similar to those seen in solid malignancies, however, are acquired in specific associations reflective of clonal evolution[70]. Smajda et al. assessed chromosomal aberrations in 208 MM patients

using conventional cytogenetics, identifying 166(66%) patients with distinct survival outcomes based upon numerical and structural chromosomal anomalies. Of these 75 patients had hyperdiploidy(chromosomes 3,5,7,9,11,15,17 and 19) and 63 had hypodiploidy(pseudo hypodiploid, hypotetraploid - associated with chromosomal structural anomalies) noted in pre-treatment BMPC analysis. A median OS of 33.8 months for hyperdiploid patients compared with 12.5 months for hypodiploid patients ( $P<.001$ ) was noted [71]

Similar results are reported by Marun et al. where they observed a five year OS difference between hypodiploid and non-hypodiploid group of 10% vs 41% respectively( $p=.0001$ )[72]. All studies noted an almost exclusive prevalence of recurrent IgH translocations including  $t(4;14)(p16.3;q32)$ ,  $t(11;14)(q13;q32)$  and  $t(14;16)(q32;q23)$  in the hypodiploid group suggesting a potential role in adverse survival outcome[69, 71-73].

The impact of aneuploidy on translocations and High-risk cytogenetic features In MM patients was assessed by Kumar et al. in their study. Patients were divided based on the presence of trisomies into standard risk or high-risk features by FISH analysis. Median OS of high-risk vs standard risk patient was 3.9 years versus not reached ( $P<.001$ ). Patients with High risk without trisomies vs high-risk with trisomies had median OS 3 years vs not reached( $p<.001$ ). Trisomies had a beneficial effect on patients with del17p or other high-risk translocations[67].

Hyperdiploid MM patients tend to be elderly with an increased predisposition for the bony disease. The mechanism of favourable impact on the patient prognosis of trisomies is not well understood however could be related to increased translation of proteins regulating cell growth and apoptosis[74]. Another possibility could be the presence of undiscovered chromosomal abnormalities specific to MM related trisomies.

## 1.7.2 IGH translocations

### 1.7.2.1 *t(11;14)(q13;q32)*

This is the most common IGH translocation in MM patients and is considered one of the primary cytogenetic events given its increased frequency in MGUS. By FISH it is identified in 10 to 20% of MM patient by FISH and portends a standard risk MM with median overall survival of 7 to 10 years[75-79]. *t(11;14)* has a higher prevalence in MM patients under 40 years of age. There is a higher proportion of IgD, IgE, IgM, non-secretory, light chain disease and increased bony involvement noted in this subgroup[80, 81]. Patients with plasma cell leukaemia(PCL) have a higher frequency of *t(11;14)*[82]. The involved breakpoints during the translocation place the proto-oncogene cyclin D1(CCND1) under IGH enhancer regulatory control. This hypothetically should accelerate the cell transition between G1 and S-phase. However, low plasma cell proliferation indexed were noted by Fonseca et al. In their study within this patient cohort[75, 78].

*t(11;14)* is conventionally a standard risk cytogenetic abnormality. However, treatment responses in this subgroup have been inferior in comparison to other standard risk abnormalities but superior to high-risk translocations.

An G et al. demonstrated diverse treatment responses to bortezomib based treatments based on CD20 expression profile in this subgroup. OS outcomes of patients expressing CD20 in comparison to non-CD 20 expressers was superior(54 months vs 16.5 months  $p=0.016$ )[83]. Another study compared outcomes of MM patients with either *t(11;14)*, Normal cytogenetics/FISH or high-risk features undergoing ASCT. This study reported a 3-year OS of 83 %, 63% and 34%( $p<0.00001$ ) respectively between the three groups[84].

Lakshman et al. In their study showed short and inferior OS of *t(11;14)* patients compared to standard risk MM patients with no impact of induction therapy on OS in this patient cohort. The median OS for non-translocation vs non-*t(11;14)* vs *t(11;14)* was 103.6( 95% CI, 85.2-112.3) vs 49.8(95% CI, 40– 60.6) vs 74.4(95% CI, 64.8–89.3)

months respectively(  $p < 0.01$ )[85]

### **1.7.2.2 *t(4;14)(9p16;q32)***

*t(4:14)* is the second most common IGH translocation seen in 10 to 15% of MM patients and about 25% of MM cell lines representing breakpoints on chromosome 4 within the proximity of FGFR3 and MMSET exon 5[86, 87]. It is also noted to be less frequent in MGUS versus SMM and MM with a higher incidence in the patient under 66 years of age[88].

One of the important translocation events is the up-regulation of FGFR3 gene transcription due to IgH promoter. It dysregulates the gene transcription with resultant increased FGFR3 signalling pathway. FGFR3 signalling activates RAS-RAF-MAPK, PI3K-AKT-mTOR, PLC $\gamma$ , protein kinase C(PKC) and STAT pathways. 25-30% MM patients with *t(4:14)* do not express FGFR3 likely due to loss of FGFR3 gene during translocation to 14q chromosome arm however MMSET is expressed in the majority of this group of patients[87, 89].

MMSET is widely expressed in other malignancies as well and has been associated with tumour aggressiveness[90, 91]. MMSET(NSD2 or WHSC1 1 in mammals) has a role in double stranded DNA damage response as it mediates methylation of Histone H4 lysine 20(H4K20) at DNA double-stranded break site and regulates the recruitment of p53 binding protein 1(53BP1) following DNA damage via H2AX-MDC11-MMSET pathway[92]. All cases of Wolf Hirschhorn syndrome invariably lose the mapped region MMSET with the loss of Wolf-Hirschhorn syndrome critical region at 4p16.3.

*t(4;14)* is undetectable using conventional cytogenetics( g banding) all spectral karyotyping due to the telomeric location of translocation and is detected by reverse transcriptase polymerase chain reaction(RT-PCR) or iFISH[78]. Interestingly, 30% of patients with *t(4:14)* lack FGFR3 expression. However, this patient population maintains a poor prognosis highlighting that likely dysregulation of both the genes is implicated in the poor overall survival[87, 89].

Study	% t(4;14), (N= total patients)	PFS t(4;14) - months	PFS all patients- months	OS t(4;14)- months	OS all patients- months	Treatment
Chang et al.[93]	15 (120)	9.9	25.8	18.3	48.1	HDT and ASCT
Gertz et al.[94]	26(153)	8.2	17.8	18.8	43.9	HDT and ASCT
Chang et al.[95]	6(40)	10.4	6.8	15.1	10.3	Bortezomib
San Miguel et al.[96]	4(682) +/- t(14;16)	19.8	21.7	Not reached (Low-risk group only)	Not reached (low-risk group only)	Bortezomib/ Melphalan/ Prednisolone
Reece et al.[97]	28 (102)	8.0 (at progression)	7.1 (at progression)	23.7	18.13	Len/Dex
Aver- Loiseau et al.[98]	14 (184)	5.5	10.6	9.4	15.4	Len/Dex
Chan H et al.[99]	75 (75)	33.5		69.6		HDT/ Chemo alone

**Table 2: Important studies evaluating outcomes of patients with t(4; 14). Adapted from Kalff et al.[100]**

### 1.7.2.3 t(14;16)(q32;q23)

t(14;16) presents in about 2- 10% of MM patients. It is cryptic to detection with classical karyotyping and was first identified using southern blot assay with an associated c-MAF translocation. MM cell lines including MM.1, JJN3, KMS 11 and ANBL6 express c-maf secondary to t(14;16).

Several studies have shown shorter PFS and OS in MM patients with t(14;16). Fonseca et al. Identified 15 t(14;16) patient in their analysis of 351 patients treated with conventional chemotherapy on Eastern co-operative oncology group clinical trial

E9486/9487. Median OS of t(14;16) vs without abnormality was 16( 95% CI 13 - 22) vs 41( 95% CI 37-48) months respectively (p =.003)[101]. Conflicting results were noted by Avet Loiseau et al. in the retrospective analysis where they did not find the statistically significant overall survival difference between similar patient groups [102]. The Gene Expression Profiling(GEP) in the TT3 Study on maintenance VRD highlighted distinct inferior outcomes in MAF/MAFB expressing patient subgroup[103].

Another recent study evaluated treatment responses in 123 t(14;16) MM patients receiving PI, IMiD drug or a combination of both as induction followed by either consultation with an ASCT or maintenance treatment. The PFS and OS for the entire cohort were 19 and 53 months respectively. PFS and OS in transplant-eligible patients, those who received ASCT compared to those who did not, was 31 vs 10 months(p=.003) and 58 vs 34 months( p=0.04) respectively. 16 months PFS and 59 months OS in ASCT ineligible patients. Maintenance treatment was noted to have favourable impact on PFS(36 vs 19 months, HR 0.56; p =.03)[104].

Clinically, increased incidence of PCL at presentation and light chain disease with subsequent renal involvement have been reported in these patient[102]. Median OS with renal insufficiency versus without renal involvement was 44.2 and 9.3 months respectively(P< 0.0001)[81]

#### **1.7.2.4 t(6;14)(p25;q32)**

Limited data is available due to a low frequency of t(6;14) Present in approximately 2-4% of MM patients. Functionally this translocation overexpressed cyclin D3 which phosphorylates RB to regulate G1/S cell cycle stage[78, 105]. It is classified as a standard risk genetic abnormality in multiple myeloma. It is present in other b-cell malignancies including non-Hodgkin's b-cell lymphoma, splenic marginal zone lymphoma and is implicated in oncogenesis of these mature b-cell malignancies[106, 107].



### **1.7.2.5 t(14;20)(q32;q12)**

Its prevalence is noted to be 1.5% in MM and SMM patients However is unexpectedly high in MGUS(5%)( $p= 0.005$ )[88]. There is a predominance of IgG isotype, advanced stage disease reported in this patient population. The juxtaposition of the MAFB gene to IGH enhancer causes increased in the gene transcription with a resultant increase in MAFB which is an AP1 superfamily transcription factor[108]. Up-regulated gene expression of MAFB has been noted in MM cell lines carrying t(14; 20) And is the likely oncogenic driver of poor prognosis in patients. Interestingly a majority of these cell lines came from patients with PCL younger than 65 years of age with the predominance of IgG isotope[109]. MGUS/SMM patients with t(14;20) appear relatively stable in the presence of this translocation; MM patients had a short median OS of 14.4 months[88].

### **1.7.3 Monosomies**

Monosomies in MM predict a poor OS outcome. Smadja et al. in their study showed an OS difference between hyperdiploid and hyperdiploid patients of 33.8 vs 12.6 months( $p < .001$ )[71].

Chromosome 13 deletions are a frequent cytogenetic abnormality with majorly monosomy 13( 85%) noted and less frequent del13p(7-15%)[110, 111]. Initial studies indicated poor OS survival for patients with monosomy 13[112, 113]. However other studies identify it as a pre-requisite to clonal expansion and likely less significant due to the confounding effects of simultaneous adverse cytogenetic aberrations. A more recent study identified a protective role of partial deletion of chromosome 13 in contrast to the adverse prognostic effect of monosomy 13[111]. This difference was likely under-highlighted due to smaller sample size of previous studies [114]. Monosomy of chromosome 2, 3, 13, 14 and 19 was associated with a significantly poor OS[72]. Shin et al. Further identified monosomy 16 and loss of y chromosome as adverse prognostic features in MM patients[115].

#### **1.7.4 Chromosome 1 amplification/deletion**

Chromosome 1 aberration present in about 40 to 50% of multiple myeloma patients. These include 1q gain or 1p loss. Specifically, 1q21 amplification is unusual in MGUS and increases in the frequency of up to 45% in SMM 43% in newly diagnosed mm and 72% in patients who have relapsed. It is generally considered a secondary event after dysregulated FGFR3/MMSET or c-MAF. About 91% of myeloma cell lines of human origin have been shown to express 1q21 amplification[116]. Several mechanisms are implicated in 1q primarily due to peri-centromeric instability causing the whole arm or jumping translocations. Shaughnessy et al. analyzed 532 newly diagnosed mm patients to identify a high-risk genetic signature subgroup with shorter duration of response. 30% of aberrant Gene expression mapped to chromosome 1q( upregulated genes) and 1p(downregulated genes)[117]. Other studies have also shown inferior OS in patients with aberrant chromosome 1 abnormalities including deletions/amplification[72, 118, 119]. CKS1B, a common upregulated gene has been shown to portend aggressive disease course with shorter progression-free survival post-ASCT[120]. Other candidate target genes upregulated include MCL-1, BCL-9, IL6R, PSMD3, PSMB4, UBE2Q and others[121]. Leone et al. examined CDKN2C deletions associated with the 1p arm in 515 MGUS, SMM and newly diagnosed MM with either hemizygous or homozygous deletions at 1p32.3(CDKN2A). Loss of CDKN2A was associated with poorer OS as compared to controls( 22 vs 38 months; p=0.003) respectively[122]. Hebraud et al. found 1p32(7.3%) and 1p22(15.1%) to be independently negative prognostic markers for PFS and OS when compared to the patient population without the 1q aberration[123]. Another associated gene deleted/mutated at locus 1p12 is FAM46C which interestingly is associated with adverse prognosis in MM patients post-ASCT[124].

#### **1.7.5 Del17p/Monosomy 17**

Loss of functional tumor suppressor gene p53(p53), is an unfavorable high-risk genetic aberration in MM identified as deletion(del17p13.1/del17p) on FISH or monosomy 17 on karyotype[125]. These are considered subsequent events given increasing

incidence with disease progression or relapse[126]. Mutations in the chromosomal region are rare(3%); however increase with disease progression[127, 128]. Almost certainly, these mutations are associated with del17p portending an adverse OS [128-130]. Del17p13.1 is frequent and have been reported in about 11% of patients with newly diagnosed MM[69, 78, 131]. MM patients with del17p have a poor prognostic outcome despite the use of newer therapeutic combinations[132].

P53(17p13.1) regulates major pathways of cellular homeostasis in stressed or damaged cells[133]. Its functional loss exists in more than 50 % of human cancers [133-136]. Functional loss of p53 in MM is associated with a poor disease outcome and frequent uncommon MM presentations including EMD and PCL [137-139]. Although some treatment response has been reported with Bortezomib based regimens[24, 25], patients with Del17p usually achieve unsatisfactory survival benefits to novel agent treatment and/or ASCT, when compared to the MM patients without Del17p[132, 140-142].

## 1.8 Microenvironment

MM cell bone marrow localization is analogous to 'seed and soil' explanation of tumor specificity for different milieu favorable for the growth of the tumor cells[143]. No significant cytogenetic/FISH abnormality delineates progression from MGUS to active myeloma which suggests the role of extrinsic factors including the microenvironment in facilitating disease progression. However,  $\geq 95\%$  aberrant plasma cells and chromosomal aneuploidy are risk factors associated with disease shift from benign to active disease[144, 145]. Several studies have identified the importance of cellular and acellular components of the bone marrow which are facilitative to the growth and development of malignant PC growth that has several mechanisms to overcome host immune defence.

### 1.8.1 Myeloma derived suppressor cells(MDSC)

MDSC are immature myeloid cells which interact with PC's promoting their proliferation and overcoming immune effects of therapeutic agents. These cells were identified over a decade ago, however, have been shown to have significant roles in cancer systems promoting treatment resistance, immune escape and anergy. Though first studied in mouse models where two phenotypes, granulocytic MDSC(G-MDSC) and monocytic MDSC(Mo-MDSC) bearing CD11b<sup>+</sup> Ly6G<sup>hi</sup> Ly6C<sup>low</sup> and CD11b<sup>+</sup> Ly6G<sup>-</sup> Ly6C<sup>high</sup> respectively were identified. G-MDSCs have increased levels of reactive oxygen species while the Mo-MDSCs have higher nitric oxide[146, 147]. In humans, G-MDSC are CD11b<sup>+</sup> CD33<sup>+</sup> HLA-DR<sup>-/low</sup> CD14<sup>-</sup> and Mo-MDSC are CD11b<sup>+</sup> CD33<sup>+</sup> HLA-DR<sup>-/low</sup> CD14<sup>+</sup>. MDSC's increase in peripheral blood and bone marrow of MM patients with increasing frequency corresponding with disease progression/relapse[148]. These cells show a prominent increase in the BM of MM patients suggesting a potential significance in the MM disease potentiation[149]. Further, Mo-MDSC's activate STAT1- dependent gene promoting nitric oxide-mediated T-cell suppression while G-MDSC likely mediates the same via increased levels of reactive oxygen species which are undetectable in Mo-MDSC's and vice-versa[147, 149, 150]. There is bi-directional crosstalk between MM cells which have been shown

to induce MDSC's which also facilitates MM cell growth in the microenvironment. Further, Gorgun GT et al. highlighted that the number of MDSC's and their immune suppressive functions were not affected with treatment with current therapeutic agents[148]

### **1.8.2 T cells and natural killer(NK) cells**

T cells dysregulation is a notable feature of bone marrow microenvironment in MM patients. There is increasing evidence supporting the accumulation of subsets of T helper cells including Th1, Th2 and Treg in MM patients when compared to the normal population. While the Th1/Th2 subset disbalance in favour of Th2 subset further facilitates an increase in Treg subset which are known to be involved in self-tolerance[151-153]. One of the noted mechanisms is via self-tolerance induced by plasma cells that behave as immature antigen presenting cells expanding the Treg cells likely inhibits anti-MM immune response[154]. MM bone marrow has increased IL6, TGF $\beta$ , and IL1 cytokine levels secreted in the microenvironment[155, 156]. These cytokines have been shown to facilitate Th17 development which secretes IL1, IL13, IL17 and IL23. MM cells express IL17 receptor and proliferate in the presence of the cytokine as shown in SCID mouse models and in-vitro experiments[157]. Importantly IL17 levels positively correlate with progressive/advancing MM disease and are implicated in enhancing secretion of angiogenic factors including VEGF, TNF $\alpha$  and microvessel density, by BM cells[158].

Natural killer cells are a separate lymphocytic lineage with cytotoxicity and cytokine-mediated effector response. Inherently they can recognize self from foreign cells via expression of MHC class one specific receptor. This recognition allows NK cells to selectively target MHC class one deficient haematopoietic stem cells while ensuring tolerance to self-cells. Other stress-induced ligands recognized by the NK cells include NKG2D, TLR and several infectious nonself ligands. In vitro, the interaction of NK cells to TLR ligands has been shown to induce IFN $\gamma$  which augments the natural killer cells cytotoxic functions[159].

A notable escape mechanism of multiple myeloma plasma cells from NK cell-mediated lysis is by shedding of MHC class I chain-related protein A (sMICA) I ligand, a recognized target of NKG2D receptors expressed on NK cell-surface[160]. Another implied mechanism of resistance is the reduced number of NKG2D expressing effector cells in MM patients[161].

### **1.8.3 Dendritic Cells**

Dendritic cells are known as the master regulator of the immune system. They are responsible for optimizing the immune response by activating the T cells via major histocompatibility complex (MHC II) class II-mediated antigen presentation. Efficient functioning of dendritic cells a high-level expression of MHC II antigen and co-stimulatory molecules (including CD86, CD83, CD80 and CD40) which are implicated in activating naïve and memory T cells[162, 163].

Several mechanisms of dendritic cell dysfunction are identified in MM patients. While phenotypic and functional abnormalities of DC from MM patients potentially causes defective immune response; Micro-environment of MM patients is known to have a higher degree of IL6, VEGF and M-CSF which compromises DC's process of T-cell antigen presentation[164-166]. Increased IL6 levels are associated with activation of P38 MAPK pathway which inhibits DC maturation and impacts migratory functions in MM patients. Further, it has been highlighted that not only a reduced number of DC in MM patients but also a reduced ability to secrete the T cell stimulatory IL12p70 which promotes T-helper cell 1(Th-1) differentiation. This affects the Th1 cytokine interferon-gamma( $\text{INF}\gamma$ ) secretion which is known to modulate differentiation of TH1 over TH2 cells[163].

### **1.8.4 Macrophages**

Macrophages are an essential component of mononuclear macrophage lineage and are hence related to the dendritic cell. They are conventionally known to differentiate

into activated macrophage 1(M1) or activated macrophage 2(M2) in response to various environmental stimuli and growth factors. M1 type has higher expression of MHC II, IL12 and tumour necrosis factor alpha(TNF $\alpha$ ) playing a specific role in nitric oxide and reactive oxygen species-mediated anti-pathogenic activity. The M2 type of differentiation in response to IL4 and IL13 is usually a facilitator of Humoral immunity and wound healing[167]. It is shown that monocytes are selectively biased towards a more distinct population of M2 type which facilitates tumour growth and resistance to chemotherapy.

Zheng et al. identified that CD68+ macrophages heavily infiltrated the bone marrow of MM patients and further conferred protection against chemotherapy-induced apoptosis by down-regulating cleavage of caspase-dependent apoptosis[168]. These tumour associated macrophages(TAM) express P/E selectin and CD18 which can interact with P- selectin glycoprotein ligand 1(PSGL-1) and intracellular adhesion molecule 1(ICAM-1) on MM cells respectively to induce multidrug resistance in MM patients by activating SRC, ERK 1/2 and c-MYC which inhibited drug-induced caspase activation[168, 169].

Macrophages are a source of cytokines including VEGF, FGFR2, IL1 $\beta$ , IL6 and IL8 stimulating the surrounding BMSC's and enhancing MM associated neovascularization[170-173]. Stimulated BMSC's in MM patients are a source of IL6 which drives MM cell proliferation. Further, via toll-like receptors (TLR) 2/6 activation, a more inflammatory phenotype of macrophages is persistently producing IL6 and IL1 $\beta$  promoting MM cell proliferation and BMSC's IL-6 production[174].

### **1.8.5 Bone marrow stromal cells (BMSC's)**

Bone marrow stromal cells are important modulators MM microenvironment. They are known to express a higher level of intracellular adhesion molecule 1(ICAM-1), vascular cell adhesion molecule 1(VCAM-1) promoting PC adhesion and activation of signalling pathways such as NF-KB, Notch[175, 176]. These pathways induce growth factors such as IL6, VEGF, IGF1 which promote MM cell survival and proliferation. Nefedova

et al. demonstrated increased sensitivity of MM cells to conventional chemotherapy by inhibiting Notch signalling[177]. Similarly, NF- $\kappa$ B inhibition is one of the targets of Bortezomib in MM[178].

Bone marrow stromal cells further communicate with PC via secreted exosomes which are between 40 to 100nm sized membranous vesicles containing miRNA that can regulate their proliferation. Recaro et al. first identified downregulated mir15a in exosomes derived from primary MM BMSC when compared to primary normal BMSC[179]. Wang et al. further demonstrated BMSC derived exosomes increase PC migration, proliferation, survival and resistance to bortezomib by reduced, increased expression of Bcl-2 and cleavage caspase 3, caspase 9 and PARP [180]. MM-derived BMSC's have been shown to support increased growth of CD184 expressing population of RPMI cell line compared to BMSC derived from non-MM patients highlighting an essential role of supporting stem cell-like MM cells[181].

### **1.8.6 Osteoblasts and Osteoclasts:**

MM microenvironment is permissive to osteoclastogenesis while selectively inhibiting osteoblast proliferation. This dynamic interplay is mediated via several pathways and signalling molecules.

#### **1.8.6.1 Molecular pathways facilitating osteoclastogenesis**

- a) **RANK/RANKL:** Receptor activator of nuclear factor(NF)- $\kappa$ B(RANK) and RANK ligand(RANKL) bone remodelling associated signalling pathway is dysregulated in MM. RANK receptor and RANKL interactions facilitate osteoclast precursors to fuse and form a mature osteoclast(OC). This interaction is inhibited by the decoy receptor osteoprotegerin(OP), which is secreted by marrow stromal cells(SC) and OB. The balance of RANK/OP is raised in MM patients, favouring increased OC formation. MM cells also degrade OP via endocytosis to further facilitate OC formation.



- b) **Notch signalling pathway:** This pathway is well described in osteoclastogenesis. Via homotypic and heterotypic interactions between adjacent MM cells and MM cells with BMSC's respectively, it promotes the production of RANKL by MM cells[182, 183]

#### 1.8.6.2 Molecular pathways associated with osteoblast inhibition

- c) **WNT pathway:** Wingless and integration-1(WNT) pathway is well described to enhance gene expression facilitating bone formation. When the canonical arm of the pathway is activated facilitating translocation of cytoplasmic  $\beta$ -catenin to the nucleus; it induces T cell factor/lymphoid enhancer factor (TCF/LFE) transcription factors resulting in gene expression of proteins implicated in bone anabolism[184].

These pathways are complemented by the simultaneous activity of various cytokines/secreted ligands/receptors and transcription factors which in MM are pro- osteoclastogenesis and catabolic. Most important ones are mentioned below:

- a) Interleukin 3: Cytokine implicated in osteoclastogenesis by inducing BM macrophages to produce Activin A (transforming growth family  $\beta$  - TGF $\beta$  family) protein which causes RANK expression. RANK further via the NF- $\kappa$ B pathway leads to osteoclast maturation[185]
- b) Interleukin 6: Important cytokine secreted by myeloid cells, known to stimulate MM cells into secreting VEGF and more IL6 which improves MM cell survival. VEGF further activates osteoclastic surface receptors stimulating differentiation[186, 187]
- c) Interleukin 7: Plays a dual role of 1) Downregulating Runt-related transcription factor 2/ core-binding factor Runt domain subunit 1(RUNX2/CBFA1) which affects non-canonical WNT pathway known to impede osteoblast-ogenesis[184, 188] and 2) Stimulates T-cells mediated RANKL production[189]

- d) Interleukin 17: Secreted by T helper cells which are likely induced by dendritic cells(predominant antigen presenting cell in BM niche of MM patients). IL17 is a known osteoclastogenic pro-inflammatory cytokine implicated in bone disease[190-192]
- e) MIP- 1 $\alpha$ /CCL-3: Pro-inflammatory cytokine expressed by stromal cells and hematopoietic stem cells further produced by MM cells. Potent osteoclast activator stimulating osteoclast differentiation. In MM, enhances plasma cell and stromal cell interaction inducing RANKL and IL6 propagating tumour burden with bone destruction[193-195]. It further activates PI3-K/Akt and MAPK pathways in MM cells, potentiating plasma cell growth, resistance to cell death and migration[196, 197]. Further evidence suggests CCL3 dysregulates the osteoblast/osteoclast balance by inhibition of osteoblastic differentiation, favouring overall bone catabolism in MM[198, 199]
- f) TNF $\alpha$ : Tumor necrosis factor-alpha is a member of TNF superfamily involved in osteoclastogenesis. TNF via RANKL stimulates osteoclast differentiation [200] which is suggestively mediated via TNF type 1 receptor(TNFr1)[201]
- g) BTK: Burton's tyrosine kinase, a non-receptor tyrosine kinase[202] implicated in osteoclast differentiation and migration towards MM plasma cell via crosstalk with CXC chemokine receptor type 4(CXCR4 – levels positively correlate to BTK expression in MM cells) and SDF-1 $\alpha$  (stromal cell produced cytokine)[203-205]. SDF-1 $\alpha$  activated BTK in MM cells while facilitating osteoclast precursors with coexpression of CXCR4 and BTK to migrate towards MM plasma cells[204, 206].
- h) Dkk: Dickkopf-1, a WNT inhibitor protein secreted by MM plasma cells which interferes with canonical WNT pathway, preventing  $\beta$  catenin nuclear translocation and ultimately preventing osteoblast differentiation[207]. Increasing levels of Dkk corresponds to increased lytic lesions and advanced MM compared to significantly lower levels in MGUS and WM(Waldenstrom macroglobulinemia)[208]. Decreased osteoblasts could favour RANK/OP ratio

to drift augmenting osteoclastogenesis and additively worsening lytic bone disease[209, 210]

- i) Activin A: TGF $\beta$  superfamily member which via canonical (ActRIIA/B receptor-mediated phosphorylation of activin receptor-like kinase 4-ALK4 induces Smad signalling with translocation of transcription factor Smad2/3/4 complex into the nucleus) and non-canonical(Akt/PI3K, MAPK/ERK, JNK and WNT/ $\beta$ -catenin) pathways induces osteoclastogenesis and inhibits osteoblast development[185, 211]
- j) Sclerostin: Anti-osteoblast protein (antagonistic to bone morphogenic protein - BMP's; adversely affecting BMP mediated osteoblastic mineralization), secreted by osteocytes by binding the LRP5/6 transmembrane receptors and preventing WNT mediated DVL-Axin-FRAT1-GSK3 $\beta$  complex formation. This causes phosphorylation of  $\beta$  catenin and proteasomal degradation via ubiquitination[212-214]
- k) Osteopontin : (OP) Is a glycoprotein secreted by different cells. In MM it has been noted to be secreted by MM cells and expressed in elevated levels by patient stromal cells. OP is increasingly expressed with evolving MM from benign MGUS to active disease with correlation to osteolytic disease burden[215-217]

The above mentioned signalling pathways/molecules along with inter and intracellular interaction have provided the basis of several pre-clinical/clinical drug testing. One of the most recent successful examples would include Denosumab. Recent data shows non-inferiority of Denosumab to Zoledronic acid as a treatment for bone disease in MM patients[218].

### 1.8.7 Vascular component and non-cellular compartment

Incremental BM micro-vessel density(MVD) has been shown with progressive disease spectrum with minimal MVD in MGUS to a progressive increase in active MM [219]. There are increased transcription factors such as hypoxia-inducible factor 1 $\alpha$ (HIF1 $\alpha$ ) which can regulate VEGFA, IL-8 and other pro-angiogenic molecules in MM patients[220]. CD138+ cells from MM patients reportedly have increased transcription of different signalling molecules including heme oxygenase 1(HOMOX1), Heat-shock protein(HSP) and XIAP(X - linked inhibitor of apoptosis)[220]. Experimentally, HSP70 inhibition reverses melphalan-induced cell adhesion mediated and acquired drug resistance. HSP70 inhibitors further induced increased apoptosis in melphalan induced drug resistance[221]. Levels of hepatocyte growth factor(HGF), TNF $\alpha$  and MVD are correlative of MM disease burden with decreased levels on treatment response[222].

Other notably deranged growth factors include VEGF, basic fibroblast growth factor (bFGF), angiopoietin-1(Ang-1), matrix metalloproteinases(MMPs), osteopontin(OPN), IL-6 and IL8[223]. Hose et al. proposed that bone marrow angiogenesis is normally under the regulation of BMPC's and can be disrupted by MM cells by stimulating angiogenesis and downregulating anti-angiogenic genes[224]. Another mechanism of vasculogenesis involves monocytic differentiation into endothelial cells when exposed to pleiotrophin(PTN) an angiogenic factor produced by MM plasma cells re-emphasizing the role of MM in facilitating a pro-angiogenic BM micro-environment[225].

The non-cellular component or extracellular matrix(ECM) forms a supporting meshwork of about 300 proteins including cellular component(fibroblasts), collagens, proteoglycans and glycoproteins, enzymes(matrix metalloproteases -MMP) and signalling molecules(cytokines and growth factors) which regulate cell survival, proliferation and metastasis along with MM cell crosstalk[226, 227]. ECM related receptors and modulating enzymes including Laminin- $\alpha$ , lysyl-hydroxylase 2, prolyl 4-hydroxylase 1, nidogen-2, MMP2 and others were shown to be upregulated in MGUS and MM compared to control patient's human fibroblast-like cells[228]. Another study reported the detection of Annexin A2(ANXA2) and Galectin-1(LGALS1) expression in

MM patients; however, not in healthy patients or MGUS patients and correlates with a decreased OS[229, 230]. Both these proteins are ECM proteins implicated in cell-cell and cell-matrix interaction and have a role in promoting MM growth and induced angiogenesis[229, 231]. Interestingly, Galectin-1 expression is downregulated by knockdown of HIF-1alpha[231].

Integrin fibronectin receptors(VLA-4, VLA-5) adherence to MM cells causes cell adhesion mediated drug resistance(CAM-DR) to doxorubicin and melphalan suggestive of direct MM cell and ECM interaction mediated conventional chemotherapy resistance[232]. Interestingly, Bortezomib can inhibit CD49d( $\alpha$ 4-integrin) a subunit of VLA-4 and can overcome CAM-DR resistance sensitizing them to other chemotherapy agents[233].

## 1.9 Clinical features at presentation

### 1.9.1 Anaemia

Kyle et al described 73% patients present with Hb<12g/dL at diagnosis of MM which is usually normocytic and normochromic[234] with sub-optimal reticulocyte response (reticulocyte index <2.5%)[235]. The degree of anaemia can also be reflective of disease activity with low borderline values usually in MGUS. Kyle et al. showed the presence of anaemia in 23% of one thousand three hundred and four MGUS patients which was attributable to non-PC activity related causes(myelodysplasia, renal insufficiency and iron deficiency)[236]. The number of patients presenting with Hb< 12g/dL increases to 76% in patients with SMM[237]. Further, 97% of patients with MM would experience some degree of Anemia[234]. Manifestations of anaemia include fatigue and poor quality of life that would worsen the prognosis for patients with concurrent cardiovascular co-morbidities.

PCs infiltrating the bone marrow can disrupt the erythroblastic islands(EBI) by direct unit displacement and further by secretion of cytokines and mediators which affect growth and development of maturing erythrocytes. Secretion of Fas ligand (FL), tumour necrosis factor related apoptosis-inducing ligand (TRAIL), further impedes normal erythropoiesis[238]. Further likely cleavage of GATA-1(transcription factor promoting erythroblastic differentiation and survival) by FL and TRAIL, would globally stunt erythroid development in MM[238, 239]. This would theoretically imply improved erythroid development post-MM treatment. Bouchnita et al. demonstrated an increase in Hb levels, erythrocyte count and marrow erythroid precursors in patients with pre-treatment 30% MM infiltration of BM who received lenalidomide/bortezomib-based treatment[240]. It would indicate that anaemia in MM is reversible with appropriate disease control. Common causes implicated in MM related anaemia would include:

- 1) Infiltration of bone marrow by the malignant plasma cells
- 2) Negative effect on growth and development of erythrocytic precursors by MM cells via FAS ligand and TRAIL-mediated cytotoxicity[238, 241]
- 3) Renal impairment/erythropoietin deficiency

- 4) Anaemia secondary to the folate/B12 deficiencies
- 5) Falsely low due to hemodilution secondary to the M protein
- 6) Chronic inflammation related to a low normal iron, high ferritin and increased hemosiderin in the bone marrow
- 7) Anaemia secondary to chemotherapy – hypoplastic
- 8) Anaemia secondary to bleeding related to amyloid deposits/coagulation defects – secondary IDA
- 9) Hemolysis/traumatic causes

Managing symptomatic anaemia involves either red cell transfusion or use of supportive treatment with erythropoietin stimulating agents(ESA). While transfusion requirements improve with therapy related disease control, Erythropoietin (EPO) levels are typically reduced in renally impaired MM[242] and would necessitate repletion for improved Hb level. Treatment with ESA is controversial in MM. Cancer-related anaemia improves subsequent to its usage along with positive correlation of subjectively improved quality of life, proportional to the degree of normalization of Hb levels [243]; there is an increased risk of hypertension, thrombosis and potential antibody mediated erythroid aplasia[244].

Current recommendations with use of ESA suggest a trial when Hb concentration is <10g/l, particularly in patients with anemia secondary to renal failure, having ruled out alternative causes of anaemia[245].

### **1.9.2 Bone disease and hypercalcemia**

Bone structural abnormalities, pain and fractures are hallmark features in MM and are well-documented features, historically notable with the case of Sarah Newbury, the first described case of MM[3]. About 60% of patients with MM present with bone pain at diagnosis with over half of those experiencing moderate to severe pain[234]. Another study highlights that risk of fractures increases by nine fold along with 69% patients developing pathological fractures post MM diagnosis predominantly in the vertebrae and the ribs[246].

MM is also shown to have the highest prevalence of hypercalcemia (serum corrected calcium >11g/dL) when compared to other osteolytic malignancies [247]. About 20% of newly diagnosis MM patients have hypercalcemia at presentation, although it is rarely the only presentation of symptomatic disease[247, 248]. Survival outcome of a patient with hypercalcemia worsen with increasing degree of hypercalcemia[247] as outlined by the Durie Salmon Staging (DSS). Further, hypercalcemia is a marker of adverse disease despite the use of newer agents[249] warranting a deeper understanding of the mechanism for effective management strategies.

Bone metabolism and subsequent remodelling is complex and is dysregulated by several mechanisms in MM predominantly augmenting the osteoclast(OC) activity while suppressing the osteoblasts(OB) in favour of a microenvironment supporting malignant plasma cell growth and development. This is clinically noted in MGUS patients who had significantly reduced bone marrow density compared to normal controls with associated derangement of osteogenesis related cytokines[250]. Clinical Abnormalities in molecular pathways including the RANK/RANKL/OPG, TNF, Notch, Wnt, RUNX2 and EphrinB2/EphB4 have been shown to increase osteoclast activity while suppressing osteoblast activity in MM as detailed elsewhere.

### **1.9.3 Renal Impairment (RI)**

Historically, renal impairment has been challenging to classify given the range of functional kidney functional damage at presentation. End-stage renal disease is likely most commonly seen secondary to MM and requires dialysis in about 1.5% patients [251]. A certain degree of Acute Kidney Injury(AKI) and Chronic Kidney Disease(CKD) is reported between 20-50% of patients with MM during the disease course[234, 252-254]. A serum creatinine(Cr) of > 2mg/dl(173 $\mu$ mol/L) or reduced creatinine clearance (CrCl) at <40ml/min is the usual cut off to detect renal impairment in MM patients[255] identifying 20% patients renally impaired when newly diagnosed with MM[254, 256].

CrCl estimation is usually advised using either the Modification of Diet in Renal

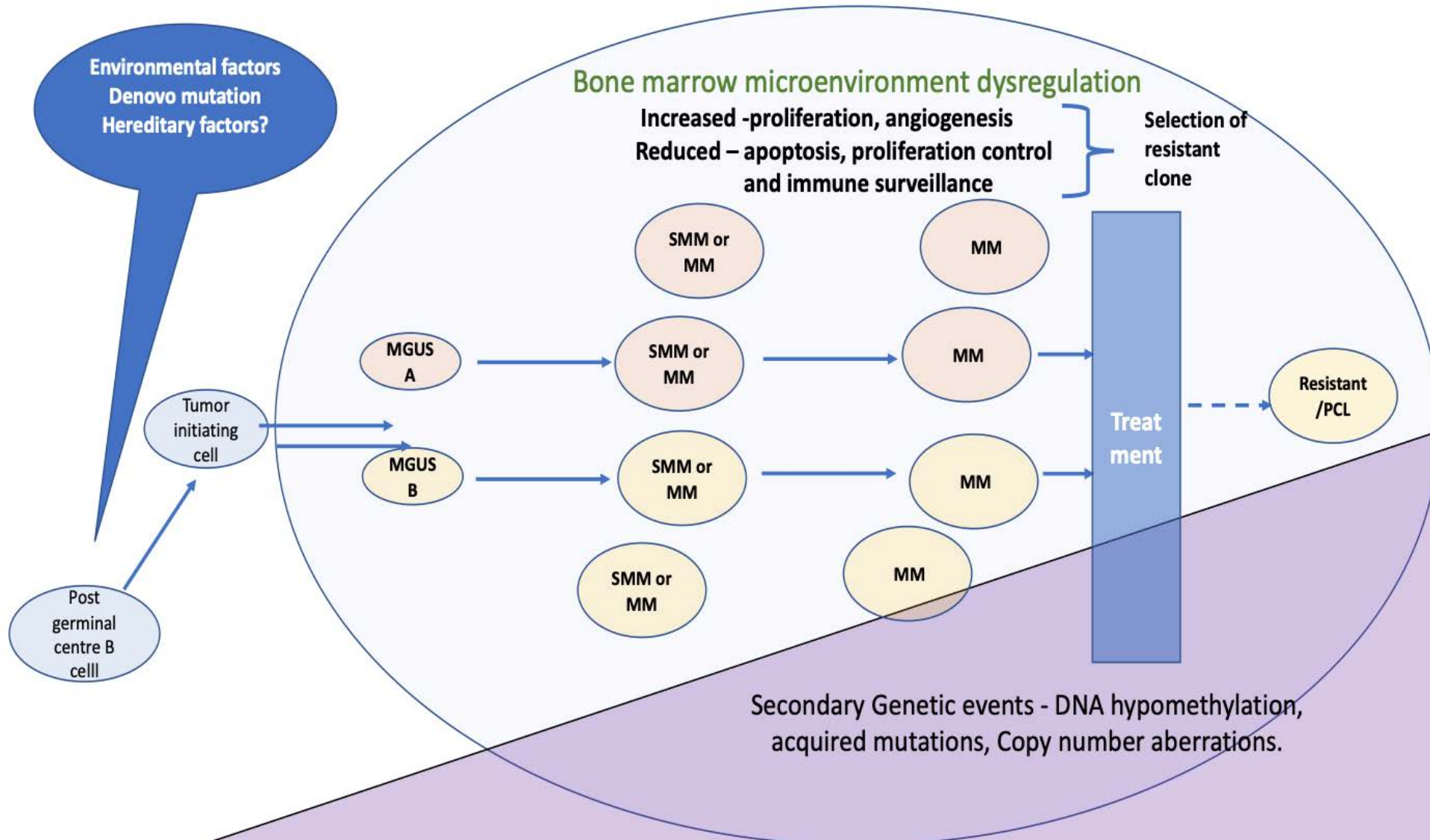


Disease(MRDR) or the Chronic Kidney Disease Epidemiology Collaboration(CKD-EPI) formulae, with a preference towards the latter given its higher sensitivity towards the estimation of GFR[257]. Estimated GFR(eGFR) is known to be confounded by individual factors such as changes in body mass, diet and proximal tubule dysfunction. The potential use of Cr and serum cystatin would be superior in estimating GFR along with the added advantage of estimation of MM disease burden[258]. Further, AKI is more common than CKD in newly diagnosed MM patients, and RIFLE(risk, injury, failure, loss and end-stage kidney disease) criteria are recommended for assessment[257, 259].

### **1.9.3.1 Pathogenesis – RI**

MM Monoclonal light chains are freely filtered in the glomerulus. They are endocytosed and catabolised in the proximal tubular cells. Overwhelming of this scavenging mechanism promotes the appearance of the light chains in the tubular fluid where they combine with Tamm-Horsfall (Uromodulin – a glycoprotein secreted by the medullary ascending loop of Henle)[260]. The interaction of complementary determining region of the light chains and discrete binding sites on uromodulin leads to the formation of casts and aggregates which in turn cause distal tubular obstruction[261]. This upsets the usual ratio of kappa ( $\kappa$ ) to lambda ( $\lambda$ ) light chains from median 0.6(range 0.26-1.65) to 1.1(range 0.37-3.1)[262, 263]; burdening the reticulo-endothelial system for serum free light chains (sFLC) clearance. Another determinant of light chain cast formation would be the difference of Isoelectric point (I<sub>p</sub>) of the light chain potentially influencing the pattern of light chain deposition with higher I<sub>p</sub>'s associated with light chain deposition disease [264]. RI in this setting can be worsened by the simultaneous use of nephrotoxic drugs/dyes, hypercalcemia and dehydration[265].  $\lambda$  light chains usually show a greater renal involvement with amyloid and renal damage while  $\kappa$  light chains have been a more frequent cause of light chain deposition disease (LCDD) and rare Fanconi's syndrome[266, 267]. Kappa light chains have a higher propensity of liver deposition[268].

2.0 Fig. 5 Overview of factors implicated in development of MM and treatment resistance



## **2.1 Disease spectrum**

### **2.1.1 Monoclonal Gammopathy of Undetermined Significance(MGUS)**

It is widely accepted that MGUS precedes MM and exists in about 3 % of population over 50 years of age with increasing prevalence at 5.3% of those over 79 years of age and factors such as race(black population> white population> Asian population), sex (male >female), familial history and occupational risk[236, 269]. MGUS in itself is benign with presence of  $\leq 3\text{g/dL}$  serum M protein with  $\leq 10\%$  bone marrow monoclonal plasma cells, no CRAB( C= hypercalcemia, R= renal impairment from Monoclonal protein (M), A = Anemia with disease-related Hb  $< 10\text{g/dL}$  and B = bone involvement - Fractures/lytic lesions) features and no 'M' protein or only modest light chain in the urine[270, 271]. The risk of progression to MM is 10% at ten years and 36% at about 40 years from diagnosis(not accounting for death due to competing risk). The progression risk is higher in IgM MGUS versus non-IgM MGUS. This risk progressively worsens with the presence of risk features including an abnormal serum free light chain ration (sFLC) and high serum M protein. Given the potential risk of progression of MGUS to MM despite prolonged dormancy, these patients need clinical follow-ups at regular intervals[236].

### **2.1.2 Smouldering multiple myeloma(SMM):**

This biological state of MM spectrum of development is important to highlight given the increased potential of progression to symptomatic MM. Kyle et al. first successfully defined SMM in 6 patients analogous to smouldering acute leukaemia where bone marrow contains a higher proportion of blast cells; however, patients are typically asymptomatic for a prolonged duration of time. They further suggested a wait and watch approach towards management of SMM. Although newer studies show high-risk SMM with rapid progression to active MM within two years from diagnosis[273]. Hence early diagnosis, shorter follow up periods and proactive treatment of high-risk SMM is vital to improving OS despite the previously suggested universal watch and wait

approach.

Currently, SMM is defined as the presence of serum M protein of  $\geq 3\text{g/dL}$  with 10% to 60% clonal bone marrow PCs with no CRAB features or Myeloma defining event (MDE){ including: a) Bone marrow plasma cells [BMPC's], b) involved: uninvolved serum free light chain[sFLCs]  $\geq 100$ , c)  $>1$  magnetic resonance imaging[MRI] proven disease related lesion of the disease[274]. It is a distinct entity from MGUS given the higher potential to progress to active MM (50% progression at five years, 3% progression rate for the next five years and 1% stable progression rate from 10 years onwards). SMM increasingly recognizes higher risk dormant MM with the potential of transforming to active disease in 40% of patients with these features per year[275]. Further, no role of Ig heavy or light chain subtype in increased susceptibility to PD is noted[276].

## 2.2 Diagnosis and risk stratification

### 2.2.1 Revised diagnostic criteria

Traditionally MM was diagnosed based on CRAB features along with 10% or more monoclonal plasma cells in the bone marrow. However, several studies identified high-risk features (now termed MDE's) which are associated with a high rate of progression from indolent disease to active MM[273, 276-279]. Further, improved sensitivity of detection of myelomatous bone lesions has led to the inclusion of more advanced imaging modalities to be acceptable in the workup of MM as mentioned below

- a) Clonal BM plasma cells  $\geq 10\%$  or biopsy-proven bony or extramedullary plasmacytoma with one or more of the following:
  - i) Hypercalcemia – Serum calcium  $> 2.75\text{mmol/l}$ ( $>11\text{mg/dl}$ ) by  $0.25\text{mmol/l}$  ( $>1\text{mg/dl}$ ) : 'C'
  - ii) Renal dysfunction: Serum creatinine  $> 177\mu\text{mol/l}$ ( $>2\text{mg/dl}$ ) or creatinine clearance  $< 40\text{ml/min}$ : 'R'

- iii) Anemia: Hb value <100g/l(10g/dL) or >20g/l(2g/dL) below lower limit of normal : 'A'
- iv) Bone lesion:  $\geq 1$  osteolytic lesion on Skeletal survey, CT, PET-CT: 'B'

b)  $\geq 1$  of the following biomarkers:

- i) Involved: uninvolved sFLC ratio  $\geq 100$
- ii)  $\geq 1$  focal lesion on MRI scans ( lesion to be  $\geq 5$ mm in diameter)

*\*International Myeloma Working Group Diagnostic Criteria for Multiple Myeloma (IMWG) criteria[280]*

The CRAB features must be MM associated, and other causes of the same should be excluded. The renal disease of MM should be biopsy proven if causes other than light chain cast nephropathy could be implicated for the cause of renal dysfunction, including light chain deposition disease, amyloid light chain amyloidosis and membranoproliferative glomerulonephritis which can occur with M protein and would require disease-specific management approach[280, 281]

Solitary plasmacytoma's with normal bone marrow findings and no disease-related features are usually managed with localized radiotherapy 40-50Gy to the affected area[280, 281].

### **2.2.2 Disease staging and assessment for risk**

Given the disease heterogeneity, it is vital to risk stratify MM patients for prognostication. Two widely used risk stratification scores are used, the Durie and Salmon system(DSS) and the International Staging System(ISS). Both systems utilized different variables to risk stratify patients predictive of overall survival.

### 2.2.2.1 Durie Salmon Staging(DSS)(table 3)

DSS was introduced in 1975 estimated MM tumour burden based upon the Hb, calcium and M protein levels in the patient's serum along with MM-related bone lesions. The score obtained from these measurements qualified patients into Stage I -low(<math>0.6 \times 10^{12} /m^2</math>), Stage III - high(<math>1.2 \times 10^{12} /m^2</math>) and stage II - intermediate(in between stage I and III) tumour mass. Further subclassification into Group A or B was based on serum creatinine of <math>< 2</math> or <math>> 2</math>mg/100ml respectively. The system provides useful initial assessment and post-therapy response by evaluating a change in tumor burden (table 3).

However, the drawback of this system is subjectivity in evaluating the lytic lesions[282]. It is uncertain how independently prognostic is the number of lytic lesions with the disease as studies evaluating the same are limited. Further, the system primarily considers the host disease burden for assessing the tumour burden. With growing applicability of knowledge of disease biology and understanding of MM being a heterogeneous disease of clonal population, modest estimation of tumour disease burden would underappreciate the high-risk features associated with the tumour biology itself, particularly in high-risk SMM. With newer MM therapies, where the disease 'bulk' might be reduced, the remaining resistant clonal MM plasma cell would be challenging to risk stratify using this system of classification.

Stage	Criteria	Tumor mass
I	All criteria's: Hb >10g/dL(100g/L) Normal Calcium or <math>< 12</math>mg/dL (3mmol/L) IgG<math>< 5</math>g/dL IgA ,3g/dL No or single bone lesion Monoclonal urinary protein > 12g/24hours	Low tumour mass <math>< 0.6 \times 10^{12} /m^2</math>
II	Between stages I and II	Intermediate tumour mass
III	Any of the following: Hb <math>< 8.5</math>g/dL(85g/L) Calcium > 12mg/dL (3mmol/L)	High tumour mass >math>1.2 \times 10^{12} /m^2</math>

	IgG > 7 g/dL(466.9µmol/l), IgA > 5g/dL(333.6µmol/l) Monoclonal urinary protein > 12g/24h Multiple osteolytic lesions, fractures	
Subclass	A – Creatinine <2mg/dL(<177µmol/l) B – Creatinine >=2mg/dL(>=177µmol/l)	

**Table 3: DSS staging system**

### 2.2.2.2 Durie Salmon Staging(DSS) plus staging(table 4)

The system improved upon observer bias by including advanced imaging including MRI/PET scans to their previous criteria's. DSS plus also aimed to prevent unnecessary treatment of MGUS and early stage smouldering MM while identifying high-risk disease subgroups( > 20 bone lesions or extramedullary disease) and accurately staging oligosecretory/non-secretory MM [283].

Durie/Salmon PLUS myeloma staging system - Integration of imaging		
Durie/Salmon STAGE	Plus Upstage	MRI/PET Number of lesions
I B		I 0-4
II A or B		II 5-20
III A or B		III > 20
B: Creatinine > 2mg/dL and/or Extramedullary disease(EMD) on PET or MRI		

**Table 4: Durie Salmon Staging plus staging**

### 2.2.2.3 International Staging system(ISS)(table 5)

Is a simplified risk stratification system utilizing two variables, serum  $\beta$ 2microglobulin( $\beta$ 2M) and serum albumin.  $\beta$ 2M is reflective of tumour burden along with renal function[284, 285]. Serum albumin production by the liver is likely affected by the effects of increased IL-6 associated with the bone marrow microenvironment in MM patients. on liver[285]. This system segregates patients based on the levels of both of these variables; the normal value is associated with better patient prognosis.

Stage	Criteria
I	Serum $\beta$ 2M <3.5mg/l(296.8nmol/L) and serum albumin $\geq$ 3.5g/dl(35g/L)
II	Neither stage I or III
III	Serum $\beta$ 2M $\geq$ 5.5mg/l(466.49 nmol/L)

**Table 5: ISS staging system (2005) [284]**

#### 2.2.2.4 Revised International Staging System (R-ISS)(table 6)

Cytogenetic abnormalities have significant prognostic implications for patients with MM. As MM is a disease, delineating it merely by using biochemical measures would be inadequate when individualizing patient treatment. Several studies identified poor overall survival (OS) in MM patients harbouring del17p, t(4;14) along with a high Lactate dehydrogenase (LDH)[286-288] at diagnosis. This 5% cohort of MM patients at diagnosis respond poorly to treatment with early relapse and mortality within two years of diagnosis. ISS was therefore revised to increase the sensitivity of this prognostication index.[107, 289, 290]

**Table 6: Revised ISS[107]**

Prognostic factor	Criteria
ISS Stage	
I	Serum $\beta$ 2-microglobulin < 3.5 mg/L, serum albumin $\geq$ 3.5 g/dL
II	Not ISS stage I or III
III	Serum $\beta$ 2-microglobulin $\geq$ 5.5 mg/L
Cytogenetics by interphase FISH (iFISH)	
High risk	Presence of del(17p) and/or translocation t(4;14) and/or translocation t(14;16)
Standard risk	No high - risk CA
LDH	
Normal	Serum LDH < the upper limit of normal
High	Serum LDH > the upper limit of normal
R-ISS stage	
I	ISS stage I and standard-risk CA by iFISH and normal LDH
II	Not R-ISS stage I or III
III	ISS stage III and either high-risk CA by iFISH or high LDH



## 2.3 Response assessment

Quality and Duration of responses have significantly improved with newer drug combinations and high-dose therapy with autologous stem cell transplantation (HDT/ASCT). Patients achieving IMWG defined complete remission(CR)(Table. 7) have been shown to relapse slowly and have significantly longer OS[291]. Response categorization to chemotherapy in MM has incorporated investigative methods with a sensitivity of detecting 1 MMPC in  $10^{5-6}$  cells in the bone marrow. The disease burden in this context is called minimal residual disease (MRD).

The international myeloma workgroup(IMWG) have sub-divided the depth of response achieved based upon newer methodology with higher sensitivity including multicolour flow cytometry, Ig Allele–specific oligonucleotide–based quantitative polymerase chain reaction(ASO-PCR), next-generation sequencing(NGS) and MRI/PET-CT[292]. These advanced modalities detect MRD(table 7b) which is undetectable by conventional methods where the maximal attainable response is stringent complete response sCR(table.7a). The rationale for assessing MRD is due to the evidence of prolonged PFS for patients in CR who are MRD negative compared to those who are in CR but MRD positive. MRD negativity on long-term follow up has shown to be relapse free up to 10 years, identifying possible ‘cured’ population of MM patients who enjoy a longer PFS and OS [293, 294].

Utilization of MRD in routine clinical practice still has a few hurdles to cross. Being specialized, these diagnostic tests are not available worldwide. MRD is a surrogate marker for OS and can be used to compare treatment options. However standardization of interpretation of MRD results in the context of timing and frequency of repeat results, and its optimum utilization in applying to different clinical contexts including high-risk disease, appropriate timing of re-commencing/changing treatment, the definition of loss of MRD status and more issues need to be resolved[295].

Response subcategory	Response criteria
mCR	CR & ASO-PCR -ve' sensitivity 10 <sup>5</sup>
iCR	sCR & -ve BM aberrant PC's by MPF(>4 colours) on analysing 1x10 <sup>6</sup> BM cells
sCR	CR + Normal FLC & -ve clonal PC by IHC or 2-4 colour flowcytometry
CR	-ve urine and serum IF and resolution of soft tissue plasmacytoma and <=5% BMPC's
(VGPR)	M protein in serum and urine by +IF & - SPEP or >= 90% reduction of serum M protein + urine M protein<100mg/24hrs
PR	>=50% reduction of serum M protein and reduction in 24hrs urinary M protein by >=90% to <200mg/24hrs. If non/oligosecretory MM then >50% reduction in PC's when baseline PC's were >=30% Along with above if plasmacytoma noted at diagnosis, >= 50% reduction in size is required
PD	Increase of 25% from lowest confirmed response value for: a) Serum M protein (absolute increase must be >=0.5g/dL b) Serum M protein increase>=1g/dL if the lowest M component was >=5g/dL c) U M protein(absolute increase to be >=200mg/24hrs)

**Table 7a: Adapted from ESMO 2017 guidelines[274].**

mCR: molecular complete response; iCR: immunophenotypic CR; sCR: stringent complete response; CR: complete response; VGPR: very good partial response; PR: partial response; PD: progressive disease; -ve: negative ASO-PCR: allele-specific polymerase chain reaction; MPF: multiparametric flowcytometry; FLC: free light chain ratio; IHC: immunohistochemistry; IF: immunofixation; SPEP: serum protein electrophoresis.

Response subcategory	Response criteria
Sustained MRD -ve	MRD -ve In BM(NGF/NGS) & by imaging confirmed a year apart. Evaluate to specify the duration of MRD -ve.
Flow MRD -ve	No monoclonal PC by NGFC on BMA using Euroflow standard operation procedure for MRD detection in MM( or an equivalent validated method) with a minimum sensitivity of 1 in 10 <sup>5</sup> nucleated cell or higher
Sequencing MRD -ve	No clonal PC by NGS on BMA where the presence of a clone is defined as less than two identical sequencing reads obtained after DNA sequencing of BMA using the Lymphosight platform(or validated equivalent method) with a minimum sensitivity of 1 in 10 <sup>5</sup> nucleated cell or higher
Imaging + MRD -ve	MRD -ve as per NGFC or NGS + PET-CT disappearance of tracer uptake from baseline or decrease to < mediastinal blood pool SUV or decrease to less than that of surrounding tissue

**Table 7b: Adapted from ESMO guidelines 2017[274]**

MRD: minimal residual disease; -ve: negative; NGF: next generation flowcytometry; NGS: next-generation sequencing; NGFC: next generation flow cytometry; BMA: bone marrow aspirate; PET-CT: positron emission tomography-computed tomography.

## 2.4 Treatment

Significant advances with the introduction of newer and targeted therapeutics have increased treatment options for MM patients. As multiple myeloma is still incurable, primary treatment objective is to induce the best possible response for a prolonged duration with minimal toxicities. To accomplish this, synergistic drug combinations are widely used in the treatment of MM.

### 2.4.1 Treatment drugs

#### 2.4.1.1 Proteasome inhibitor(PI)

##### 2.4.1.2. Bortezomib (First generation)

Bortezomib is a specific 26S proteasome inhibitor which forms a reversible but strong covalent bond between dipeptide boronic acid moiety and threonine proteases' 20s subunit. It has several important mechanisms of action including:

1. Mitochondrial dysfunction; cytochrome c release; reactive oxygen species production
2. Modulate JNK kinase activity causing enhanced caspase 3 and 8 activation
3. Immune-mediated cell damage
4. Dysregulation of cellular stress response including NF-kB
5. Derangement of apoptotic protein including unfolded protein response(UPR)
6. Elevate p53 levels
7. Reduces cytokine production
8. Impairment of transcriptional factors
9. Cell cycle proteins dysfunction

Owing to a multitude of targets and synergism with a lower dose of steroids and other newer targeted agents, it is widely used in combination for all disease treatment stages as a single agent or as drug combinations for treatment of MM[296]. Significant dose-

limiting side effects include motor and sensory neuropathy, cytopenia/s, gastrointestinal symptoms including nausea, vomiting, diarrhoea[178].

#### **2.4.1.3 Second generation proteasome inhibitor - Carfilzomib**

Carfilzomib is an epoxyketone which irreversibly binds to N- terminal threonine residues of the catalytic proteasomal subunits with prolonged proteasome inhibition as compared to the reversible boronic acid binders (Bortezomib and Ixazomib). It received approval in Europe for use in MM patients who received 1-3 prior treatment lines in 2015. Dimopoulos et al reported a median progression-free survival of 18.7 months( 95% CI 15.6 - not estimable) versus 9.4 months( 8.4 - 10.4) In patients receiving Carfilzomib with dexamethasone(KD) versus bortezomib with dexamethasone respectively in the interim analysis[HR 0.53(95% CI 0.44-0.65);p<0.0001] of ENDEAVOR study. Common side effects In Carfilzomib group anemia (14%), hypertension(9%), thrombocytopenia(8%) and pneumonia(32%) [297]. The Aspire study compared Carfilzomib lenalidomide and dexamethasone (KRD) to lenalidomide and dexamethasone alone and noted significantly improved PFS of 26.3 vs 17.6 months in the respective study arms(p=.0001). Comparable grade 3 or higher adverse events were reported in either of the arms, and increasing trend of treatment discontinuation was noted in the lenalidomide and dexamethasone[298].

#### **2.4.1.4 Ixazomib (MLN9708)**

Ixazomib is a second-generation proteasome inhibitor with boron containing proteasome inhibitor reactivity. It has a short 20s proteasome dissociation T1/2 likely improving its tissue distribution. In preclinical models, it showed superior antitumor activity as compared to bortezomib due to improved pharmacokinetics, tolerability and bioavailability in comparison to bortezomib[299]. The anti-tumour activity of ixaz-omib was further replicated in mouse models of B-cell and plasma cell malignancies leading to initial phase trials in relapsed/refractory MM[300-303].

The Tourmaline-MM1 Study was a double-blinded placebo-controlled trial for 722 relapsed and or refractory impatient who received either ixazomib with lenalidomide

and dexamethasone(treatment) or placebo with lenalidomide and dexamethasone(placebo) arm. An overall response rate(ORR) of 78% vs 72% were noted in the treatment versus placebo A group with a more prolonged median progression-free survival(PFS) of 20.6 months versus 14.7 months ( $p=0.01$ ) reported in the two groups respectively. Similar rates of adverse events were noted between the two groups. Grade 3 thrombocytopenia(12%), rash(36%) and low grade gastro-intestinal adverse events were reported in the treatment group[304].

## **2.4.2. Immunomodulatory agents**

### **2.4.2.1 Thalidomide**

Thalidomide was initially marketed as a sedative and anti-emetic during pregnancy and subsequent withdrawal due to teratogenicity in newborn babies. Thalidomide belongs to the immunomodulatory(IMiD) group of drugs with strong anti-myeloma activity. It is a synthetic glutamic acid product with poor water solubility. Some of the important mechanisms of action include[305, 306]:

1. Anti-angiogenic activity secondary to inhibition of cytokines such as basic fibroblast growth factor(BFGF)
2. Immunomodulatory effects
3. Inhibition of NF- $\kappa$ B signalling inducing apoptosis via caspase-8 / death receptor pathway
4. Inhibition of Cereblon(cerebral protein with E3 ubiquitin ligase complex) and E3 ubiquitin ligase complex – Speculated role in teratogenicity

Rajkumar et al. investigated thalidomide in combination with dexamethasone (treatment) vs dexamethasone alone(control) when treatment options for newly diagnosed MM patients were limited. This phase 3 randomised trial reported higher response rates in the treatment versus control arm ( 63% vs 41%;  $P=0.017$ ). Grade 3 deep vein thromboembolism(DVT), rash, cardiac adverse events and neuropathy were significantly higher in the treatment group[307]. Significantly higher overall response rate(ORR) of 63% vs 46%( $P<0.001$ ) and the median time to progression (TTP) 22.6

versus 6 months( $p < 0.001$ ) were reported in another multicenter random-ized double-blind phase III comparing treatment versus control arm as in the previous study[308]. Its side effect profile includes peripheral neuropathy which is exacerbated when used in combination with bortezomib with increased incidence on higher thalidomide dose[309]. There is an increased risk of venous thromboembolism, mainly when used in combination with high dose steroids. It is now used less preferably when compared to the newer generation of IMiD's due to better treatment tolerability and response[309]. Increased risk of arrhythmias has also been reported in the OPTIMUM study as compared to Dexamethasone[310].

#### **2.4.2.2 Lenalidomide**

Lenalidomide an analogue of thalidomide with lesser toxicity likely due to its structural difference from thalidomide by single carbonyl ring and an amino acid group. It is an anti-proliferative immunomodulator which induces apoptosis and stimulates T and NK disrupting the MM - microenvironment crosstalk [311, 312].

MM 009 and 010 studies showed the superiority of lenalidomide in combination with dexamethasone(treatment) when compared to dexamethasone alone(control). The MM009 study showed significantly higher response rates with a median time to progression of 11.1 months vs 4.7 months( $p < 0.001$ ) and median overall survival different of 29.6 vs 20.2 months( $p < .001$ ) between the treatment and the control arms respectively. MM 010 study also reported along longer time to progression, higher response rates and significantly improved OS between the treatment and the control arms. Common side effects included neutropenia, thrombocytopenia and increased risk of venous thromboembolism in the treatment group.

Lenalidomide is widely used as an induction agent given higher response rates in combination with proteasome inhibitors(transplant eligible) or combination with dexamethasone in transplant ineligible/frail elderly patient. It is also used for maintenance in patients with a higher risk of relapse.

### **2.4.2.3 Pomalidomide**

Pomalidomide is a thalidomide analogue which has significant anti-MM activity. Like other IMiDS it is an immunomodulatory agent which binds cereblon component of E3 ubiquitin ligase complex. This impacts B and T cell development due to ubiquitination and subsequent proteasomal degradation of downstream transcription factors Ikaros and Aiolos. It is also known to enhance NK and T cell activation causing antibody-dependent cellular cytotoxicity[313, 314].

Pomalidomide was approved for use in relapsed / treatment refractory MM patients in Europe based on evidence from MM-003 trial. This multicentre open-label, randomised phase III trial included patients refractory or relapsed and refractory to minimally two previous lines of treatment including lenalidomide and bortezomib. The treatment arm received pomalidomide with low-dose dexamethasone and the control arm received high-dose dexamethasone alone. Median PFS after treatment on was 4 months( 95% CI 3.6-4.7) versus 1.9 months( 95% CI 1.9-2.0). Neutropenia(48%), pneumonia(13%) were higher in the treatment group vs the control group[315].

## **2.4.3 Monoclonal Antibody**

### **2.4.3.1 Daratumumab**

Daratumumab is an anti-CD 38 IgG1k human monoclonal antibody. CD38 is selectively highly expressed on malignant plasma cells, with limited expression on other hematopoietic cells. Its tumoricidal effect is mediated by antibody dependent cell-mediated cytotoxicity, complement dependent cytotoxicity and antibody-dependent cellular phagocytosis. It was first approved as monotherapy based upon the results of MMY2002, and GEN501 studies, where a significant proportion of patients showed a clinical response to single-agent daratumumab after having relapsed or been refractory to at least two or more prior treatments including PI and IMiDs[316, 317].

MMY3003 and MMY3004 are important trials that evaluated daratumumab in



combination with either lenalidomide with low-dose dexamethasone or bortezomib with low-dose dexamethasone with comparative arms of lenalidomide with low-dose dexamethasone and bortezomib with low-dose dexamethasone respectively. Patient populations in both studies had been previously heavily treated and relapsed on several lines of therapy. The MMY3003 showed a PFS advantage of 83.2% vs 60.1% in daratumumab group vs the control group at 12 months respectively. Higher response rates were also noted in the daratumumab group versus the control group ( 92.9% vs 76.4%;  $p < 0.001$ ). Cytopenias were the most common side effects other than grade 1 to 2 infusion-related reactions in 48%[318]. The MMY3004 reported a 12-month PFS of 60.7% in the daratumumab arm versus 26.9% in the control arm. The median PFS was 7.2 months in the control arm vs not reached after a median follow-up of 7.4 months. Higher rates of responses were noted in the daratumumab arm vs the control arm( 83% vs 63%  $p < .001$ )[319]. Similar toxicity profile or noted in this study as a report by MY3003.

#### **2.4.3.2 Elotuzumab**

Elotuzumab recombinant monoclonal immunoglobulin G1(IgG1) antibody which selectively binds its epitope located within C2 domain of anti-signalling lymphocyte activation molecule family 7 (SLAMF7). This family of receptors have an imminent role in regulating immune responses. MM plasma cells universally express high levels of SLAMF7 along with some expression reported on NK cells, all B cells and activated T cells. The binding of elotuzumab to SLAMF7 on MM signals CD16 receptor-driven NK cell crosslink to the plasma cell activating ADCC causing targeted MM plasma cell death. However, functional NK cells are required for elotuzumab mediated ADCC[320, 321].

ELOQUENT 2 Phase III randomized clinical trial assessed the efficacy of elotuzumab in combination with lenalidomide and dexamethasone and (elotuzu-mab) purchase lenalidomide and dexamethasone alone(control) in patients who had a documented relapse after one two three lines of previous therapies. The rationale for this combination being lenalidomide role in upregulating NK cells. The median PFS in the elotuzumab group was 19.4 months versus 14.9 months in the control group which

was statistically significant( $p < 0.001$ ). Significantly higher response rates were also noted at 79%(elotuzumab) vs 66%(control)( $P < 0.001$ ). Compared to daratumumab lesser infusion-related grade one or two reactions were reported(10%)[322].

ELOQUENT 3 study evaluated a combination of elotuzumab pomalidomide and dexamethasone(elotuzumab) versus pomalidomide and dexamethasone(control) alone in patients previously refractory to a PI and lenalidomide. The primary endpoint of the study was to assess PFS which was 10.3 months in the elotuzumab group versus 4.7 months in the control group( $p = 0.008$ ). Elotuzumab group saw double the overall response rate as compared to the control group(53% vs 26%). The toxicity profile was favourable in the elotuzumab group with a lesser number of grade 3 or 4 neutropenia and anaemia as compared to the control group. The study highlighted a significantly lower risk of progression or death in this cohort of patients[323].

## **2.4.4 Histone deacetylase inhibitor(HDAC)**

### **2.4.4.1 Panobinostat**

Panobinostat is a histone deacetylase(HDAC) inhibitor that augments processes regulated by histone acetylation. Preclinical studies with xenograft models showed increased anti-tumor potential in MM of panobinostat in combination with bortezomib and dexamethasone[324]. Phase III Panorama trial results using panobinostat in combination with bortezomib and dexamethasone in comparison to dexamethasone and bortezomib for patients with relapsed or refractory and relapsed MM. There was a modest 4-month survival advantage with the triplet with significant grade 3-4 gastrointestinal toxicity, thrombocytopenia and fatigue[325, 326]. Panobinostat's use based upon the trial results would require significant deliberation where the benefit of the treatment strongly outweighs the significant side-effect profile.

## **2.4.5 High dose therapy(HDT)/Autologous stem cell transplantation (ASCT)**

HDT with ASCT has been a crucial therapeutic milestone for patients with multiple myeloma where the median survival of patients with conventional chemotherapeutic was in the range of about 3.5 years[327-329]. The role of transplantation before the advent of novel agents was vital for inducing prolonged remission and improving OS [Attal et al. showed an overall estimated survival of 52% at five years post ASCT compared to 12% with conventional chemotherapy( $p=0.03$ )] [330]. Novel agents have not only improved outcomes as an induction regimen compared to conventional regimens but have also shown similar survival trends as compared to ASCT and have opened options for delayed transplantation[331]. Further triplet combinations of PI and IMiDs with steroids are preferred given a higher frequency of more profound response and PFS advantage[332, 333].

However, there is a definite advantage of ASCT over either standard/conventional chemotherapeutic approaches separately and should be considered for all newly diagnosed MM patients transplant eligible. This has been shown in a recent meta-analysis examining the role of ASCT in the context of novel agent use. This study showed that high dose melphalan followed by autologous stem cell transplant was associated with superior PFS when compared to chemotherapy alone. They further showed superior PFS with either tandem ASCT or ASCT with VRD when compared to ASCT alone. The study more importantly reflects higher complete remission rates in the ASCT group[334]. This is important given no significant increase in treatment-related mortality ASCT group and particularly considering long-term effects and cost of continued/recurrent chemotherapy. Role of upfront tandem ASCT is likely more important in the context of high-risk cytogenetics subgroup given suboptimal treatment response in this patient subgroup compared to standard risk group even in the era of novel agents[335, 336].

### **2.4.5 Conventional chemotherapy**

MP(melphalan, prednisolone), M-2 protocol (melphalan with cyclophosphamide, prednisolone, carmustine and vincristine) and VCMP( vincristine, cyclophosphamide, melphalan, prednisolone) are some of the historical treatment options used in MM. These treatment options had significant toxicity profile including high steroid-related cushingoid effects, no significant benefit for bony disease and further, in some instances such as MP, incremental bone marrow failure rates. VAD(vincristine, doxorubicin, dexamethasone), perhaps is the most reasonable for usage due to the fast response rates as induction before HDT/SCT. The OS benefit of either combination therapy(COT) or MP based was found to be similar at 29 months, highlighting a lack of incremental response with either approach compared to the use of newer generation of targeted therapies. In relapsed MM, the prognosis was dismal if patients relapsed on CCP usage with VAD and cyclophosphamide( hyper-CVAD)[337]. Use of MP might still be appropriate in the elderly patient with resistant disease/inadequate response to newer therapeutic combinations or limiting toxicity profiles, where some disease control is required, permitting a quality of life.

## **2.5 Treatment strategies**

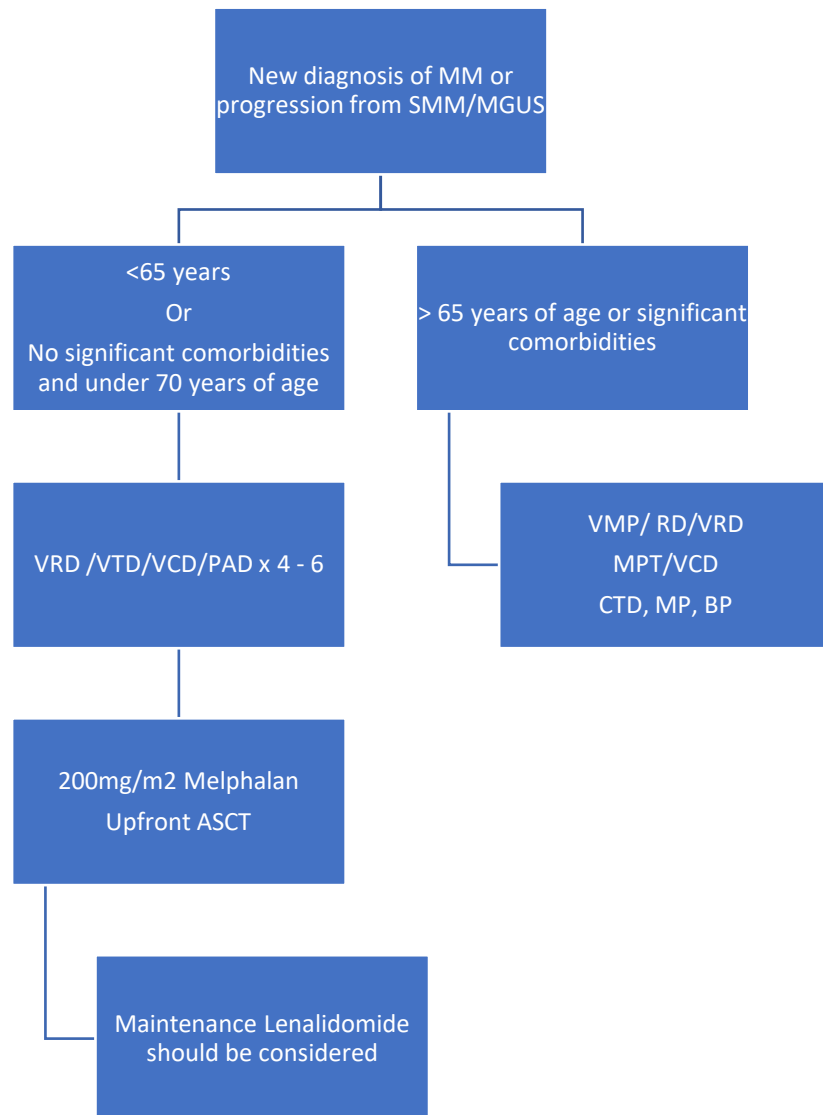
### **2.5.1 Newly Diagnosed MM**

#### **2.5.1.1 Transplant eligible/<65 years(Fig.6)**

Current treatment approach for patients under 65 years of age with good performance status should be given 4 to 6 cycles of usually triplet combination induction therapy to achieve deep response followed by HDT/conditioning with Melphalan 200mg/m<sup>2</sup> or 140mg/m<sup>2</sup>(older patients or renal impairment[338]) and ASCT involving by re-introduction of at least 2 million peripheral blood progenitors(CD34+) cells[339]. Subsequently, IMiD based maintenance therapy should be considered. Allogeneic transplantation is avoided due to significant toxicity and prolonged need for immunosuppression and higher mortality risk.

#### **2.5.1.2 Transplant ineligible/>65 years(Fig.6)**

A transplant is a considerable option for non-frail, older patients with younger biological age and no significant comorbidities[340]. Preferable options include VMP or Rd. until disease progression. Recently bortezomib is also used in combination with Rd given a significant improvement of OS in this patient sub-group. In the case of pre-existing neuropathy, Bendamustine with prednisolone can also be considered.



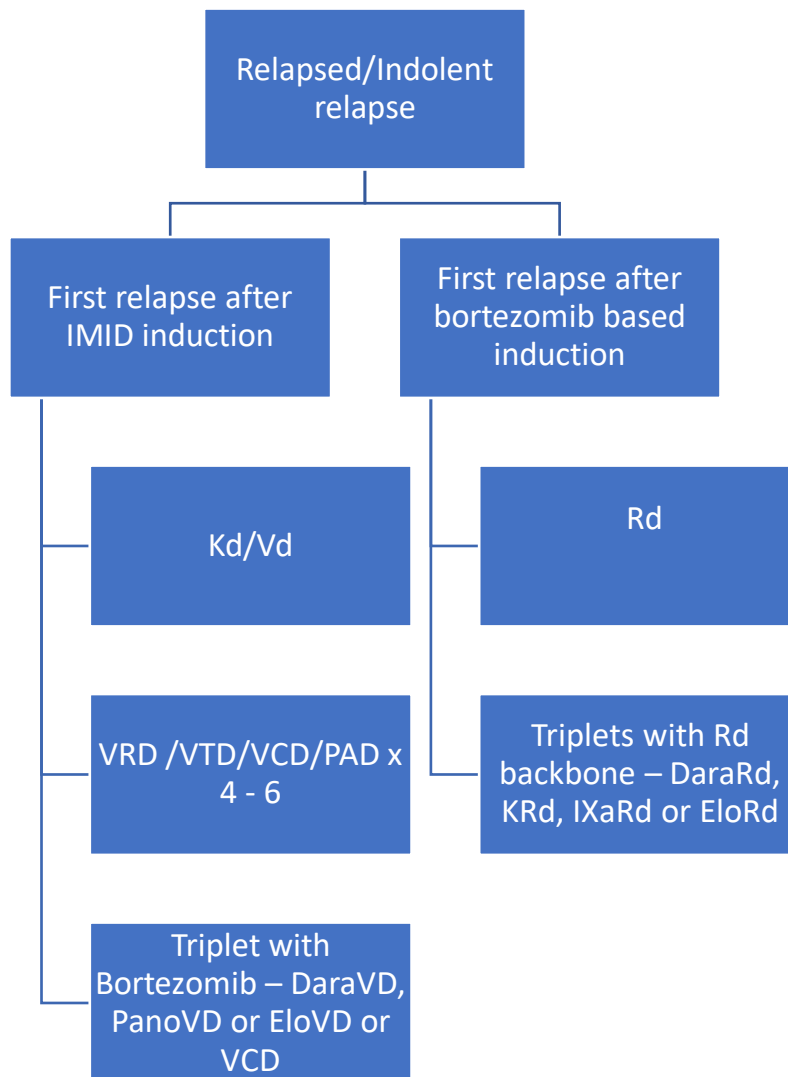
**Fig6: Treatment pathway for newly diagnosed MM patient[339]**

VRD- bortezomib, revlimid, dexamethasone; VTD-bortezomib, thalidomide, dexamethasone; VCD- bortezomib, cyclophosphamide, dexamethasone; PAD- bortezomib, doxorubicin, dexamethasone; VMP- bortezomib, melphalan, prednisolone; RD- revlimid, dexamethasone; MPT- melphalan, prednisolone, thalidomide; CTD- cyclophosphamide, thalidomide, dexamethasone; MP- melphalan, prednisolone; BP- bendamustine, prednisolone

### 2.5.1.3 Relapse/refractory MM (fig.7a and b)

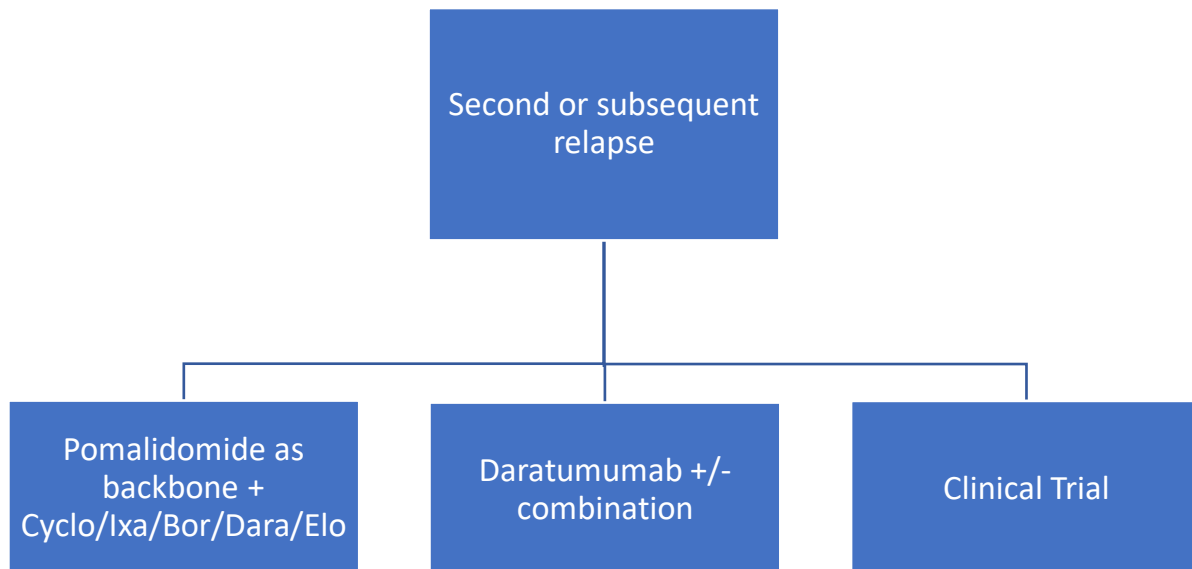
Based on the induction regimen, treatment options on relapse should be chosen from an alternative drug group in combination with steroids (fig.7). Relapse management varies based on previous treatment exposure, drug resistance and the number of treatment cycles. There is a significant role of newer PI's and mAb-based approach in

this patient population as the disease usually becomes progressively resistant. The potential rationale of clinical trial eligibility should be explored.



**Fig7a: Treatment pathway for relapsed MM patients[339]**

VRD- bortezomib, revlimid, dexamethasone; VTD- bortezomib, thalidomide, dexamethasone; VCD- bortezomib, cyclophosphamide, dexamethasone; PAD- bortezomib, doxorubicin, dexamethasone; RD- revlimid, dexamethasone; DaraVD- bortezomib, daratumumab, dexamethasone; PanoVD- panobinostat, bortezomib, dexamethasone; EloVD- elotuzumab, bortezomib, dexamethasone; KRd- carfilzomib, lenalidomide, dexamethasone



**Fig7b: Treatment options after 2<sup>nd</sup> relapse[339]**

Cyclo- cyclophosphamide; Ixa- Ixazomib; Bor- Bortezomib; Dara- Daratumumab; Elo- Elotuzumab

### 2.5.2 Plasmacytoma:

Solitary plasmacytoma in the absence of systemic involvement with MM can be managed with localized radiotherapy. Careful monitoring is required to assess for systemic disease progression[339].

### 2.5.3 Supportive care:

80% of MM patients have skeletal involvement while about 60% of these will experience a disease related fracture during their disease course. There is increased osteoclastogenesis and reduced osteoblast activity in MM. Along with systemic treatment, administration of bisphosphonates has strong evidence in MM patients, particularly with lytic lesions. Either monthly pamidronate or Zoledronic acid can be given up to two years. A dental review is recommended before bisphosphonate usage owing to the risk of osteonecrosis along with monitoring of the renal function before bisphosphonate usage[339, 341].



#### **2.5.4 Emergency care:**

Cord compression would be an acute emergency for patients with involvement of vertebral column. Orthopaedic decompression may be needed along with localized radiotherapy with high dose dexamethasone which should be promptly initiated on diagnosis[339]. Tumour lysis syndrome should be pre-empted in patients with significant disease burden/renal impairment and managed with adequate hydration allopurinol/ rasburicase.

### **3.0 Aims of thesis**

- I) Identification of molecular target for aurora kinases and t(4;14) translocation with fgfr3 specificity in MM**
  
- II) Study of natural history of 17p loss in MM:**
  - Ila) De-novo**
  
  - Ilb) Acquired**

**3.1 Aim I) Identification of molecular targets of aurora kinases and t(4;14) translocation with fgfr3 specificity in MM**

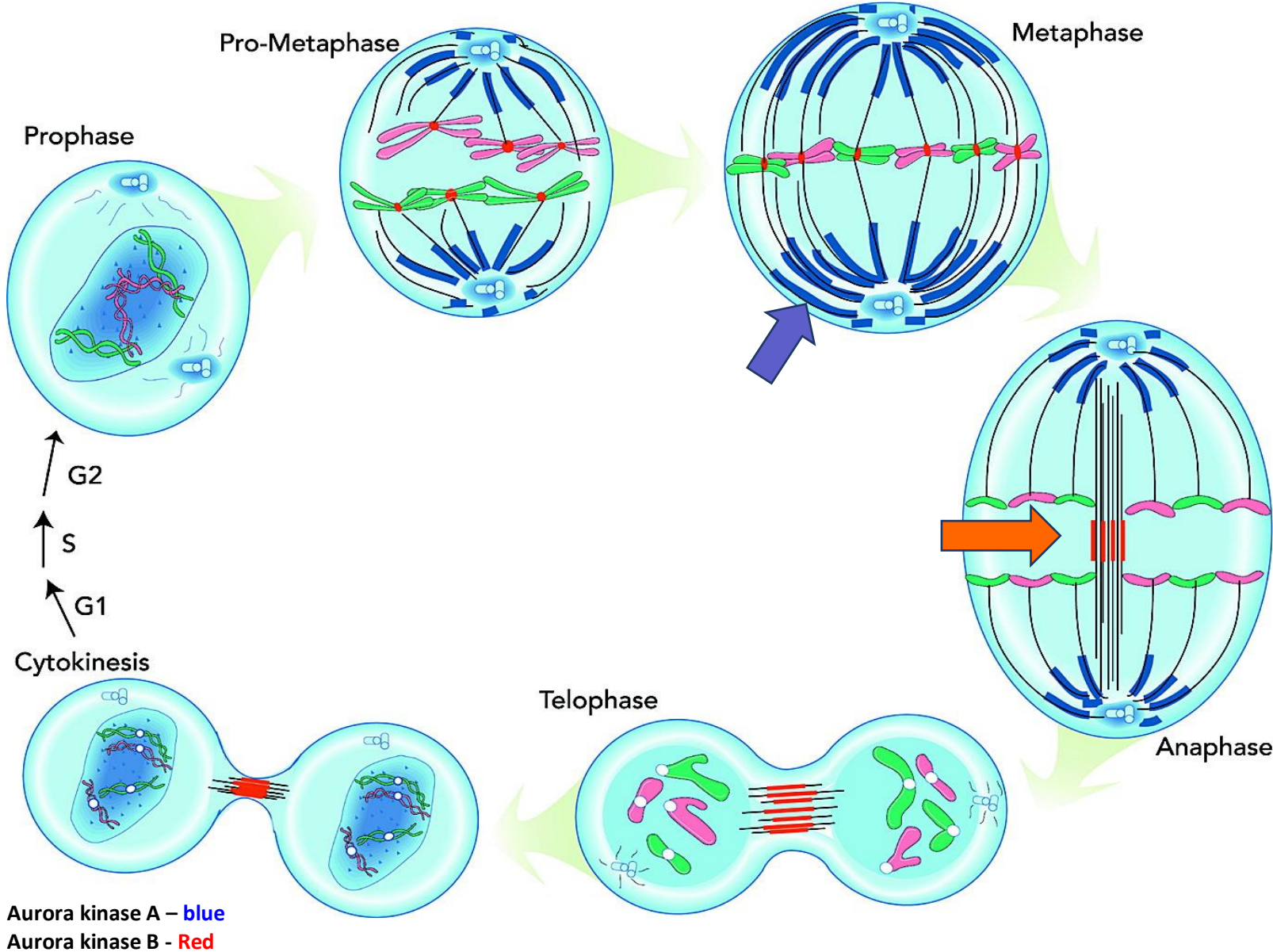
### 3.1.1 Introduction

Aberrant expression of proteins involved in cell division is a constant feature in multiple myeloma (MM), especially in high risk disease. Aurora kinases are proteins that are important mediators of cell division whose levels are up regulated in MM. Functional loss of Aurora kinases results in genetic instability and dysregulated division leading to cellular aneuploidy and growth arrest. We investigated the role of Aurora kinase inhibition in MM, using a small molecule inhibitor A1014907.

Low nanomolar A1014907 concentrations induced aneuploidy in all MM cell lines across various cytogenetic abnormalities by inhibiting Aurora Kinases A and/or B. However, A1014907 induced more pronounced dose dependent apoptosis in cell lines with t(4;14) translocation. t(4;14) translocation is observed in about 20 % of patients with MM with two-thirds of these demonstrating constitutively activate FGFR3. Further investigation on the mechanism of action of A1014907 revealed potent FGFR3 pathway inhibition in sensitive cell lines as demonstrated by inhibition of p-PLC, p-Stat3 and p-PKC levels.

Examining the effect of A1014907 on patient cells in vitro also showed preferential apoptotic induction by the drug in patients with t(4;14) translocation. Thus, our results showed that aurora kinase inhibition led to cell cycle arrest and aneuploidy with minimal apoptosis whereas inhibiting both aurora kinase and FGFR3 activity induced potent apoptosis in MM cells. Finally, combining A101 with dexamethasone showed potent synergy in inducing cytotoxicity in both the sensitive and resistant MM cell lines. Our results warrant clinical evaluation of A1014907 in combination with dexamethasone in MM patients with t(4;14) translocation.

3.1.2 Fig. 8 Progression of cell through G2/M phase and Aurora Kinases expression[1]



### **3.1.3 Materials and Methods**

#### **3.1.3.1.1 Cell lines**

MM cell lines MM1S, MM1R, RPMI8226, OPM2 and U266, were kindly provided by Dr Jonathan Keats (TGen, Phoenix, AZ). Kas6 was kindly provided by Dr John Lust (Mayo Clinic, Rochester, MN). KMS11, KMS18, KMS28BM, KMS34, LP1, OPM1, JIM2, JIM3 and INA6 were kindly provided by Dr Leif Bergsagel (Mayo Clinic, Scottsdale, AZ). H929 was purchased from ATCC (Manassas, VA, USA) and DOX40 was kindly provided by Dr William Dalton (Moffitt Cancer Center, Tampa, FL, USA). ALMC1, ANBL6 and KP6 were kindly provided by Dr Diane Jelinek (Mayo Clinic, Rochester, MN).

All cell lines except Kas6, KP6, ANBL6, ALMC1 and INA6 were cultured in RPMI 1640 media (Mediatech Inc., Manassas, VA) containing 2 mM L-glutamine (Invitrogen, Grand Island, NY), 100 U/mL penicillin, 100 µg/mL streptomycin and 10% foetal bovine serum (Mediatech, Inc.). Kas6 and INA6 were cultured in same media but in the presence of 4 ng/mL of IL6 and 1 ng/ml IL6 respectively (R&D Systems, Inc., Minneapolis, MN). ALMC1, ANBL6 and KP6 were cultured in IMDM media (Invitrogen) containing 2mM L-glutamine (Invitrogen), 100 U/ml penicillin, 100 µg/mL streptomycin, 10% foetal bovine serum (Mediatech, Inc.) and supplemented with 1 ng/ml IL6 (R&D Systems).

#### **3.1.3.1.2 Bone marrow aspirate**

Bone marrow aspirates were obtained from MM patients after informed consent under a protocol approved by the Mayo Clinic Institutional Review Board in adherence with the Declaration of Helsinki. CLL B-cells were obtained after informed consent under a protocol approved by the Mayo Clinic Institutional Review Board in adherence with the Declaration of Helsinki.

Bone marrow (BM) aspirates from patients with MM were obtained after informed

consent under a protocol reviewed and approved by the Mayo Foundation Institutional Review Board in accordance with the Declaration of Helsinki and the applicable federal regulations.

### **3.1.3.1.3 Bone marrow aspirate preparation**

Bone marrow aspirate samples were lysed using ACK lysis buffer solution containing NH<sub>4</sub>Cl (8,024mg/l), KHCO<sub>3</sub> (1,001mg/l) and EDTA Na<sub>2</sub>·2H<sub>2</sub>O (3.722 mg/l) for the lysing of red blood cells.

Procedure: Lysis reagent was prepared as per manufacture's protocol.

All reagents of the lysis buffer mentioned above were dissolved in 850 mL of H<sub>2</sub>O and pH maintained between 7.2–7.4. Further 1litre of H<sub>2</sub>O was added for completion of lysis reagent.

1. Fresh blood undergoes cytopspin and then supernatant discarded
2. An equivalent volume of ACK lysing buffer was added to the pellet remaining with care to keep similar volume of buffer to the pellet the remaining pellet.
3. Gently shake the tube for 30-60 seconds to resuspend the pellet
4. Fill tube with medium that does not contain serum and centrifuge.
5. Again remove supernatant and retain pellet.
6. This process can be repeated one more time if necessary.
7. Proceed with normal cell protocol.

The lysed samples further underwent CD138 antibody conjugation to magnetic beads using RoboSep (StemCell Technologies, Vancouver, BC, Canada) for separation of CD138+ MM cells. The isolated MM cells were checked for purity using a slide-based method to confirm more than 95% purity. MM cells were placed in medium A for use in the respective assays. For the generation of bone marrow stromal cells (BMSCs), CD138-negative MNCs were placed in 25-mm<sup>2</sup> culture flasks in RPMI 1640 medium containing 20% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

BM stromal cells (BMSCs) and coculture Freshly obtained BM aspirates were subjected to Ficoll–Paque gradient separation to isolate mononuclear cells (MNCs). These cells were placed in 25 mm<sup>2</sup> culture flasks in RPMI-1640 media. Once the BMSC were confluent, they were trypsinized and passaged as needed.

### **3.1.3.2 Drug**

A1014907 was synthesized and provided by Abbott Laboratories Ltd under a Material Transfer Agreement (MTA). Stock solutions were made in DMSO, diluted in RPMI-1640 and subsequently stored at –20° C. The diluted A1014901 was subsequently used for all experiments. Drug dilutions were made using the equation  $M1V1 = M2V2$

### **3.1.3.3 Cell Viability Assay**

Colorimetric assays were also performed to assay cell viability in the presence of A1014907. 4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) was utilized to assess for viable cells as a percentage of control. Cells from individual cell lines were plated in the 96 well plates at a count of 20,000 cells in 100µl of conditioned media (RPMI-1640 containing 10% Fetal bovine serum, 100u/ml penicillin and 100mg/ml of streptomycin) with varied concentrations of A1014907 for 72-hour. In the last four hours of the 72 hours, all wells containing cells were pulsed with 10 µL of 5 mg/mL 3 MTT. The 96-well plates were incubated at 37°C away from light for 4 hours, followed by addition of 100 µL isopropanol that contained 0.04 HCl. Absorbance readings at a wavelength of 570 nm (with correction using readings at 630 nm) were taken on a spectrophotometer (Molecular Devices, Sunnyvale, CA). Each experiment was performed in triplicate and all experiments repeated three times.



### **3.1.3.4 Cell proliferation assay**

DNA synthesis was measured by tritiated thymidine uptake [ $^3\text{H}$ -TdR] (Perkin Elmer, Boston, MA). MM cells (20,000cells/well) were incubated in 96-well culture plates (Costar, Cambridge, MA) in the presence of media or varying concentrations of A1014907, for 72 hours at 37°C. Cells were pulsed with  $^3\text{H}$ -TdR (0.5  $\mu\text{Ci}$  (0.185 MBq)/well) during the last 4 hours of 72 hours time period and subsequently, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA). These harvested cells were then counted using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). Each experiment was performed in triplicate and all experiments repeated three times.

### **3.1.3.5 Bone marrow stromal cells co-culture experiments**

Bone marrow aspirates were subjected to Ficoll Paque gradient centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ), and mononuclear cells (MNCs) were separated. MNCs were placed in 25-mm<sup>2</sup> culture flasks in RPMI 1640 media (Sigma Chemical) containing 20% foetal bovine serum, 2 mM L-glutamine (Gibco), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$  streptomycin (Gibco). Once confluent, the cells were trypsinized and passaged as needed.

For the experiments, BMSCs were incubated in 96-well culture plates (approximately 2000 to 5000 BMSCs/well) in 100 mls of supplemented RPMI-1460 as described. After 18 hour incubation 20,000 MM cells were added to the wells and incubated with media alone or with different concentrations of A1014907 for 72 hours at 37°C. Cells were pulsed with  $^3\text{H}$ -TdR (0.5  $\mu\text{Ci}$  (0.185 MBq)/well) during the last 4 hours of 72 hours time period and subsequently, harvested onto glass filters with an automatic cell harvester (Cambridge Technology, Cambridge, MA). These harvested cells were then counted using the LKB Betaplate scintillation counter (Wallac, Gaithersburg, MD). Each experiment was performed in triplicate and all experiments repeated three times.

### **3.1.3.6 Morphological analysis**

Cells were fixed on glass slides using methanol. Slides were dried and deparaffinized followed by staining with Haematoxylin dye. Stained slides were rinsed, dehydrated using ethanol and xylene and finally mounted with resinous mounting medium. Microscopic images were taken at 40× magnification highlighting morphological changes post A1014907 treatment.

### **3.1.3.7 Cell cycle analysis**

Cells were treated with indicated doses of A1014907 for 24, 48 or 72 hrs. Cells were harvested, counted and washed with PBS following which 2 ml of cold 85% ethanol was added to the pellet while the tubes were vortexed. The tubes were left at 4° C overnight. Subsequently, the cells were pelleted and washed twice with PBS. The pellet was then resuspended in 0.1 ml of 5 µg/ml RNase (Sigma-Aldrich, St. Louis, MO) and incubated at 37° C for 30 minutes. PBS (0.9 ml) and 10 µl of 1 mg/ml propidium iodide (PI) (Sigma-Aldrich) were added and samples were held at 4° C till they were run on a Canto flow cytometer (BD Biosciences, San Jose, CA) and analysed using FlowJo software (Tree Star, Ashland, OR).

### **3.1.3.8 Apoptosis assay**

Apoptosis induction by A1014907 in MM cell lines and patient cells was measured by annexin V/PI staining and flow cytometry [38–40]. Briefly, cells were washed twice in Annexin Binding Buffer (ABB) (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>). 100 µl cells (10<sup>7</sup> cells/ml) were stained with 3 µl of annexin V-FITC (Caltag, Burlingame, CA) for 15 minutes at room temperature. Cells were again washed with ABB and resuspended in 500 µl ABB containing 5 µl of 1 mg/ml PI (Sigma-Aldrich) and run on a Canto flow cytometer (BD Biosciences).

### **3.1.3.9 Western blotting**

Cells were lysed in RIPA buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, 5 mM EDTA) containing Halt Phosphatase Cocktail (Thermo Fisher Scientific, Rockford, IL), 1 mM phenylmethylsulphonyl-fluoride (PMSF) (Thermo Fischer) and protease inhibitor cocktail (PIC) (Sigma Aldrich). Protein lysate concentrations were measured using BCA assay (Thermo Fisher). Equal amounts of protein were loaded on Tris-Glycine gels and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). All antibodies were purchased from Cell Signalling Technology (Danvers, MA). Antigen-antibody complexes were detected using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

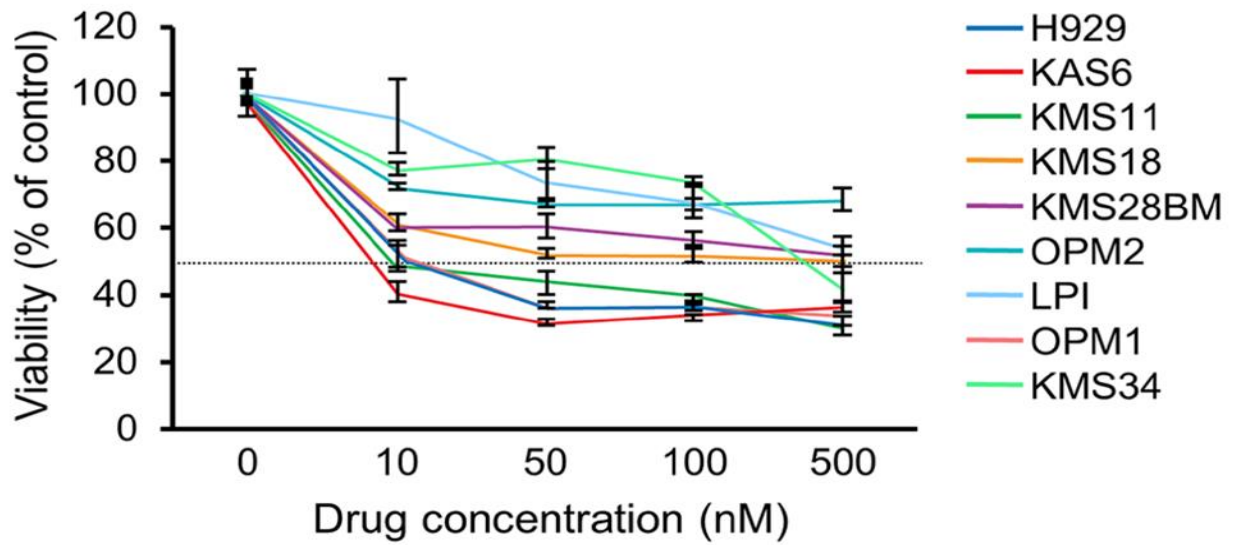
### **3.1.3.10 Receptor tyrosine kinase (RTK) array**

Human phosphor-RTK array was obtained from R&D Systems Inc, which allowed simultaneous screening of 49 different phosphorylated RTK's. The array was processed following the manufacturer's instructions. Briefly, protein extracts obtained from untreated or drug treated cells were added onto antibody coated nitrocellulose membrane. After overnight incubation at 4° C, the membrane was washed to remove excess antibodies and incubated with anti phospho tyrosine HRP Detection antibody for two hours followed by detection of the antigen antibody complex using chemiluminescence.

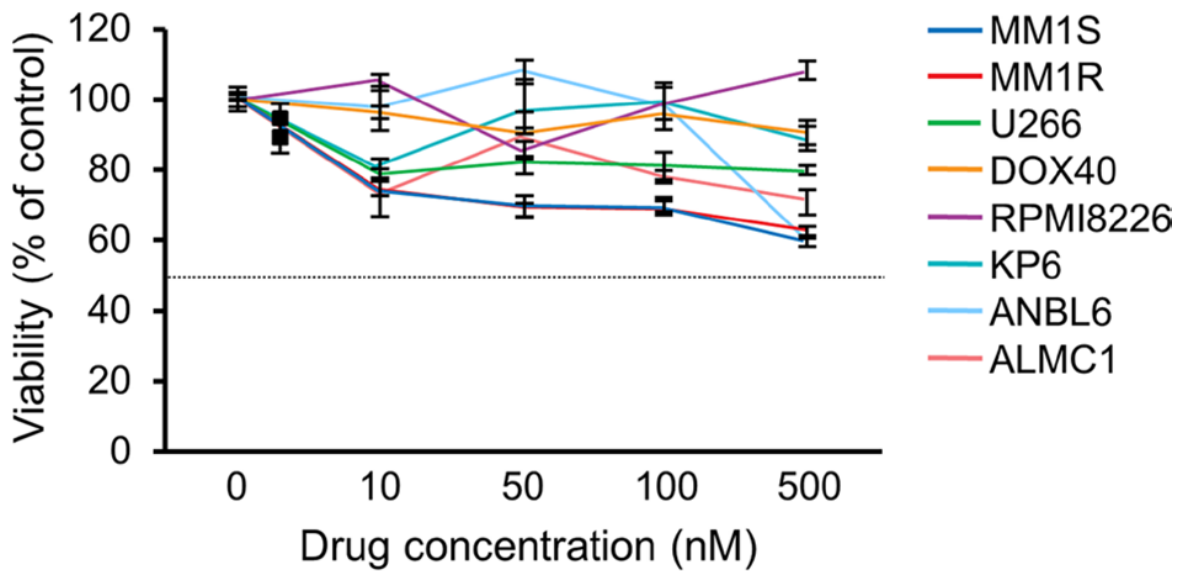
### **3.1.4 Results**

#### **3.1.4.1 Cell lines with t(4;14) translocation show increased sensitivity to A1014907(Fig 8)**

We first assessed the cytotoxic effects of A1014907 on MM cells. For this, we treated a panel of MM cell lines with various doses of A1014907 for 72 hrs and examined the cytotoxicity induced by the drug. We observed that cell lines OPM2, KMS11, KMS18, KMS28BM, H929, KAS6, LP1, OPM1 and KMS34 were significantly more sensitive to A1014907 treatment when compared to the other cell lines examined including MM1S, MM1R, U266, Dox40, RPMI8226, KP6, ANBL6 and ALMC1 (Fig 8a and b).



**Fig8a: t(4;14) cell line viability after 72 hours treatment with indicated A101 concentrations**



**Fig8b: Non t(4;14) cell line viability after 72 hours treatment with indicated A101 concentrations**

#### **3.1.4.2 t(4;14) translocation cells show increased anti-proliferative effect to A1014907(fig 9a and b)**

We next assessed the antiproliferative effects of A1014907 on MM cells. For this, we treated the same panel of MM cell lines with various doses of A1014907 for 72 and examined the effects induced by the drug. We observed that cell lines OPM2, KMS11, KMS18, KMS28BM, H929, KAS6, LP1, OPM1 and KMS34 were significantly more sensitive to A1014907 treatment when compared to the other cell lines examined including MM1S, MM1R, U266, Dox40, RPMI8226, KP6, ANBL6 and ALMC1

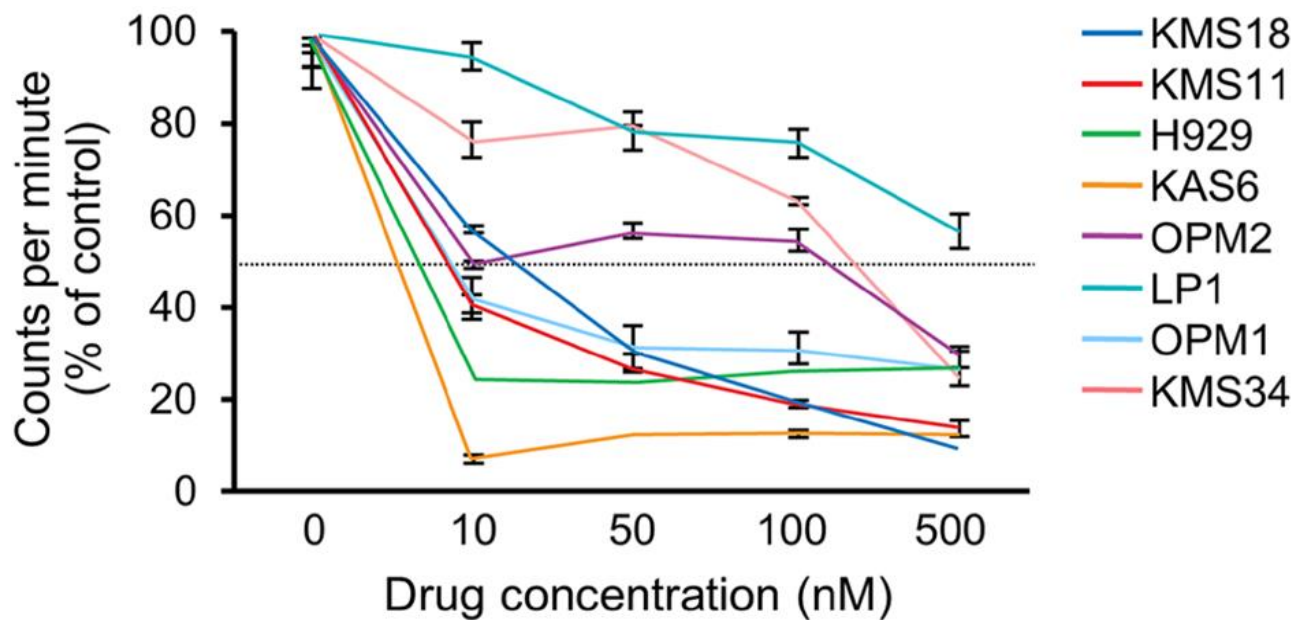


Fig9a: Anti-proliferative effects of A101 on t(4;14) cell lines after 72 hours treatment

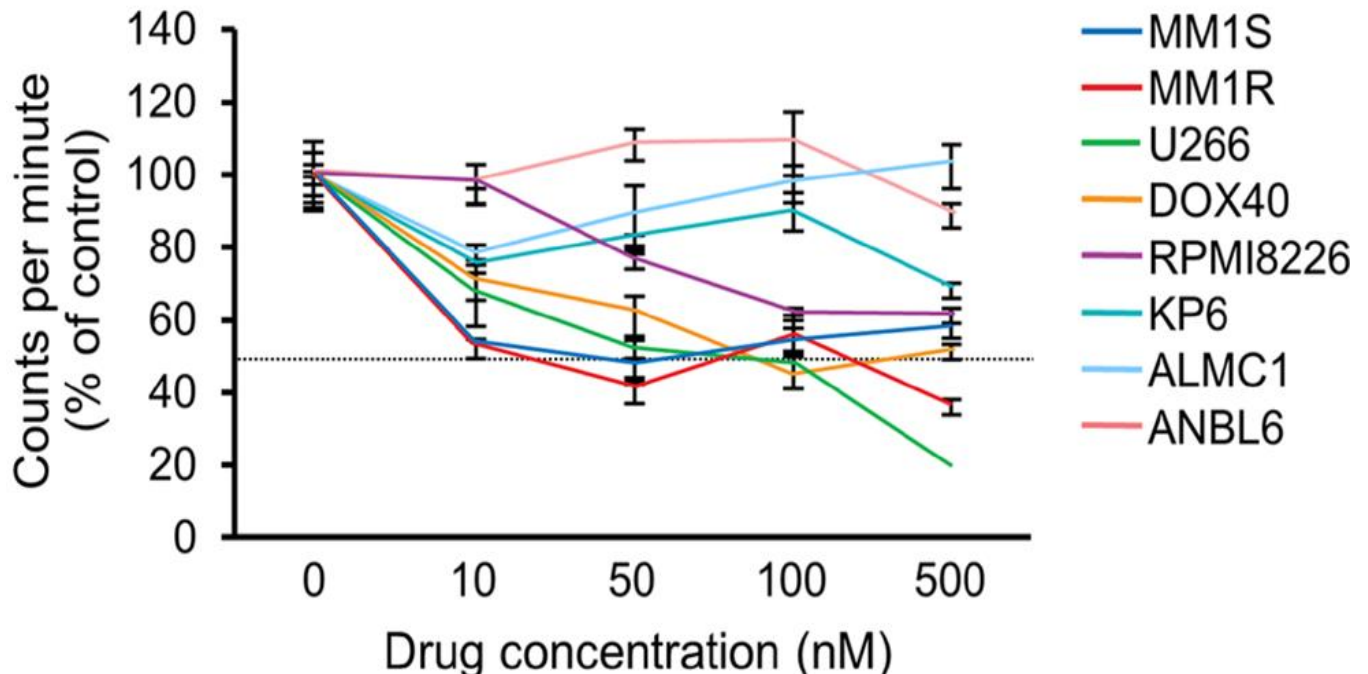


Fig9b: A101 anti-proliferative effect on non t(4;14) cell lines after 72 hours treatment

### 3.1.4.3 t(4;14) translocation cells show increased cytotoxicity and anti-proliferative effect to A1014907(fig 10. a and b)

We next jointly compared the cytotoxic(Fig 10.a) and anti-proliferative (Fig 10.b) effects of A1014907 on t(4;14) cell lines compared to non t(4;14) cells lines. The comparison of IC50 concentrations between the two sub group of cell lines showed a statistical significance using student t-test as shown below.

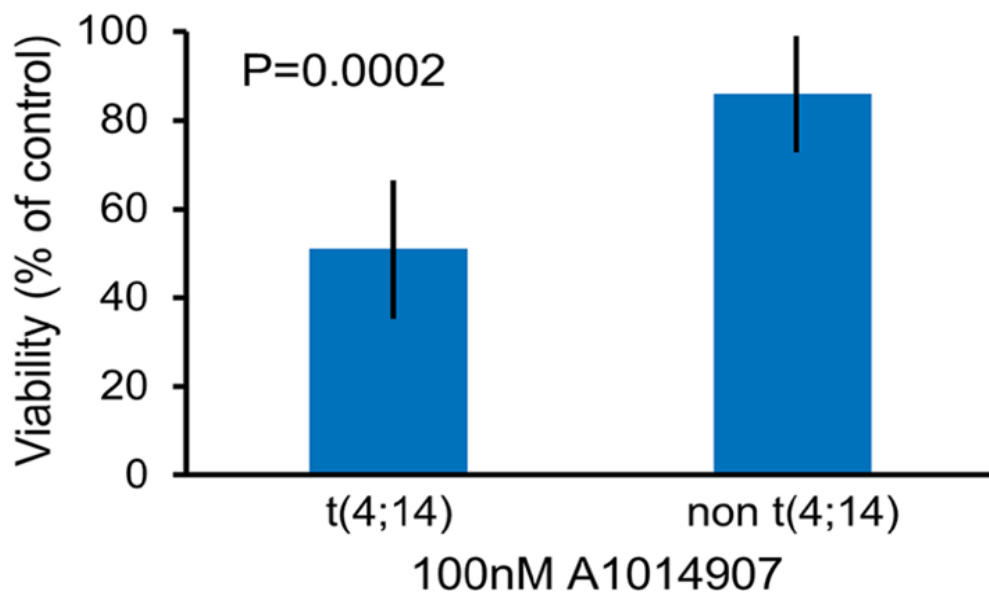


Fig10a: Significant sensitivity of t(4;14) vs non-t(4;14) cell lines to A101

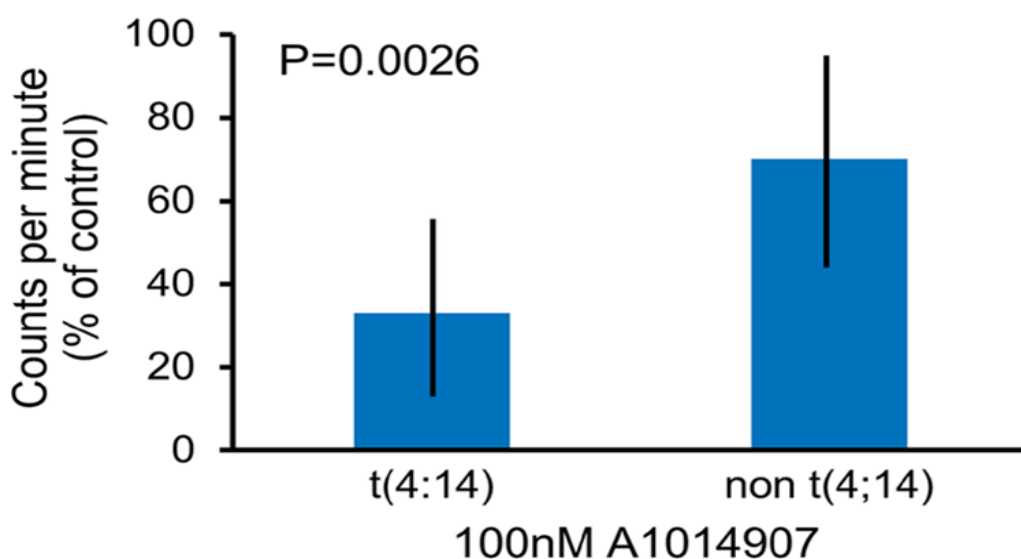
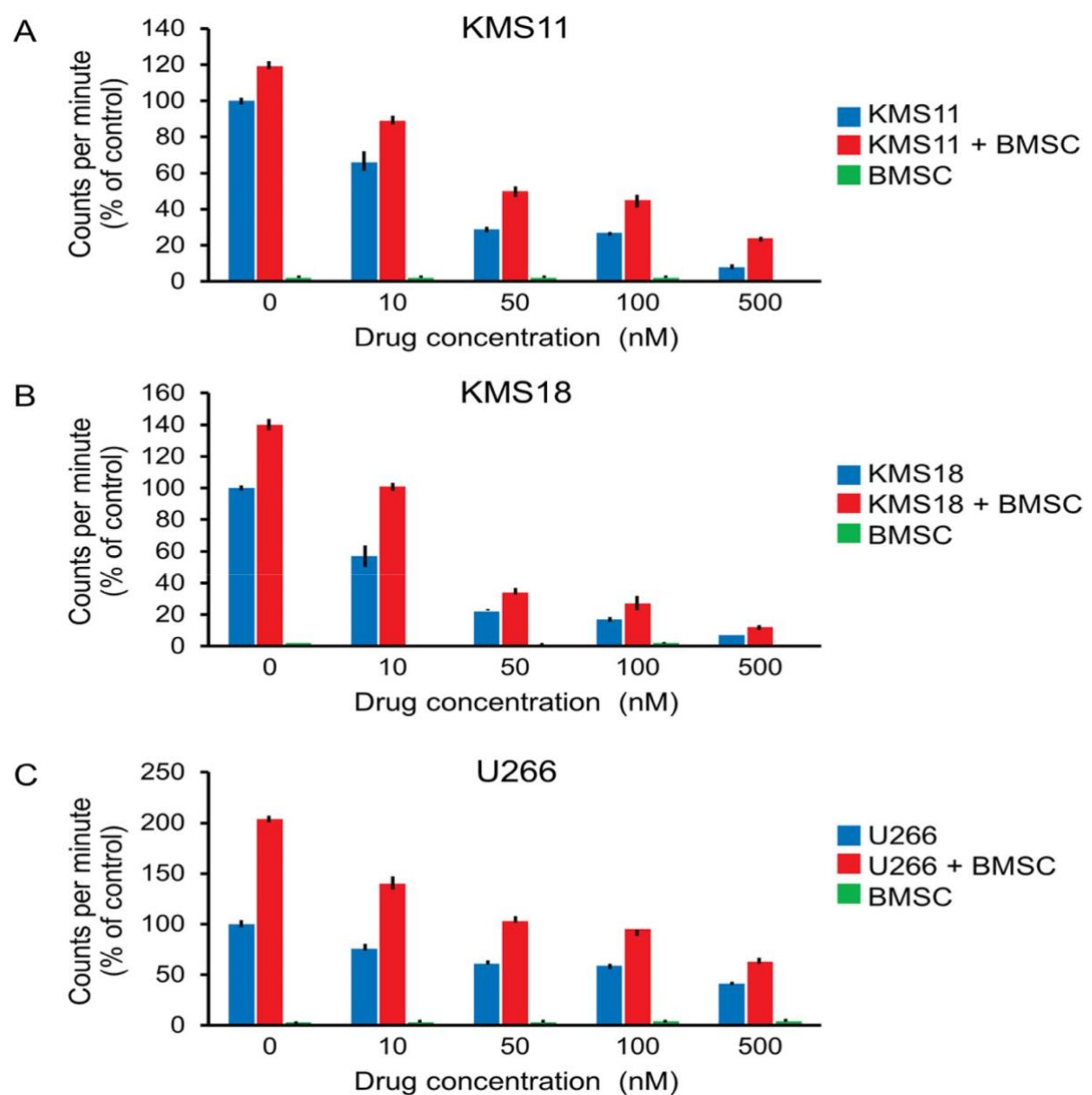


Fig10b: Significant anti-proliferative effects of A101 on t(4;14) vs non-t(4;14) cell lines



### 3.1.4.4 A1014907 is effective in the presence of tumour promoting bone marrow stromal cells (Fig 11 – A,B and C)

Bone marrow stromal cells (BMSCs) are important cellular components of the tumour microenvironment, whose interaction with MM cells contribute to disease progression and resistance to existing therapies[342]. We therefore examined if A1014907 was able to overcome the protective effects of BMSCs. We observed that A1014907 was able to inhibit proliferation of MM cells even in the presence of BMSCs in both t(4;14) (Figure 11.A and 11.B) and non t(4;14) cells (Figure 11.C)



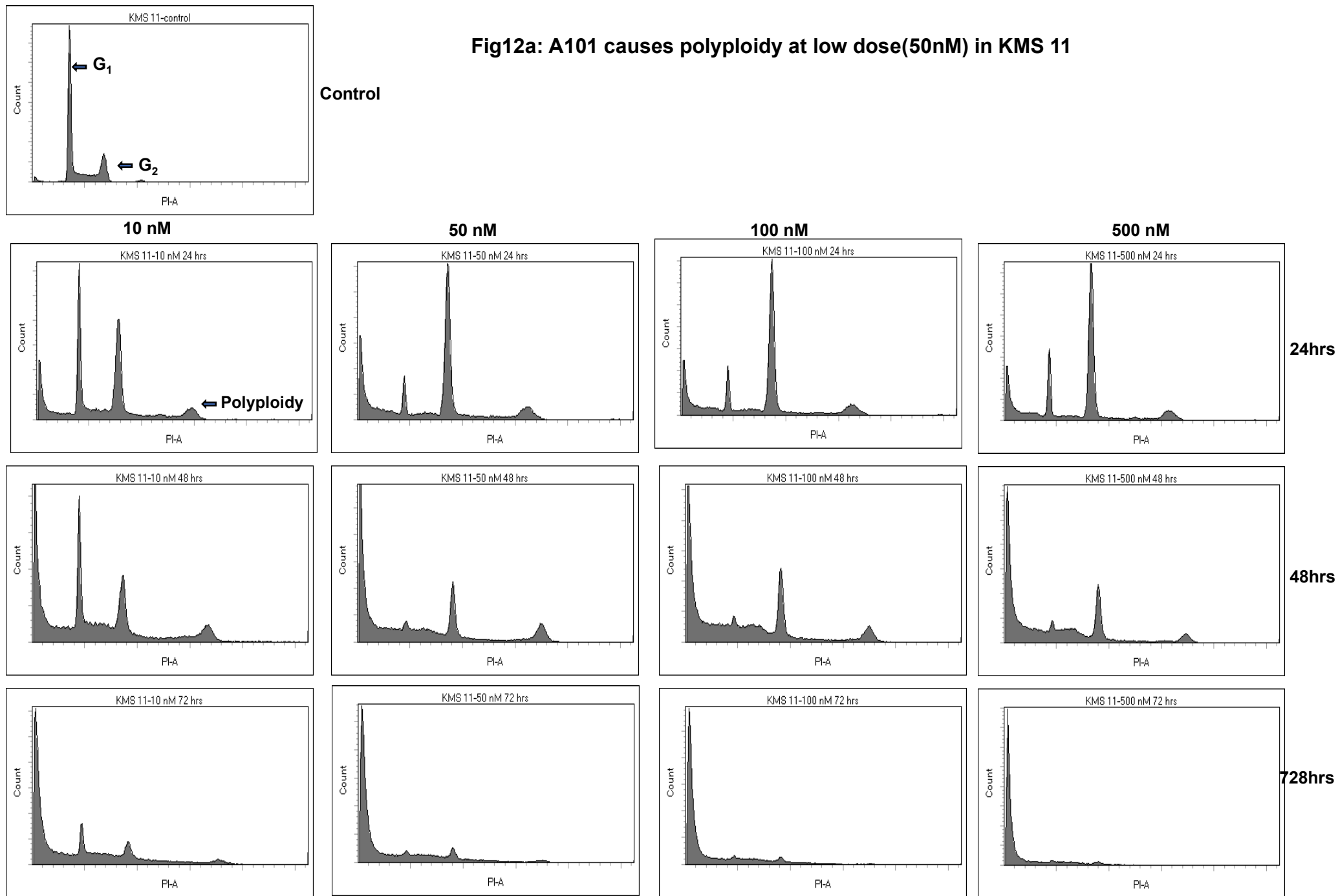
**Fig11: Increased antiproliferative effect of A101 on KMS11(A) and KMS18(B) compared to U266(C)**

#### **3.1.4.5 A1014907 induces polyploidy in all MM cell**

We next assessed if A1014907 induced cell division aberrations in both t(4;14) as well as non t(4;14) cells. For this experiment, we used KMS11, a t(4;14) and MM1S, a non t(4;14) cell line. G2M arrest and polyploidy was observed in both t(4;14)(Fig 12a) and non t(4;14)(Fig. 13a) cell lines. This was evident as early as 24 hours with 10 nM A1014907 treatment(Fig. 12 and 13).

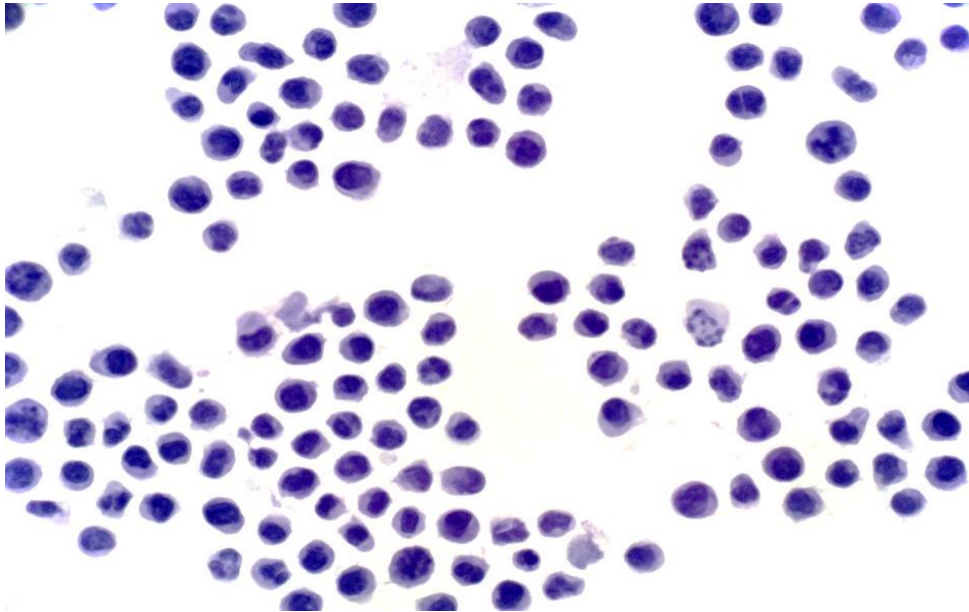
Morphological analysis showed that untreated control cells appeared to be of uniform cell and nuclear size. However, A1014907 caused the cells to increase in size with varied nuclear numbers/sizes within each cell indicative of cell division abnormality and possibly aurora kinase inhibition. (Fig. 12b and 12c ).

**Fig12a: A101 causes polyploidy at low dose(50nM) in KMS 11**

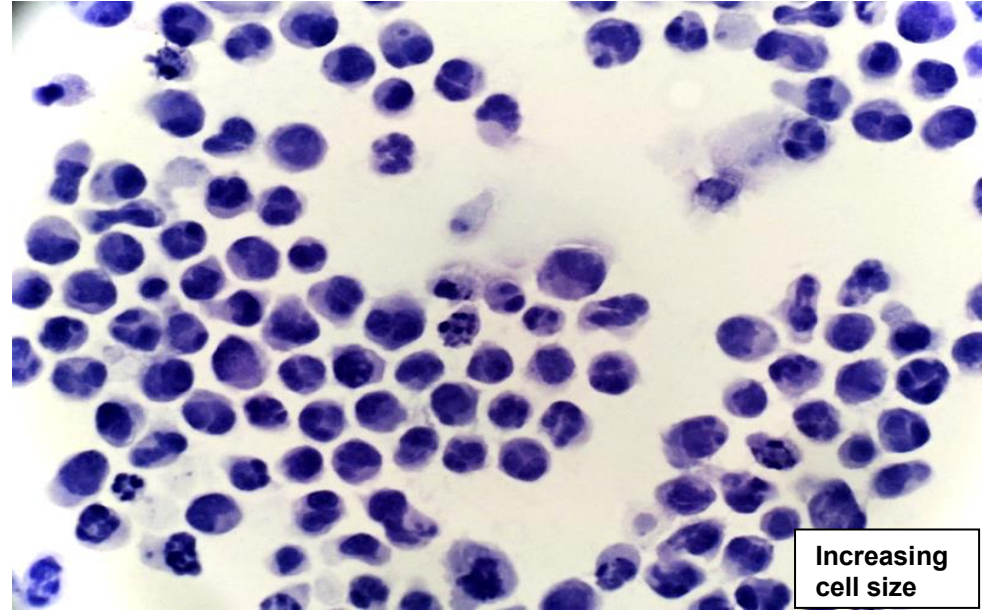


**Fig12b: A101 causes polyploidy at low dose(50nM) in KMS 11 (H& E staining)**

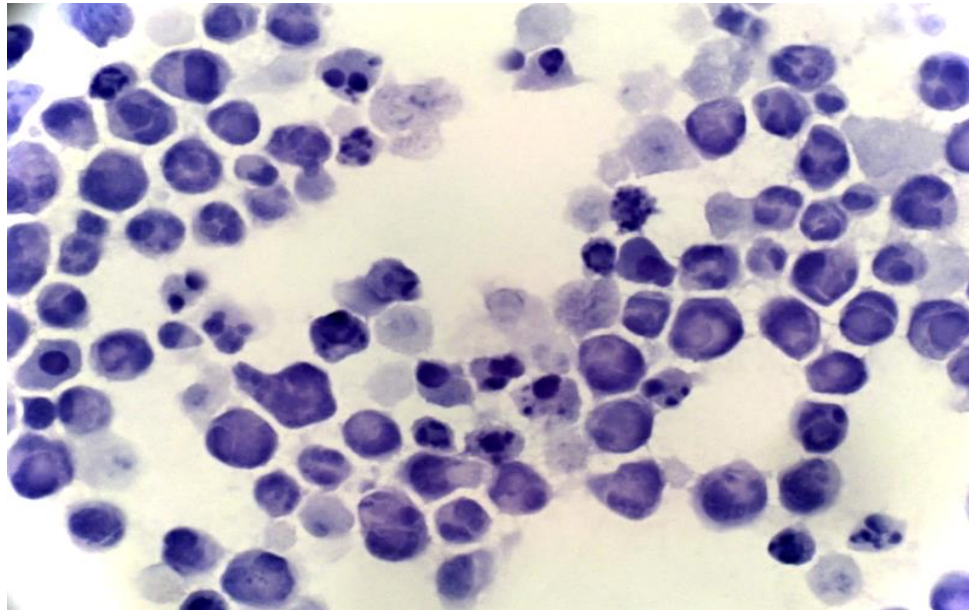
**KMS 11 72 hours Control**



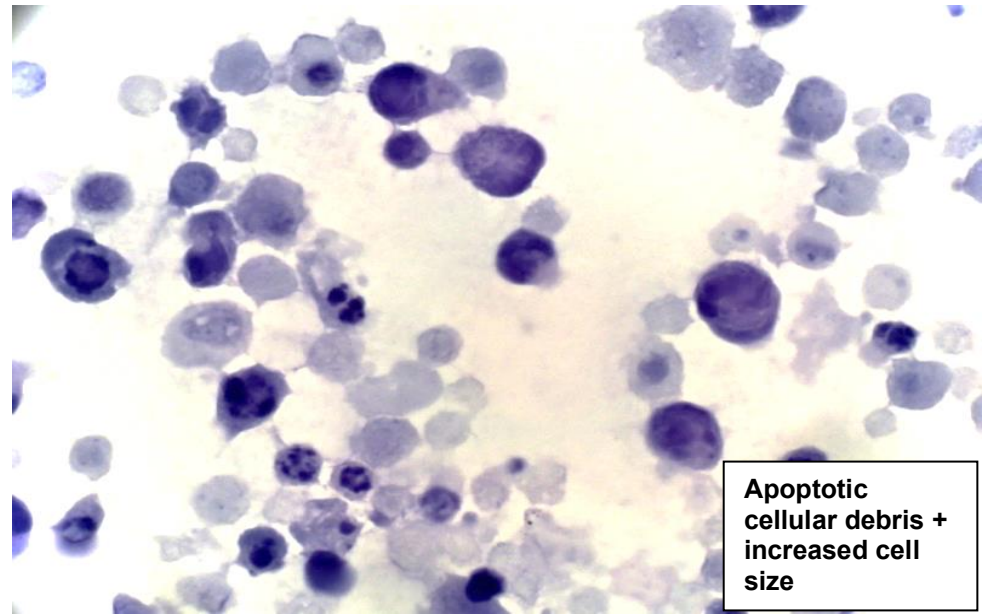
**KMS 11 24 hours 50nM A101**



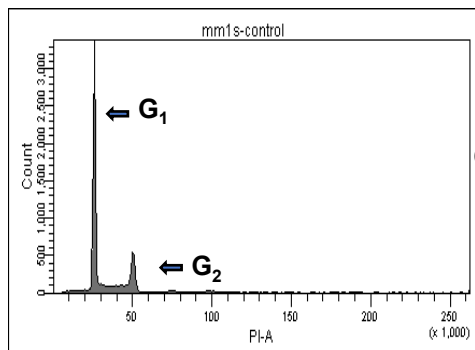
**KMS 11 48 hours 50nM A101**



**KMS 11 72 hours 50nM A101**



# Fig13a: A101 causes polyploidy at low dose(50nM) in MM1S 11



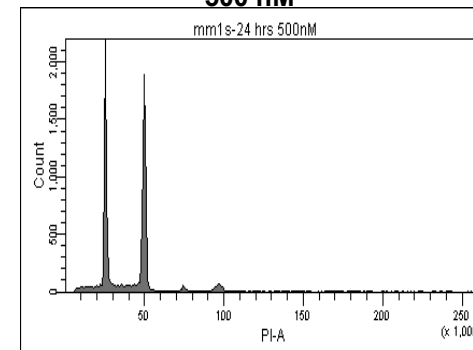
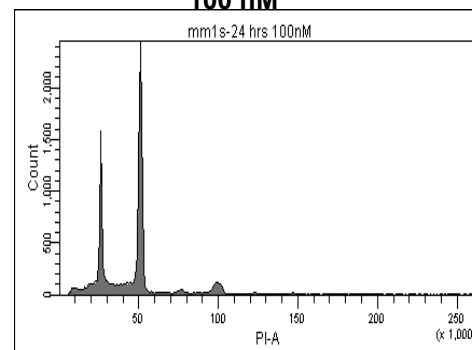
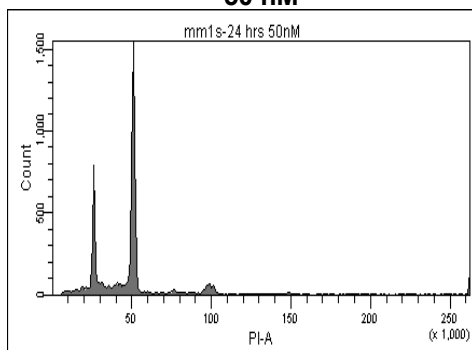
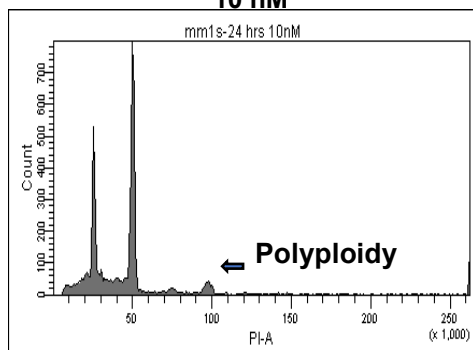
Control

10 nM

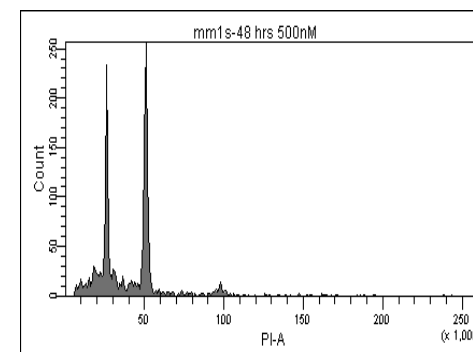
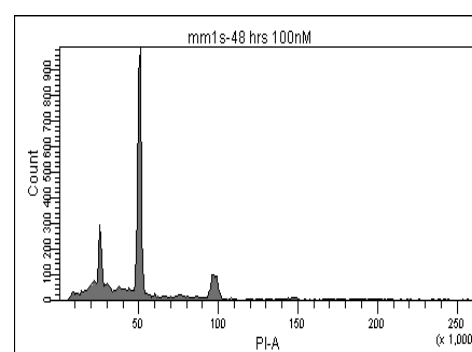
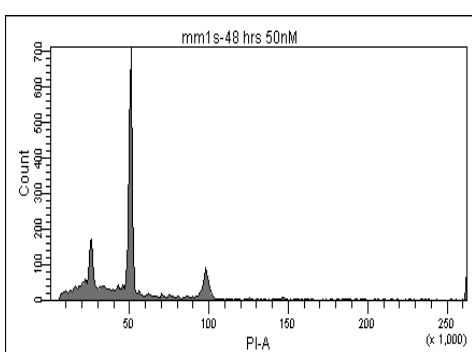
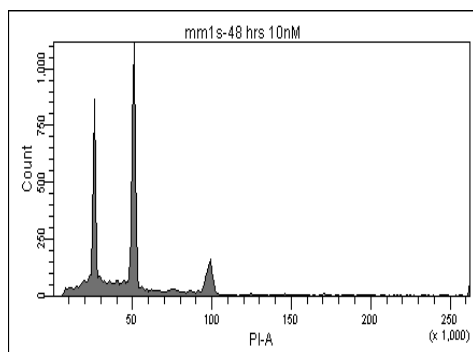
50 nM

100 nM

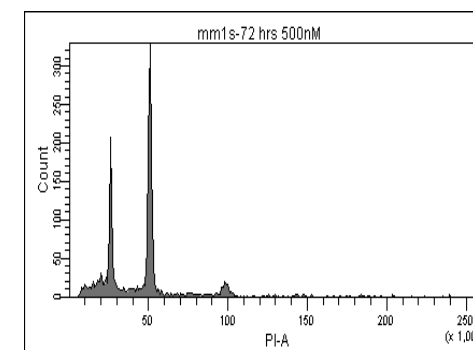
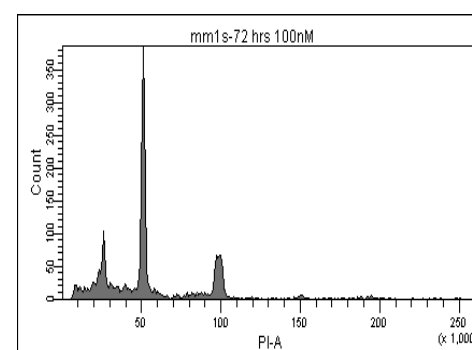
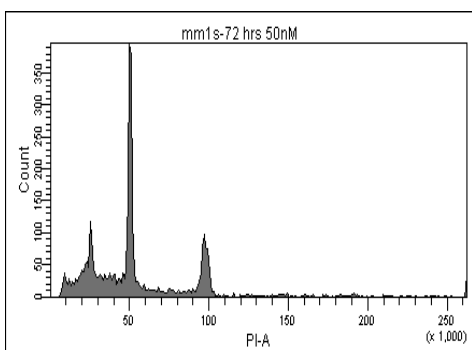
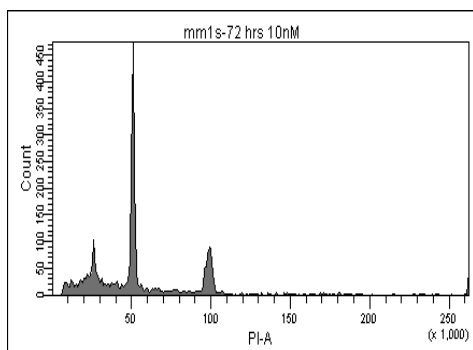
500 nM



24hrs



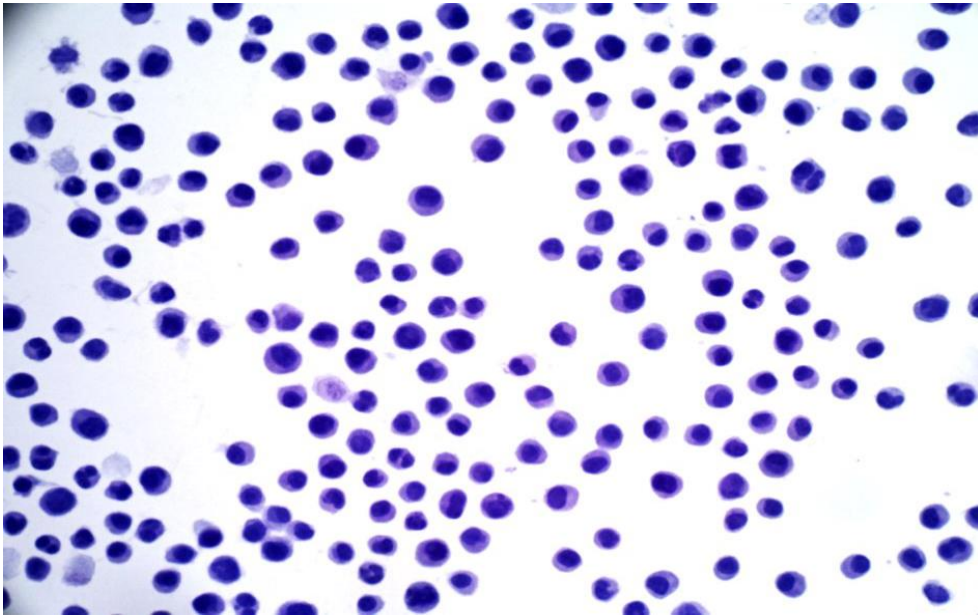
48hrs



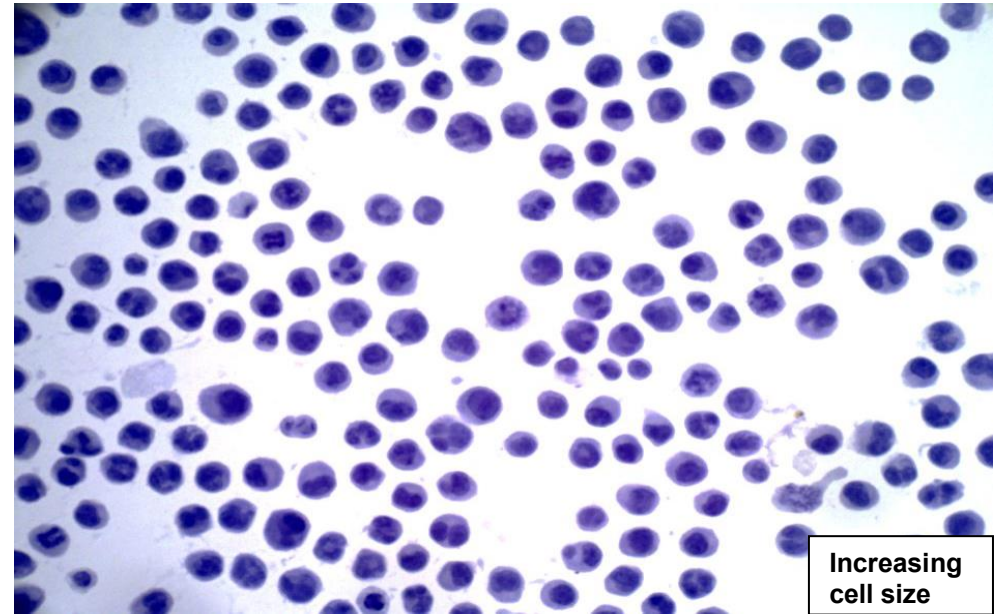
72hrs

**Fig. 13b: A101 causes polyploidy at low dose(50nM) in MMS 1S – (H&E staining)**

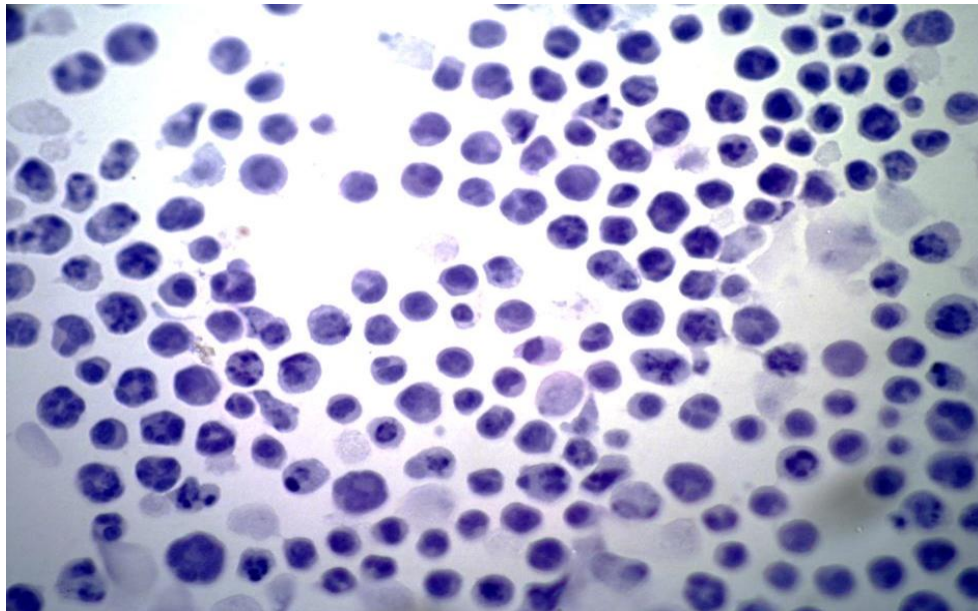
**MM1.S 72 hours Control**



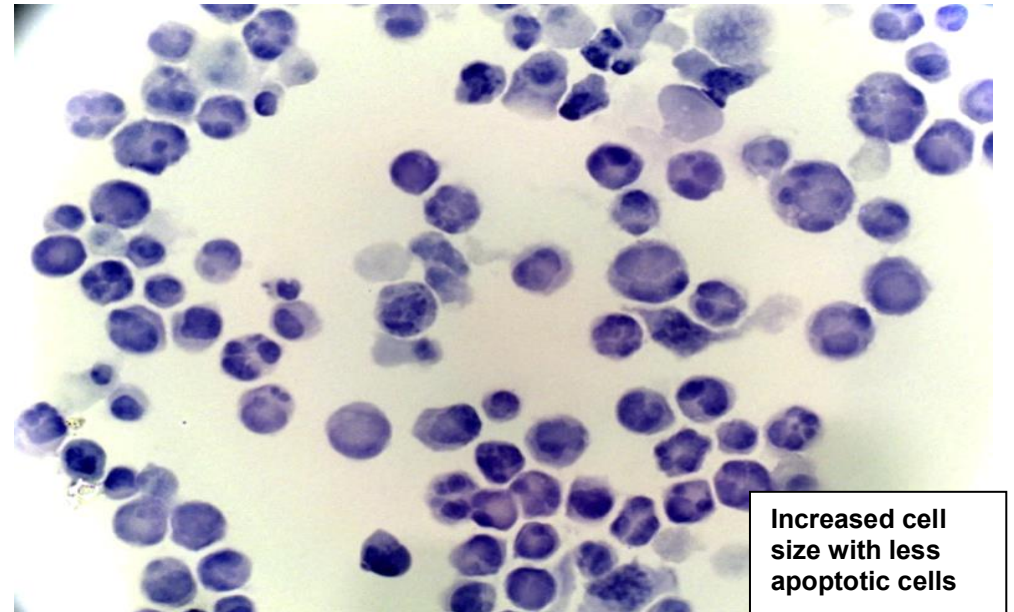
**MM1.S 24 hours 50nM A101**



**MM1.S 48 hours 50nM A101**

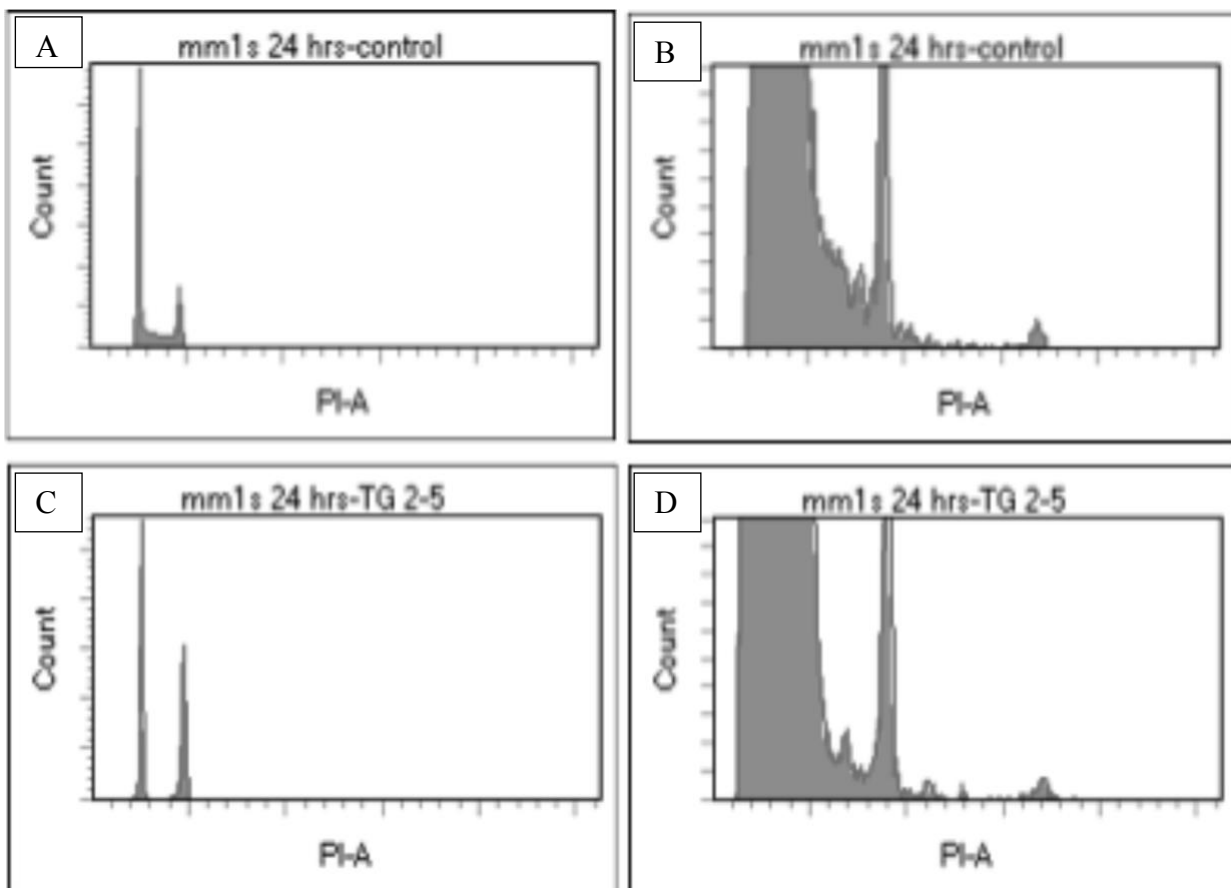


**MM1.S 72 hours 50nM A101**



### 3.1.4.6 Polyploidy is a specific A1014907 mechanism of action (Fig 14)

To confirm that the appearance of the 8N peak was due to the ability of A1014907 to induce polyploidy and not because of the drug's ability to cause G2/M arrest, we treated MM1S cells with TG101209, a Jak2 inhibitor that we had shown in an earlier study to induce G2/M arrest. Results showed that TG101209 induced G2/M arrest without causing polyploidy further confirming that A1014907 does indeed induce polyploidy (Figure14-G2M arrest examined using PI staining) .



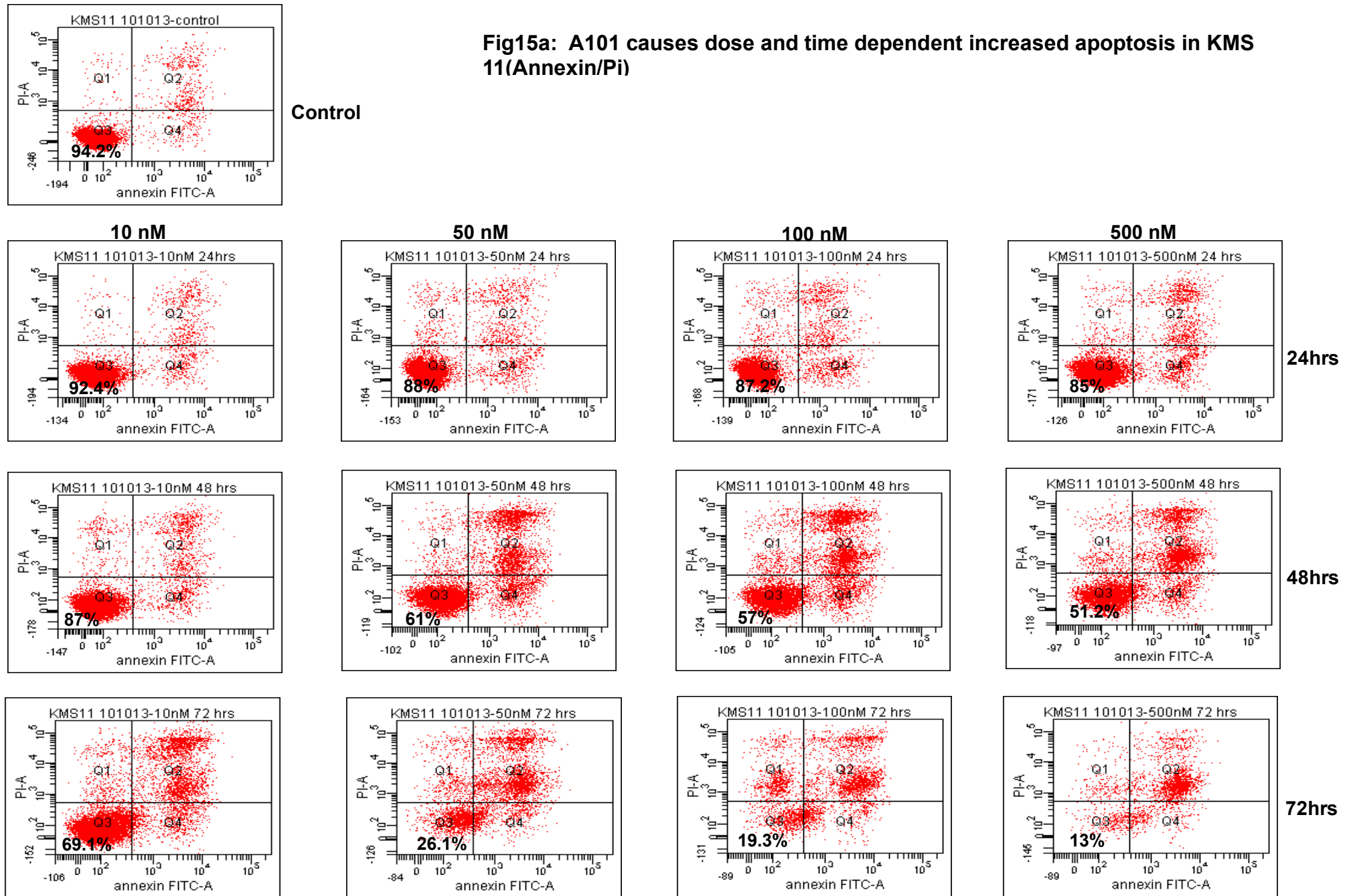
**Fig14: MM1S does not display polyploidy(A, B magnified scale); On treatment with TG101209 G2/M arrest is seen( C, D magnified scale)**

### **3.1.4.7 A101 induces dose and time dependent apoptosis in t(4;14) cell lines (Annexin/Pi staining) (Fig 15 -16)**

Given that A1014907 induced more potent cell death in cells with t(4;14) translocation than in cells lacking this translocation, we next performed assays to confirm if the cell death occurred through induction of apoptosis. For this, we treated KMS11(Fig 15a and b) and MM1S(Fig 16a and b) cells with indicated concentrations of A1014907 for various time points. We observed that A1014907 induced potent apoptotic cell death in KMS11(Figure 15a and b). However, MM1S cells showed resistance to A1014907 treatment with minimal increase in apoptosis (Figure 16a and b).

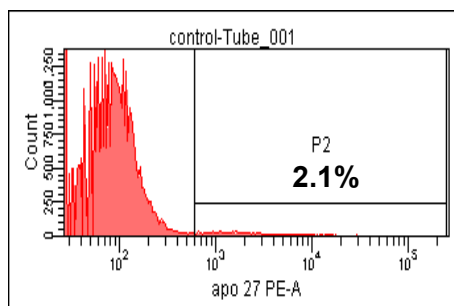


**Fig15a: A101 causes dose and time dependent increased apoptosis in KMS 11(Annexin/Pi)**



**Fig15b: A101 causes pronounced mitochondrial damage in KMS 11(Apo 2.7 staining)**

**Control**

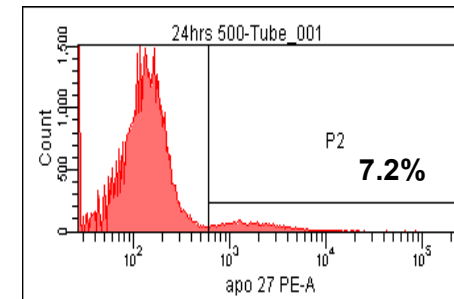
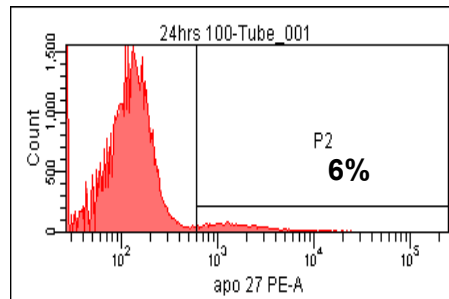
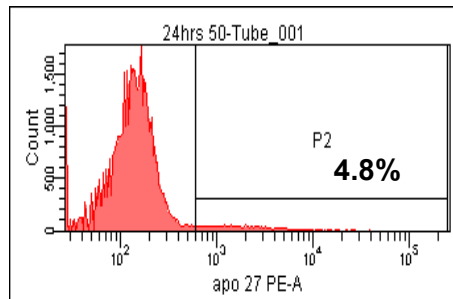
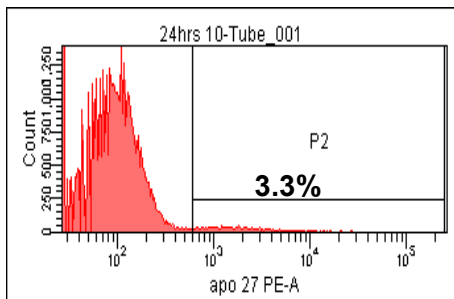


**10 nM**

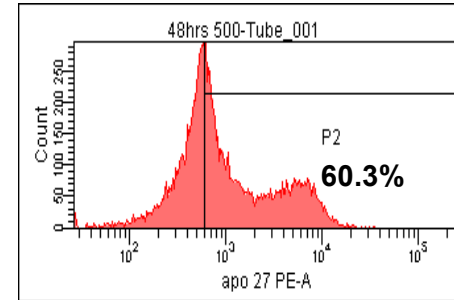
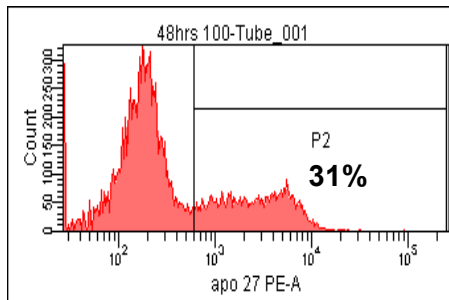
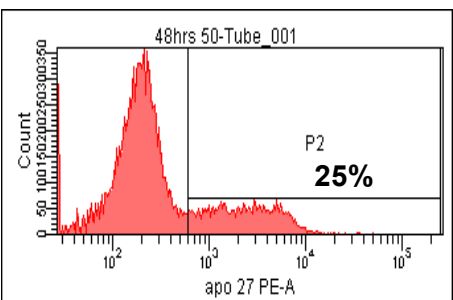
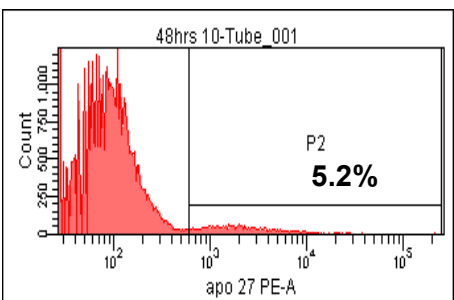
**50 nM**

**100 nM**

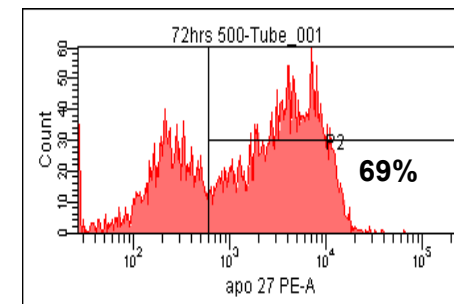
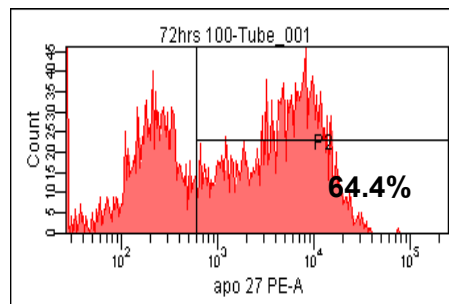
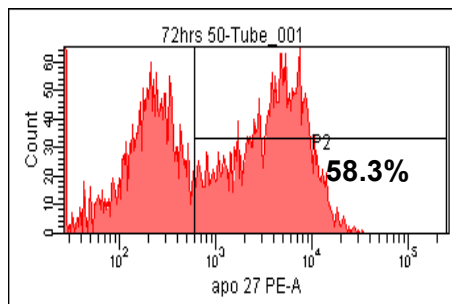
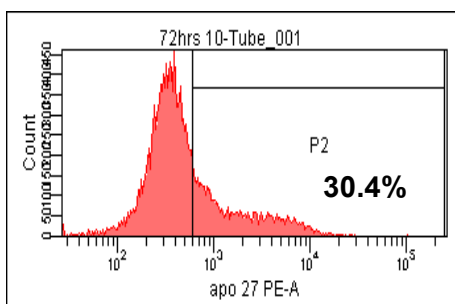
**500 nM**



**24hrs**

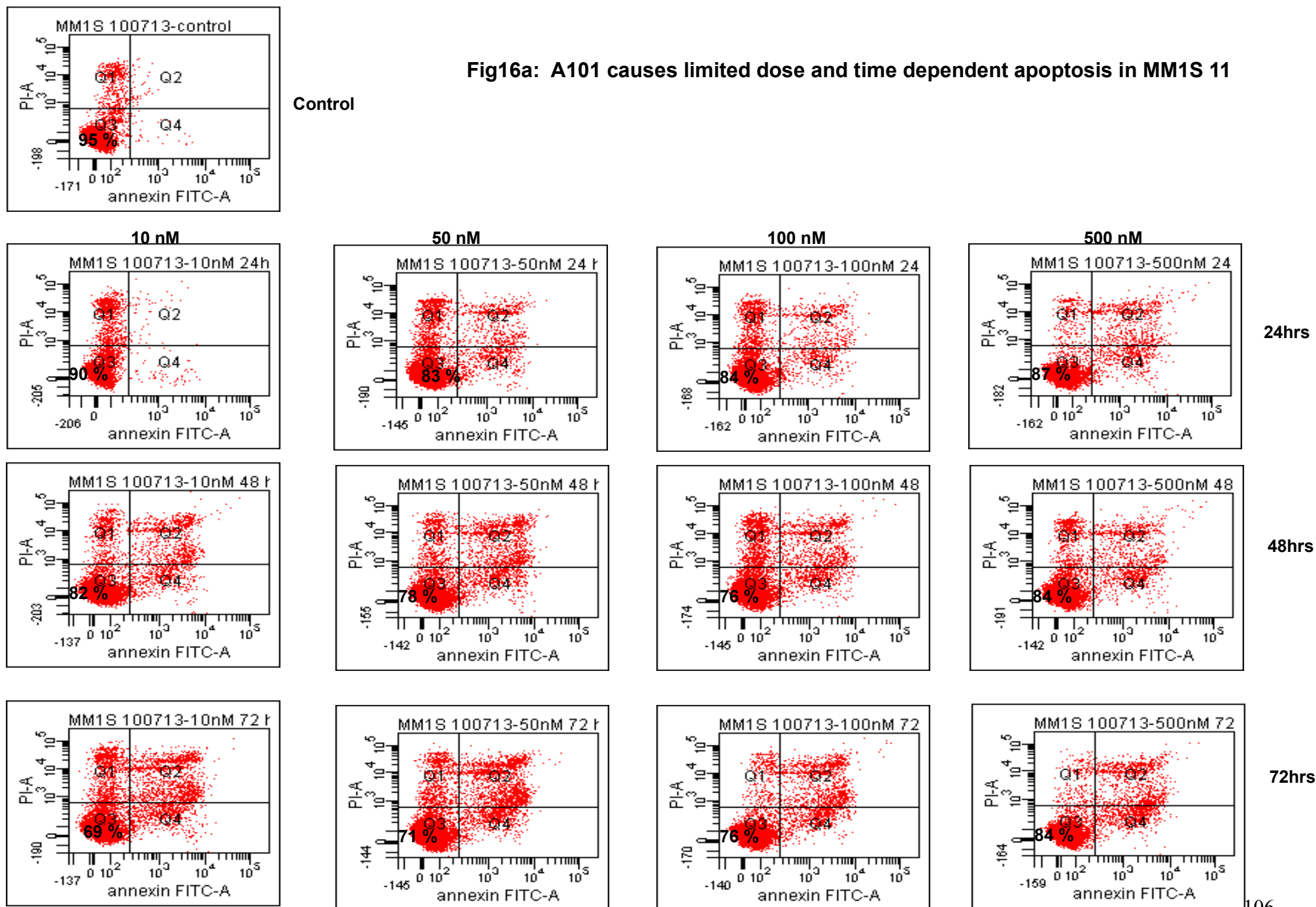


**48hrs**

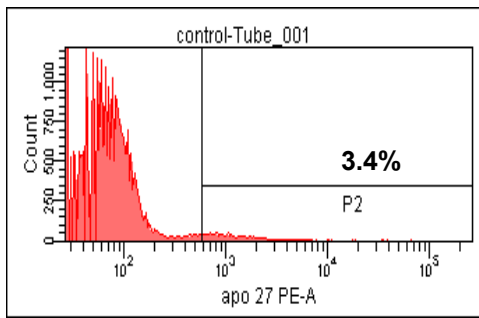


**72hrs**

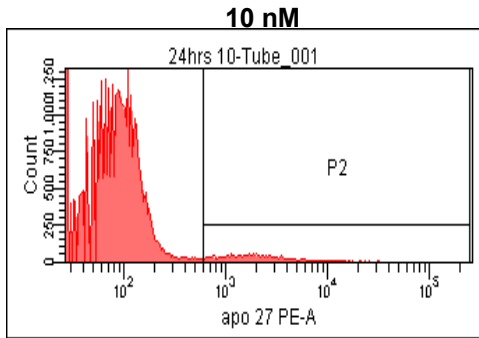
**Fig16a: A101 causes limited dose and time dependent apoptosis in MM1S 11**



**Fig16b: A101 causes limited mitochondrial damage in MM1S (Apo 2.7 staining)**



**Control**

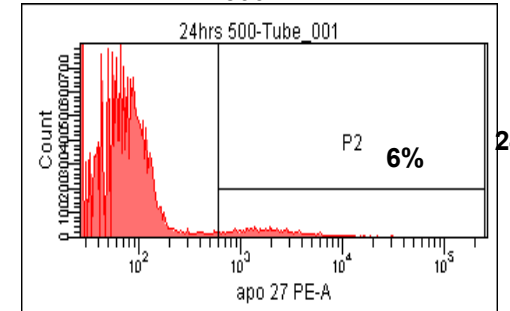
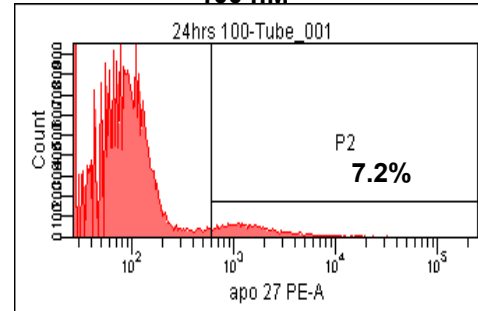
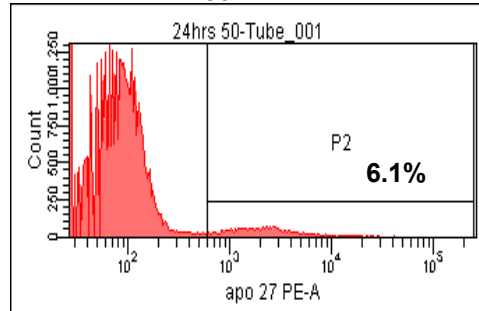


**10 nM**

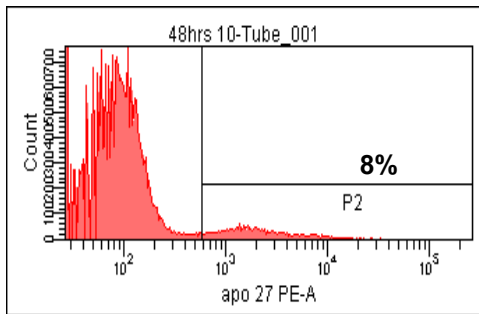
**50 nM**

**100 nM**

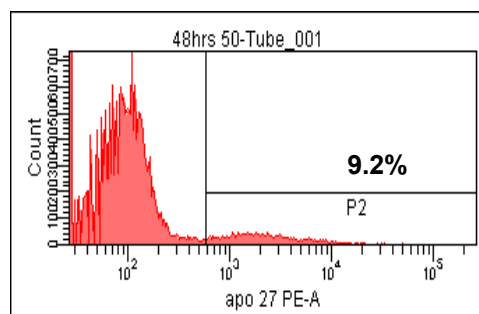
**500 nM**



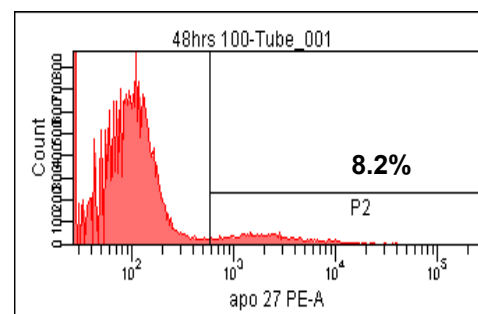
**24hrs**



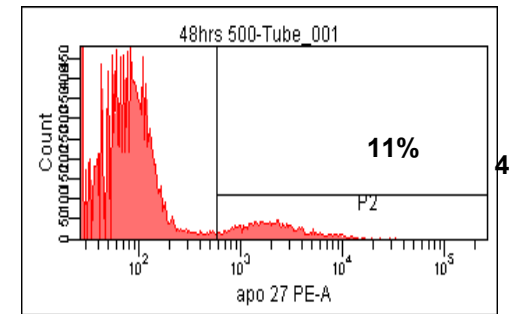
**8%**



**9.2%**

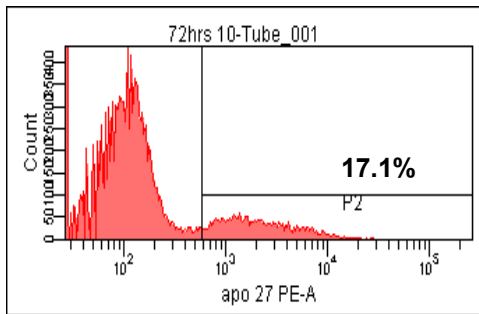


**8.2%**

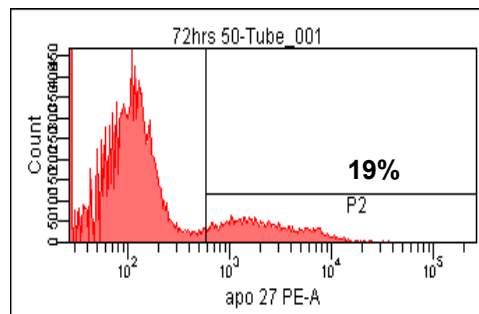


**11%**

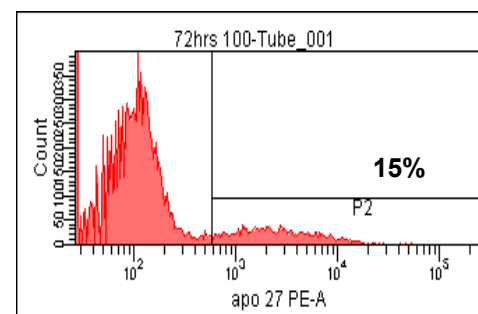
**48hrs**



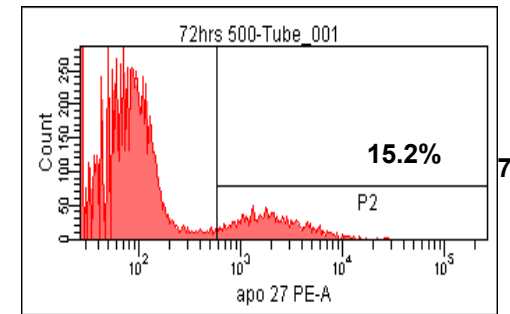
**17.1%**



**19%**



**15%**



**15.2%**

**72hrs**

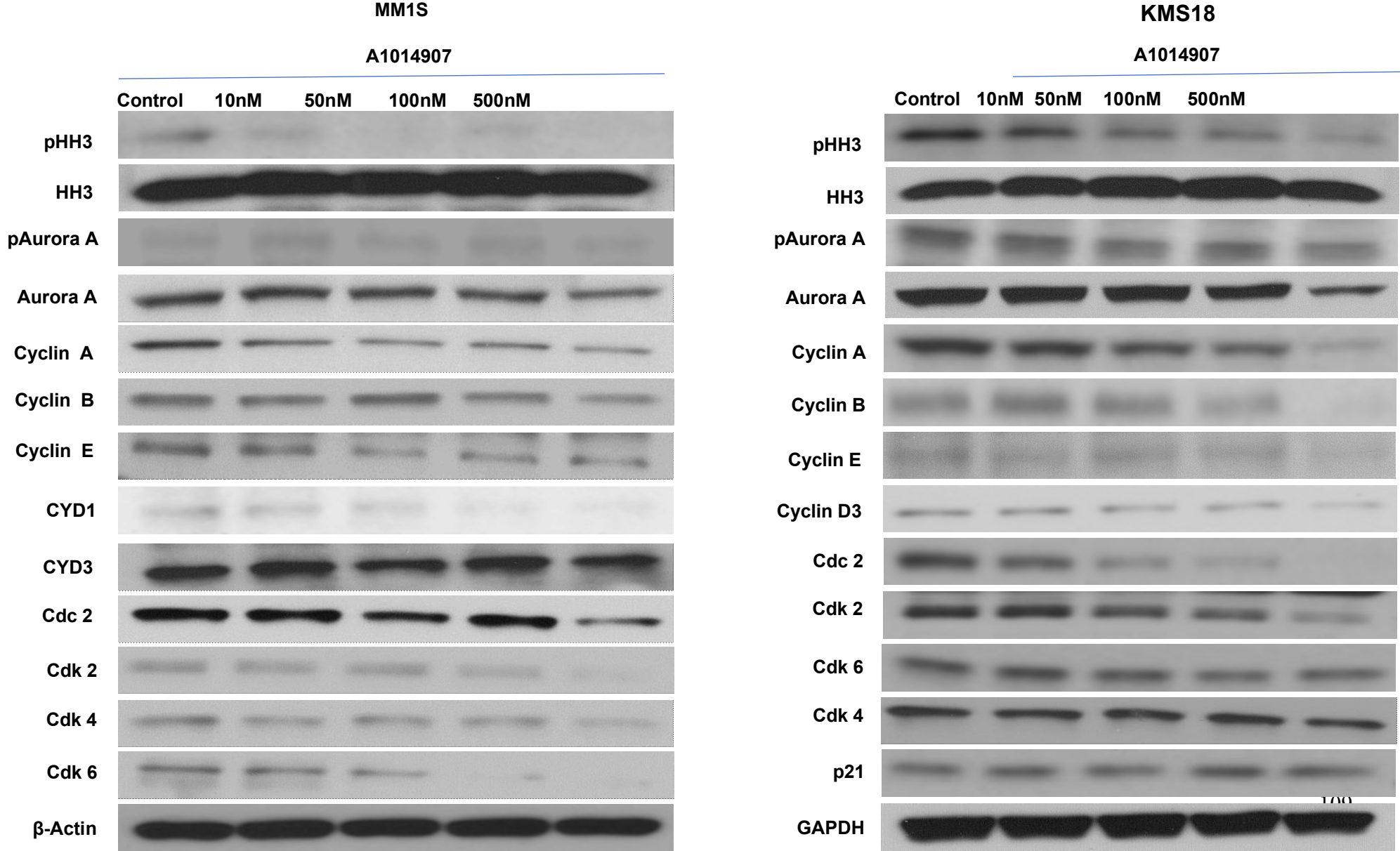
### **3.1.4.8 A1014907 inhibits Aurora Kinases A and B and proteins involved in cell cycle machinery (Fig 17)**

We next examined the mechanism of action of A1014907. We treated two t(4;14) cell lines KMS11(data not shown) and KMS18 (Fig 17.a) and one non t(4;14)(Fig 17.b) cell line MM1S cells with indicated concentrations of A1014907 and examined the expression levels of aurora kinases and proteins involved in cell cycle progression.

Down regulation of phospho Aurora A and phospho Histone H3, a substrate of Aurora B and a biomarker of mitosis in all the cell lines (Figure 17.a and b ). Surprisingly, we also observed that A1014907 caused down regulation of total Aurora A in both cell lines. We speculate that A1014907 acts by blocking aurora A phosphorylation and in addition also by inducing the degradation of Aurora A.

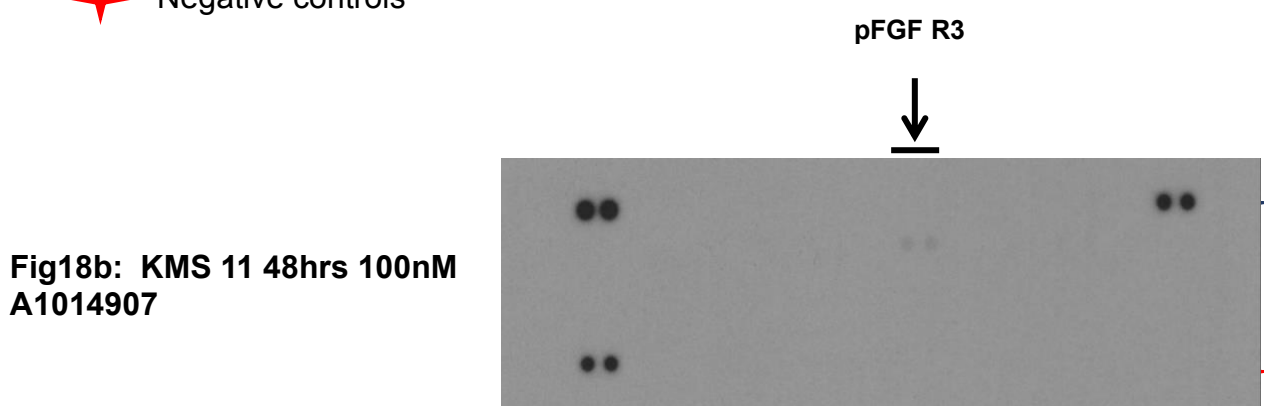
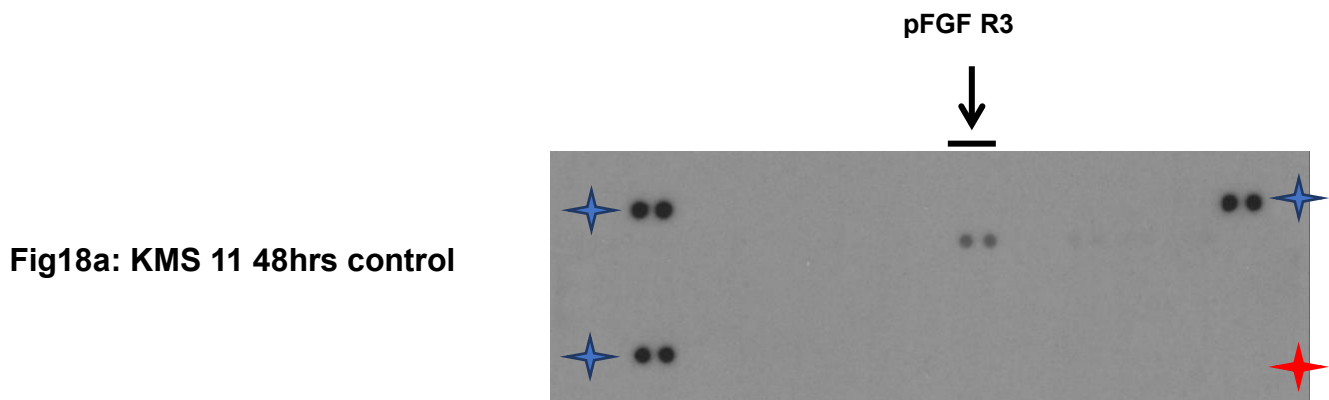
Our results from Figure and clearly showed that A1014907 caused accumulation of cells in the G2M stage of the cell cycle in addition to polyploidy. We further examined the levels of proteins involved in cell cycle progression. A1014907 treatment reduced levels of Cdc2, cyclins A and B (Figure 17.a and b). Cdc2-Cyclin A complex regulates late S phase and early M phase while Cdc2-Cyclin B complex regulates M phase of the cell cycle. Thus, our data suggests that reduction in the level of Cdc2 and its binding partners cyclins A and B could be major factors contributing to the observed G2M arrest (Fig 12.a and 13.a). Cdc2-Cyclin B complex is a key regulator of nuclear envelope breakdown. Its inhibition could further contribute to a lack of or abnormal karyokinesis, producing abnormal cells with unequal DNA content (fig 12.b and 13b).

**Fig17: A101 causes decrease in cell cycle specific Aurora kinase A and B in MM1S(fig 17a) and KMS18(fig 17b) along with reduction in cell cycle proteins**



### 3.1.4.9 A1014907 inhibits FGFR3 in t(4;14) cells (fig 18)

Our results so far suggest that A1014907 inhibits proliferation and causes polyploidy in all MM cell lines through the down regulation of Auroras A and B. However, A1014907 induced significant cell death only in MM cells with t(4;14) translocation which is associated with increased FGFR3 expression[343]. We therefore hypothesized that A1014907, in addition to being an aurora kinase inhibitor was also able to inhibit FGFR3 causing increased apoptosis in cells with t(4;14) translocation. We performed a receptor tyrosine kinase (RTK) array using KMS11 cells left untreated(fig 18.a) or treated(fig 18.b) with indicated dose of A1014907. The results showed down regulation of pFGFR3 by A1014907 (fig 18.b).

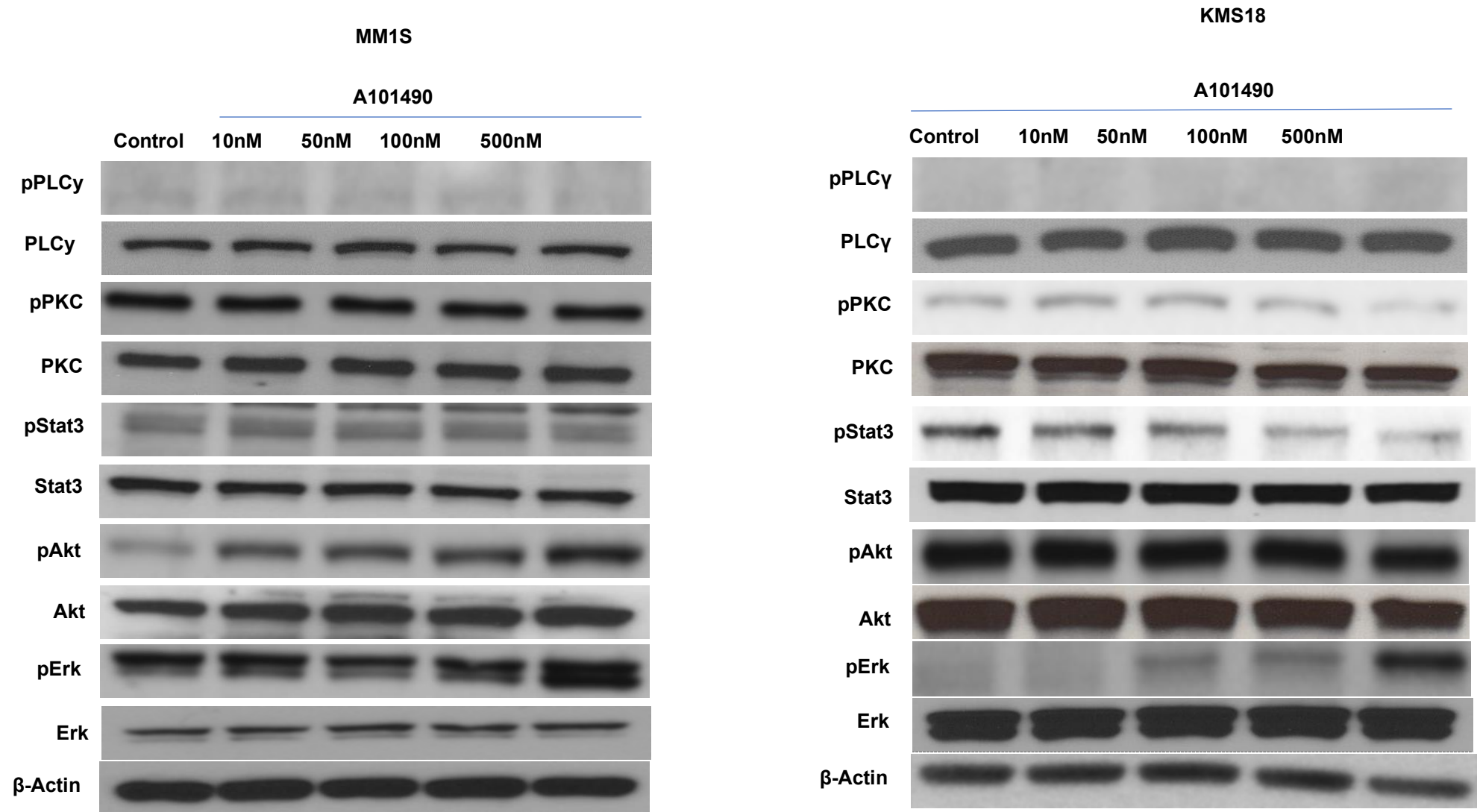


#### **3.1.4.10 A1014907 inhibits Stat3 in t(4;14) cell lines but not in non t(4;14) (fig 19)**

Activated FGFR3 can cause the up regulation of various signalling pathways including the PLC $\gamma$ /PKC, Jak2/Stat3, PI3K/Akt, and Mek/Erk pathways [100]. To better understand which of these signalling pathways are involved in A1014907 induced apoptosis, we performed western blotting using lysates from KMS11(data not shown), KMS18 and MM1S. KMS11(data not shown) and KMS18, which are both sensitive to A1014907 showed dose dependent downregulation of pStat3(fig 19.b). In MM1S, the cell line lacking FGFR3 expression, we did not observe down regulation of any of these proteins again suggesting specific inhibition of FGFR3 by A1014907(fig 19.a).



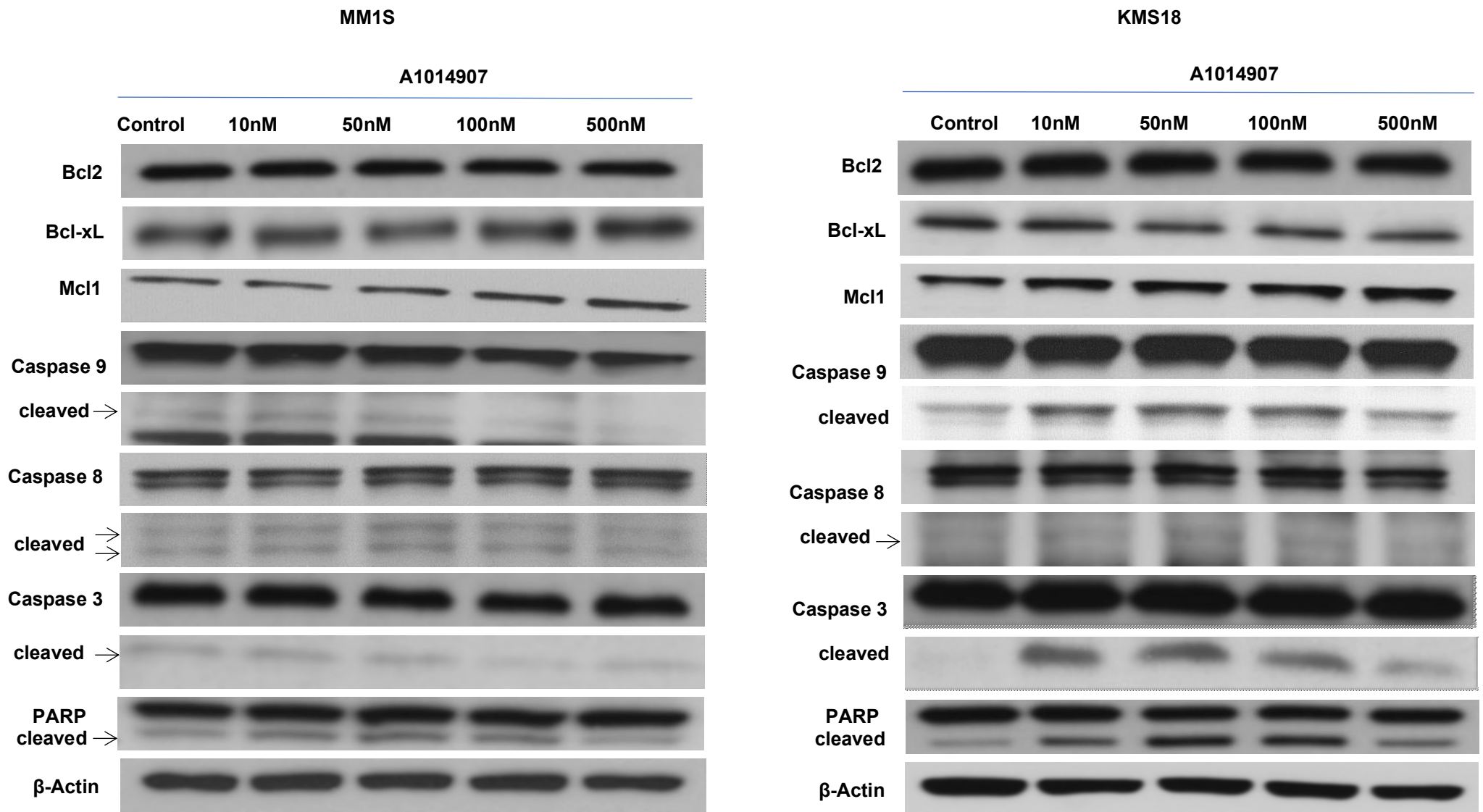
**Fig19: A101 causes dose dependent decrease in pStat3 in KMS18(fig 19b) but not in MM1S(fig 19a)**



#### **3.1.4.11 A1014907 inhibits FGFR3 in t(4;14) cells promoting apoptosis but not in non t(4;14) cells (fig 20)**

We examined levels of the Bcl2 family of anti-apoptotic proteins and observed down regulation of Bcl-2 and Bcl-Xl in KMS11(data not shown). In addition, we observed activation of the intrinsic apoptotic pathway as shown by increased levels of cleaved caspases 9 and 3 (Fig 20.b) and inactivation of PARP (Figure 20.b) all indicating increase in cell death. It must be noted that such differences in apoptotic proteins were absent in MM1S cells (fig 20.a)

Fig 20 A101 induced apoptosis via intrinsic apoptotic in KMS18(fig 20b) but not in MM1S(fig 20a)



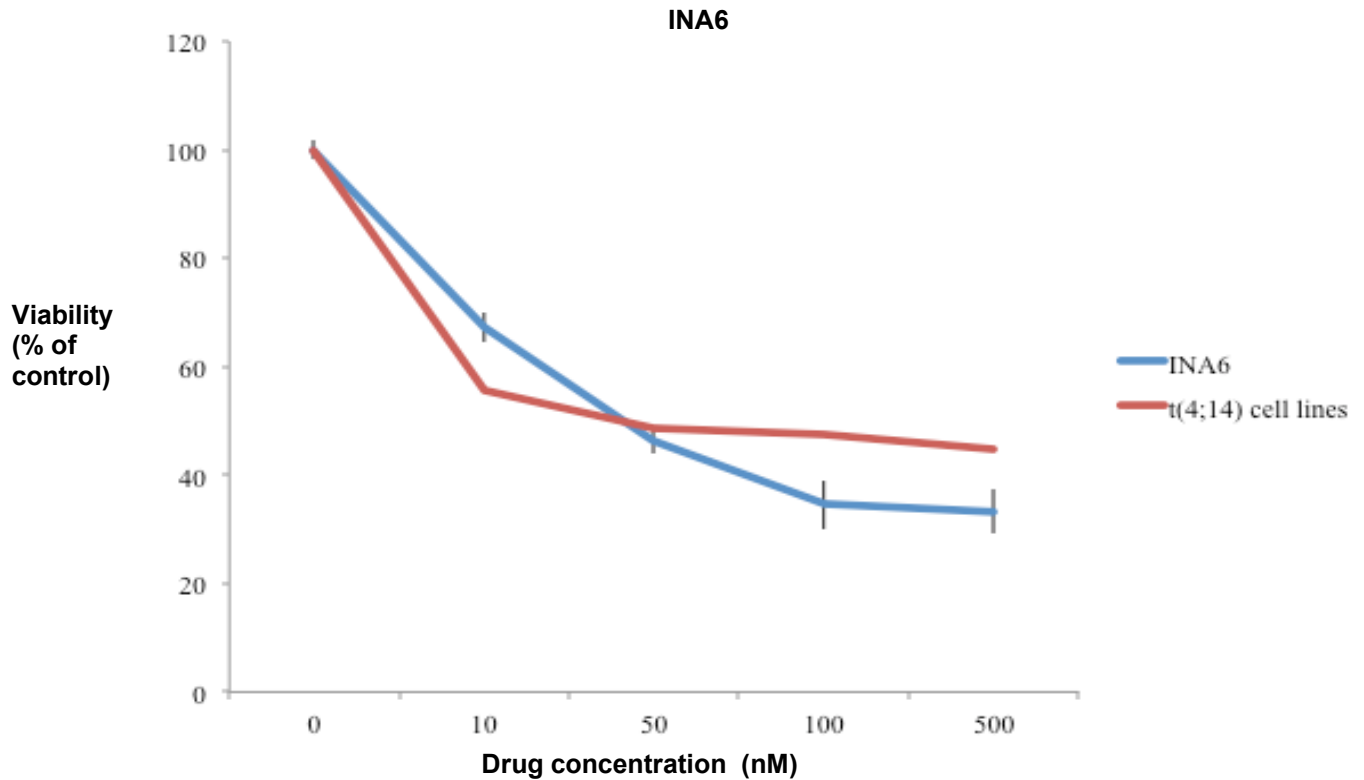
### **3.1.4.12 A1014907 activity is dependent on FGFR3 expression but not t(4;14) translocation(fig 21)**

To further confirm that A1014907 inhibits FGFR3, we checked the sensitivity of INA6, a non t(4;14) line that overexpresses FGFR3 to A1014907. We observed that INA6 was sensitive to A1014907 like the t(4;14) cell lines (Figure 12.a). To further confirm that the activity of A1014907 is dependent on FGFR3 expression and not due to the presence of t(4;14) translocation, we examined the effect of A1014907 on two MM cell lines JIM2 and JIM3, both of which are t(4;14) cell lines but negative for FGFR3 expression. A1014907 was unable to induce pronounced cell death in both these cell lines (data not shown).

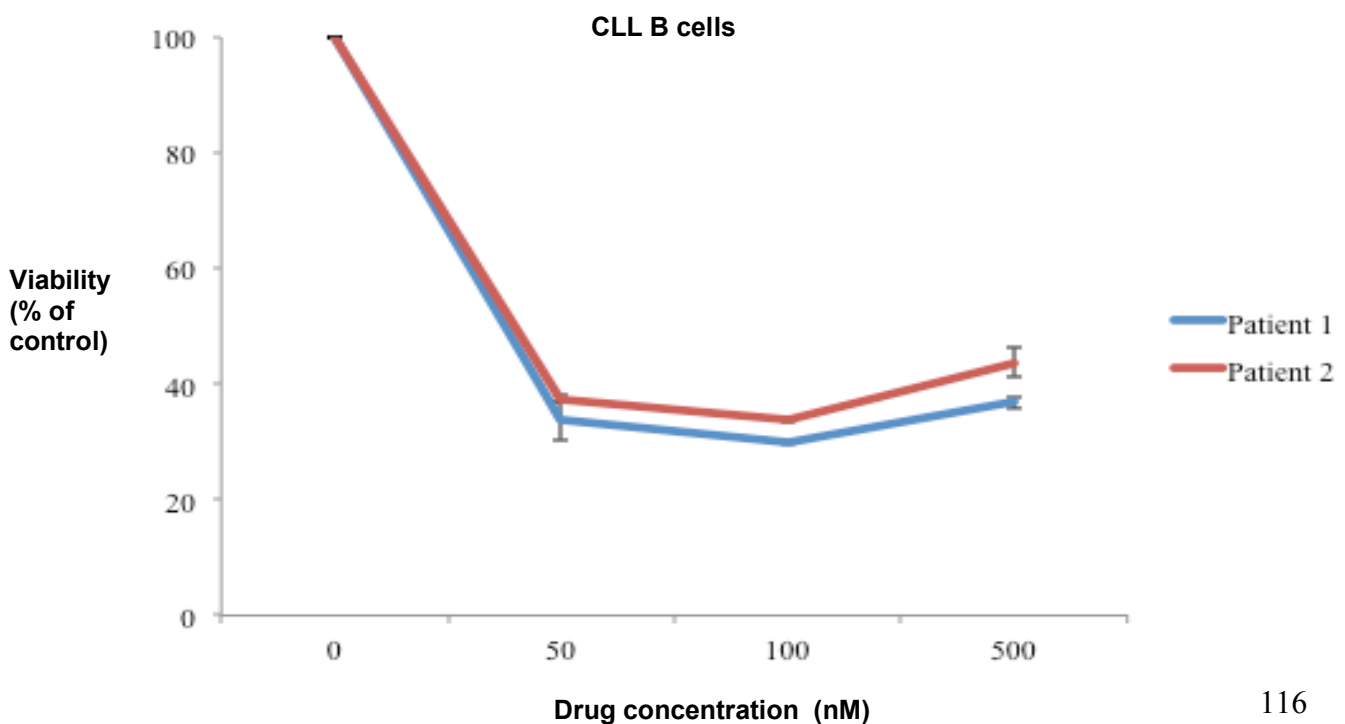
As further proof of specificity of target, we treated CLL B cells from two patients with indicated doses of A1014907. Clear induction of cell death in both these patients (fig 21b). Both the patients were positive for FGFR3 expression as determined by flow cytometry (data not shown). Taken together, we show that A1014907 induces cell death in FGFR3 expressing MM cells. FGFR3 expression could therefore serve as a biomarker to predict for sensitivity to A1014907

**Fig21: A1014907 activity is dependent on FGFR3 expression but not t(4;14) translocation**

**Fig21a: Comparison of INA6 with t(4;14) cell lines.**



**Fig21b: CLL patients expressing FGFR3 t(4;14) are sensitive to low dose(50nM) A101. These results are comparable to those seen in INA6 and t(4;14) cell lines**



**Aim II: Natural history of 17p loss in MM**

## 3.2 Aim II: Natural history of 17p loss in MM

### Background

Loss of functional p53, forms crucial component of prognostically unfavorable of high risk genetic aberrations seen in Multiple Myeloma (MM). This loss is usually monoallelic and is distributed heterogeneously amongst ethnically diverse population with no predilection for age and is associated with reduced treatment responses, shorter PFS and OS. The mutations of the tumor suppressor gene p53 are rare events (3%) in MM patients[50]. Its deletions (Del 17p13.1) are more frequent and have been reported in about 11 % [69, 344] of patients with newly diagnosed Multiple Myeloma and increase in frequency as the disease advances [125].

P53(17p13.1) regulates major pathways of cellular homeostasis in stressed or damaged cells. Its functional loss exists in more than 50 % of human cancer [125, 133, 135, 345-349] making it one of the most studied tumor regulator proteins. Functional loss of p53 in MM is associated with a dismal disease prognosis and has a higher association with uncommon MM presentations including extramedullary disease and plasma cell leukemia [69, 101, 138, 139, 344, 350-352]. Although some treatment response has been reported with Bortezomib based regimens[141, 353], patients with Del17p usually achieve unsatisfactory survival benefits to novel agent treatment and/or Stem cell transplantation (SCT), when compared to the MM patients without Del17p[67, 69, 94, 97, 344, 354, 355].

Given the aggressive disease course and poor survival outcomes, it is essential to premeditate optimal strategy for patients with del17p. Further, predictability of the patients at risk of developing del17p is essential as the therapeutic options are limited and newer alternatives need to be explored. Due to inherent lower frequency of the abnormality, it is challenging to sufficiently study natural history of patients with del17p. With our study we aim to identify and study the outcomes of this unique cohort of patients with MM

### **3.2.1 Aims of the study**

**Identify and study the natural history, treatment responses and outcomes of patients with del17p either at diagnosis(de-novo) or acquired during the disease course in MM patients:**

**Aim IIa : Study natural history of patients with de-novo del17p:**

- **Identify and assess impact of factors affecting outcomes**

**Aim IIb: Study natural history of patients with acquired del17p:**

- **Identify and assess factors predictive of acquisition of del17p**



## **3.3 Aim Ila: Study natural history of patients with de-novo del17p**

### **3.3.1 Patients**

#### **Patients identification :**

We reviewed the Dysproteinaemia database at Mayo Clinic, Rochester and electronic medical records, to identify patients with MM who underwent FISH testing between 2004 and August 2016 and demonstrated del (17p) at diagnosis or within 6 months of the diagnosis of MM. De novo del(17p) was defined as del(17p13.1), which includes the p53 gene region, and/or monosomy for chromosome 17. Relative loss of 17p was defined as del (17p) in presence of trisomy or tetrasomy involving chromosome 17.

#### **Exclusion criteria**

We excluded all patients who had MM with an amyloid related systemic syndrome (n = 4) or PC leukaemia before the index FISH (n = 31), or for whom details about initial therapy were not available (n = 11)42.

#### **Patient selection**

Three hundred and ten (310) patients satisfied the inclusion criteria. For each patient with del(17p), we identified two patients with MM matched for age and time period of diagnosis, who did not have del(17p) by FISH within 6 months from diagnosis and satisfied the other inclusion criteria. We subdivided the control group (n = 620) into a high-risk translocation (HRT) group [with t(4;14), t(14;16) or t(14;20)] (n = 79) and a standard- risk (SR) group (n=541) for comparing the outcomes.

The Mayo Clinic Institutional Review Board approved the study. The study was conducted in accordance with the Declaration of Helsinki and the Health Insurance Port- ability and Accountability Act guidelines of 1996.

## **Patient data**

We collected data regarding demographic characteristics, pre-treatment laboratory parameters, treatment administered, best response to induction, progression, and survival status at final data cut-off by retrospective chart review. The data cut-off date was 31 January 2018. In all patients, diagnosis of MM was made based on the standard criteria, which were in use during the defined period<sup>42,43</sup>.

## **Patient assessment criteria**

We used the international staging system (ISS) to risk stratify patients where serum beta-2-microglobulin and albumin levels were available before starting treatment<sup>4</sup>. High PC proliferative rate was defined by a PC labelling index of  $\geq 1.5\%$  or a monotypic PC- S-phase fraction of  $\geq 3\%$  in the flow cytometric PC proliferation study<sup>44</sup>. We assessed the best response to induction using the International Myeloma Working Group (IMWG) consensus response criteria<sup>45</sup>. Clinical benefit rate (CBR) was defined as the proportion of patients who obtained at least a minor response as the best response to induction. We defined 'early SCT' as SCT done within 12 months of starting treatment for MM.

### **3.3.2 Outcome measures**

Our primary outcome was OS, defined as duration from diagnosis of MM to death due to any cause, patients being censored if they were alive at the last follow-up<sup>46</sup>. Secondary outcome measures included best response to induction therapy and PFS. We defined overall response rate (ORR) as the proportion of patients attaining a partial response (PR) or better following induction. We defined PFS as the duration from the initiation of treatment to first progression or death due to any cause and we censored patients who were alive without progression at their last follow-up<sup>46</sup>.

### **3.3.3 Methods**

#### **3.3.3.1 FISH**

Bone marrow aspirate samples enriched for mono- nuclear cells by the Ficoll method were used for preparing cytopsin slides. Cytoplasmic immunoglobulin staining was used to identify plasma cells and the FISH analysis was performed as described previously from our institu- tion using the following probes: 3cen (D3Z1), 7cen (D7Z1), 9cen (D9Z1), 15cen (D15Z4), 11q13 (CCND1- XT), 13q14 (RB1), 13q34 (LAMP1), 17p13.1 (p53), 17cen (D17Z1), 14q32 (IGH-XT), 14q32 (3'IGH,5'IGH), 4p16.3 (FGFR3), 16q23 (c-MAF), 6p21 (CCND3), 20q12 (MAFB), 1p (p73), and (1q22)8. The cut-points for a positive test were 7 and 9% for deletion 17p13.1 and monosomy 17, respectively. Hyperdiploidy was defined as presence of trisomy/tetrasomy of  $\geq 2$  odd-numbered chromosomes.

#### **3.3.3.2 Statistical analysis**

We summarized categorical variables as proportions and continuous variables as medians (range). We used Fisher's exact test to compare categorical variables and the non- parametric Mann–Whitney U and Kruskal–Wallis tests as appropriate to compare continuous variables between groups. We estimated PFS and OS using the Kaplan–Meier method and used the log-rank test to compare them between groups. We used the Cox proportional hazards model to identify baseline factors affecting PFS and OS. A two-tailed p-value  $< 0.05$  was considered significant for all statistical tests. We used JMP® Pro 12.0 software package (SAS Institute Inc., Cary, NC, USA) for all statistical analyses.

### **3.3.4 Results**

#### **3.3.4.1 Baseline characteristics( table 8)**

A comparison of the baseline demographic and laboratory characteristics across the patient groups is given in Table 1 and the other cytogenetic abnormalities detected are given in Table 2. A higher proportion of patients with del(17p) had a higher PC proliferative rate and elevated lactate dehydrogenase (LDH) at diagnosis.

<b>Table 8. Demographic and laboratory characteristics of the study population at diagnosis (n=930)</b>					
<b>Characteristic</b>	<b>Del(17p) (n=310)</b>	<b>All control patients (n=620)</b>	<b>High-risk translocation (n=79)</b>	<b>Standard-risk (n=541)</b>	<b>P*</b>
Age at diagnosis, median (range)	64.1 (33.8-90.9)	64.2 (35.2-91.0)	60.4 (37.1-81.2)	64.8 (35.2-91.0)	0.787; 0.060
Age ≥65 years, n (%)	147 (47.4)	296 (47.7)	29 (36.7)	267 (49.3)	0.945; 0.108
Female gender, n (%)	122 (39.3)	242 (39.0)	41 (51.9)	201 (37.1)	0.924; <b>0.044</b>
Haemoglobin, g/L, median (range), (n=907)	<b>10.7(4.7-16.8)</b>	<b>11.2 (5.8-16)</b>	<b>10.6 (5.8-14.7)</b>	<b>11.2 (5.9-16.0)</b>	<b>0.015; 0.005</b>
Calcium, mg/dL, median (range), (n=849)	9.7 (7.7-16.8)	9.6 (7.1-17.1)	9.4 (7.7-16.6)	9.6 (7.1-17.1)	0.089; <b>0.268</b>
Creatinine >2 mg/dL, n(%), (n=883)	52 (18.4)	82 (13.8)	14 (18.7)	68 (12.9)	0.071; 0.121
Bone disease at diagnosis, n (%)	243 (78.4)	474 (76.4)	48 (60.8)	426 (78.4)	0.562; <b>0.003</b>
Lytic lesions, n (%)	205 (66.1)	419 (67.6)	45 (57.0)	374 (69.1)	0.658; 0.092
Pathological fractures, n (%)	50 (16.1)	104 (16.8)	6 (7.6)	98 (18.1)	0.852; 0.050
Vertebral compression fractures, n (%)	108 (34.8)	403 (65.0)	18 (22.8)	199 (36.8)	1.0; <b>0.048</b>
Bone marrow plasma cell percentage, median (range), (n=920)	<b>50 (2-100)</b>	<b>50 (2-100)</b>	<b>50 (10-100)</b>	<b>46 (2-100)</b>	<b>0.033; 0.013</b>
High plasma cell proliferative rate†, n (%) (n=504)	<b>42 (30.0)</b>	<b>59 (16.1)</b>	<b>7 (16.3)</b>	<b>52 (16.1)</b>	<b>&lt;0.001; 0.003</b>
M-protein level, g/dL, median (range), (n=876)	2.3 (0-8.2)	2.6 (0-10)	3.7 (0-9)	2.5 (0-10)	0.154; <b>&lt;0.001</b>

M-protein isotype, n (%)					
IgG	176 (56.8)	367 (59.2)	46 (58.2)	321 (59.3)	
IgA	64 (20.7)	136 (22.0)	27 (34.2)	109 (20.1)	0.605; <b>0.031</b>
Light chain only	60 (19.3)	100 (16.1)	5 (6.3)	95 (17.6)	
Others	10 (3.2)	17 (2.7)	1 (1.3)	16 (3.0)	
Difference between involved and uninvolved free light chain , mg/dL, median (range), (n=780)	61.3 (0-2589.0)	46.2 (0-6620)	40.53 (0.4-1999.7)	46.9 (0-6620)	0.126; 0.212
Risk stratification, n (%)					
ISS I/II (n=541)	154 (62.3)	387 (69.2)	44 (63.8)	343 (70.0)	0.061; 0.092
ISS III (n=265)	93 (36.7)	172 (30.8)	25 (36.2)	147 (30.0)	
<b>Elevated LDH, (n=671)</b>	<b>49 (23.8)</b>	<b>66 (14.2)</b>	<b>7 (13.0)</b>	<b>59 (14.4)</b>	<b>0.004; 0.012</b>
<p>*p-value for Fischer's exact test or the non-parametric Mann-Whitney U or Kruskal Wallis tests as appropriate. The first value represents comparison between the de novo del(17p) and all controls and the second value represents comparison across de novo del(17p), high-risk translocation and standard-risk groups. +High plasma cell proliferative rate was defined by a plasma cell labelling index of ≥1.5% or a monotypic plasma cell S-phase fraction of ≥3%. ISS-International staging system, LDH- lactate dehydrogenase.</p>					

### **3.3.4.2 Baseline cytogenetics( table 9)**

HRTs were more likely to coexist in the del(17p) group (24.2%) when compared to the control group (12.7%) ( $P < 0.001$ ). Among patients for whom testing for del(1p) and del(1q) were available, they were detected at a similar frequency in the del(17p) (31.9%) and the control group (31.1%) ( $P = 0.908$ ). Overall, any high-risk abnormality other than del(17p) occurred in 31.6% patients with del (17p) and 17.7% patients in the control group ( $P < 0.001$ ). Out of 310 patients in the del(17p) group, 246 (79.4%) patients had del(17p13.1) and 41 (13.2%) patients had monosomy 17. Two patients (0.6%) had concurrent del (17p13.1) and monosomy 17. Relative loss of 17p was present in 21 (6.8%) patients; 3 of these patients had tetraploidy while others had trisomy/tetrasomy.

<b>Cytogenetic abnormality</b>	<b>De novo del(17p) (n=310)</b>	<b>All control patients (n=620)</b>	<b>High-risk translocation (n=79)</b>	<b>Standard-risk (n=541)</b>	<b>P*</b>
t(4;14)	48 (15.5)	49 (7.9)	49 (62.0)	-	<b>&lt;0.001</b>
t(6;14)†	4 (1.7)	7 (1.5)	-	7 (1.7)	1.000
t(11;14)	45 (14.5)	131 (21.1)	-	131 (24.2)	<b>0.016</b>
t(14;16)	24 (7.7)	22 (3.2)	22 (27.8)	-	<b>0.009</b>
t(14;20)†	3 (1.2)	9 (1.9)	9 (13.8)	-	0.760
Unspecified immunoglobulin heavy chain (IgH) rearrangement/ IgH variable region deletion	19 (6.1)	56 (9.0)	-	56 (10.3)	0.159
Hyperdiploidy (trisomy or tetrasomy)	154 (49.7)	345 (55.6)	31 (39.2)	314 (58.0)	0.002; 0.094
Del (13q) and/or monosomy 13	200 (64.5)	257 (41.4)	64 (81.0)	193 (35.7)	<b>&lt;0.001; &lt;0.001</b>
Del (13q)	39 (12.6)	51 (8.2)	11 (13.9)	40 (7.4)	<b>0.045; 0.017</b>
Monosomy 13	163 (52.6)	213 (34.3)	57 (72.5)	156 (28.8)	<b>&lt;0.001; &lt;0.001</b>
1q gain‡	21 (29.2)	38 (31.1)	11 (68.7)	27 (25.5)	<b>0.872; 0.003</b>
Del (1p) ‡	4 (5.6)	1 (0.8)	1 (6.2)	0 (0)	<b>0.064; 0.021</b>

\*p-value for Fischer's exact test. Comparison between del(17p) and all controls when only one value is present and the second value when present represents comparison across del(17p), high-risk translocation and standard-risk groups. †Calculation is limited to patients who had iFISH after May 2009 (n=716) when probes for these abnormalities were introduced. ‡Calculation limited to patients who had iFISH after August 2014 (n=194) when probe for 1q gain and del (1p) were introduced.



### **3.3.4.3 Patient Follow-up**

The median follow-up for all the patients was 63.5 months (95% CI, 58.3–67.5); 54.5 (95% CI, 49.8–66.9), and 65.7 (95% CI, 59.2–71.3) months for the del(17p) and control groups, respectively. At data cut-off, 169 (54.5%), 32 (40.5%) and 174 (32.2%) patients, respectively in del(17p), HRT and SR groups had died.

### **3.3.4.4 Induction therapy (Fig 22a)**

The major classes of induction therapy received by patients in the del(17p), HRT, and SR groups are shown in Fig. 1a. Patients with del(17p) and HRT were more likely to receive a PI-containing regimen (71.1 and 73.4%, respectively) when compared to SR patients (51.8%) ( $P < 0.001$ ). Best response to induction was evaluable in 289 (93.2%), 79 (100%) and 534 (98.7%) patients, respectively, in the three groups.

### **3.3.4.5 Response to induction (Fig 22b)**

The best responses obtained during induction in these patients are shown in Fig. 1b. ORR was lower in patients with del(17p) (76.5%) compared to those patients with HRT (87.3%) or SR disease (84.8%) ( $P = 0.006$ ). Among patients who received PI + IMiD-based induction, the ORRs were 85.4, 94.7, and 97.1% in the three groups ( $P = 0.009$ ), while VGPR or better rates in the three groups were 52.7, 78.9, and 63.7%, respectively ( $P = 0.054$ ). Similarly, in patients who received a PI-containing regimen, the ORRs were 78.7, 93.1, and 84.4%, respectively, in the three groups ( $P = 0.026$ ) and VGPR or better rates were 28.2, 23.8, and 32.2%, respectively ( $P = 0.615$ ).

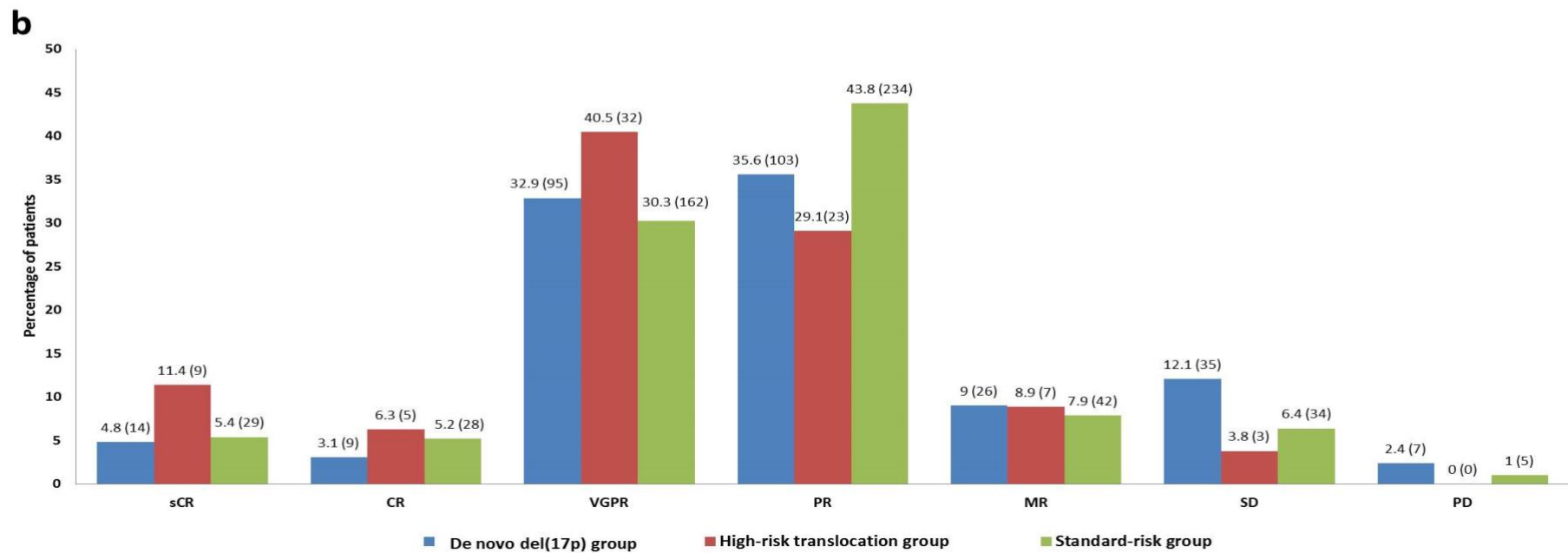
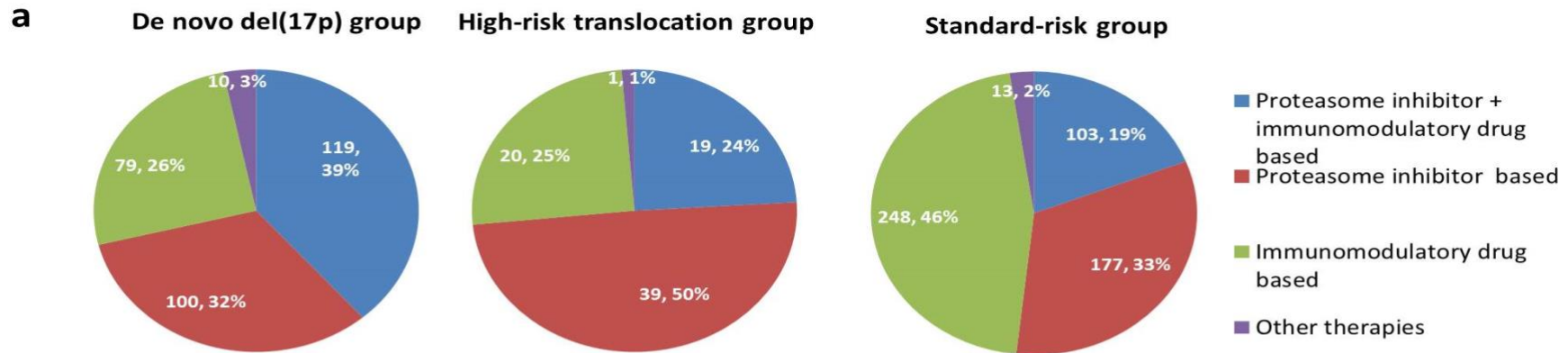
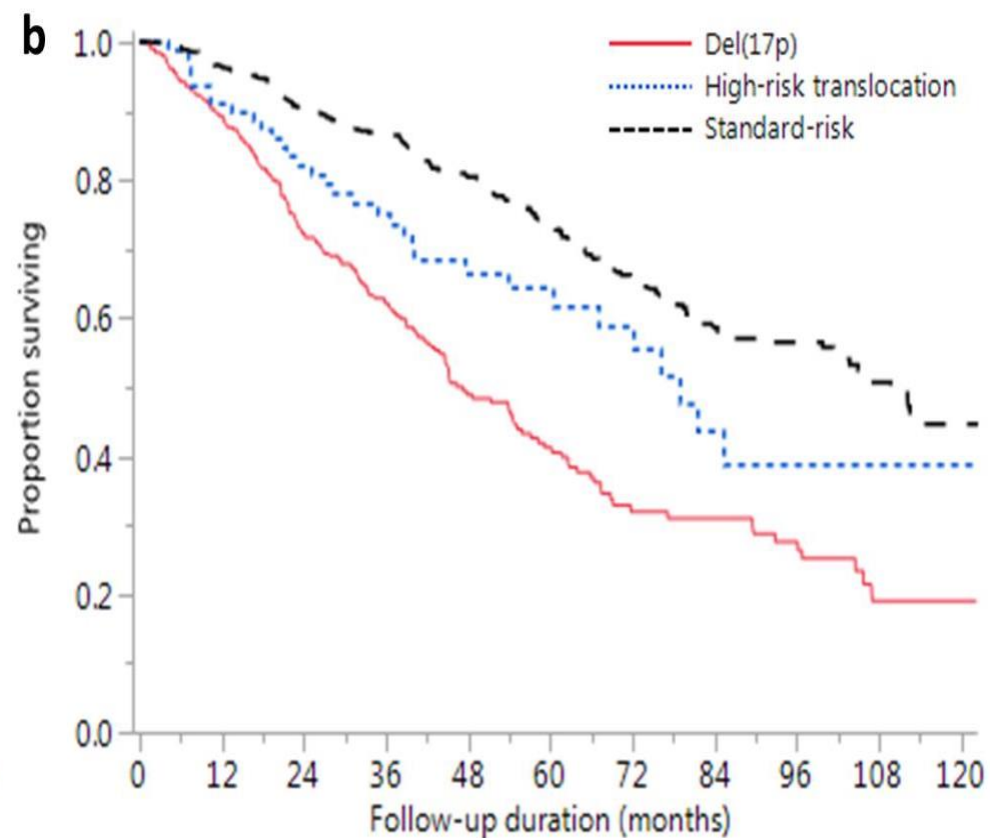
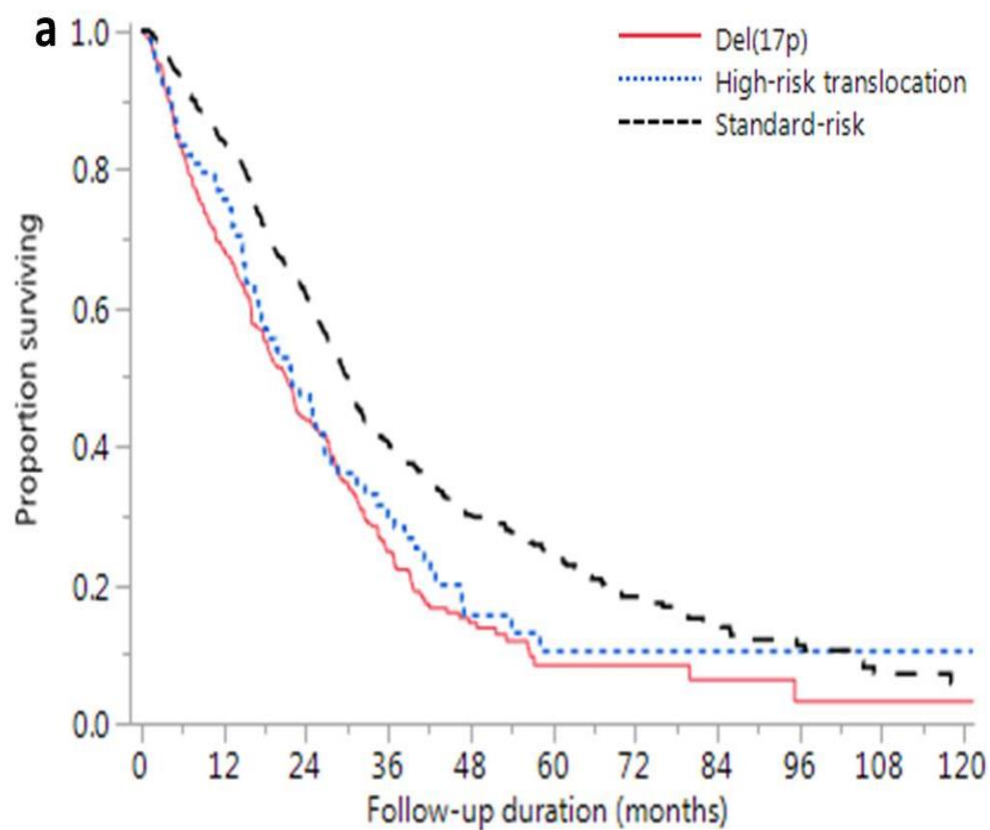


Fig 22: a) Drug treatment usage in different risk groups; b) Grouped percentage response by IMWG criteria

### 3.3.4.6 Survival outcomes(Fig 23)

The estimated median PFS for del(17p), HRT and SR groups were 21.1 months (95% CI, 17.8–23.9), 22.0 months (95% CI, 16.7–26.8) and 30.1 months (95% CI, 27.5–31.5) respectively (P = 0.437 for del(17p) vs. HRT and P < 0.001 for del(17p) vs. SR) (Fig. 23a). The estimated median OS for the three groups were 47.3 months (95% CI, 42.7–55.9), 79.1 months (95% CI, 60.5-not reached[NR]), and 109.8 months (95% CI, 99.9–125.6), respectively, (P = 0.007 for del(17p) vs. HRT and P < 0.001 for del(17p) vs. SR) (Fig. 23b). The median PFS for patients with relative loss of 17p was 22.1 months (95% CI, 8.7–51.8) and was comparable to 21.2 months (95% CI, 17.8–25.0) seen in patients with del17p or monosomy 17 (P = 0.485). Similarly, the median OS for the two groups were comparable: 48.7 months (95% CI, 32.1-NR) vs. 47.3 months (95% CI, 41.6–55.9) (P = 0.603), justifying their inclusion in the del (17p) group.

Fig 23: a) PFS; b) OS of patients with del17p compared to other high risk translocations and standard risk patients



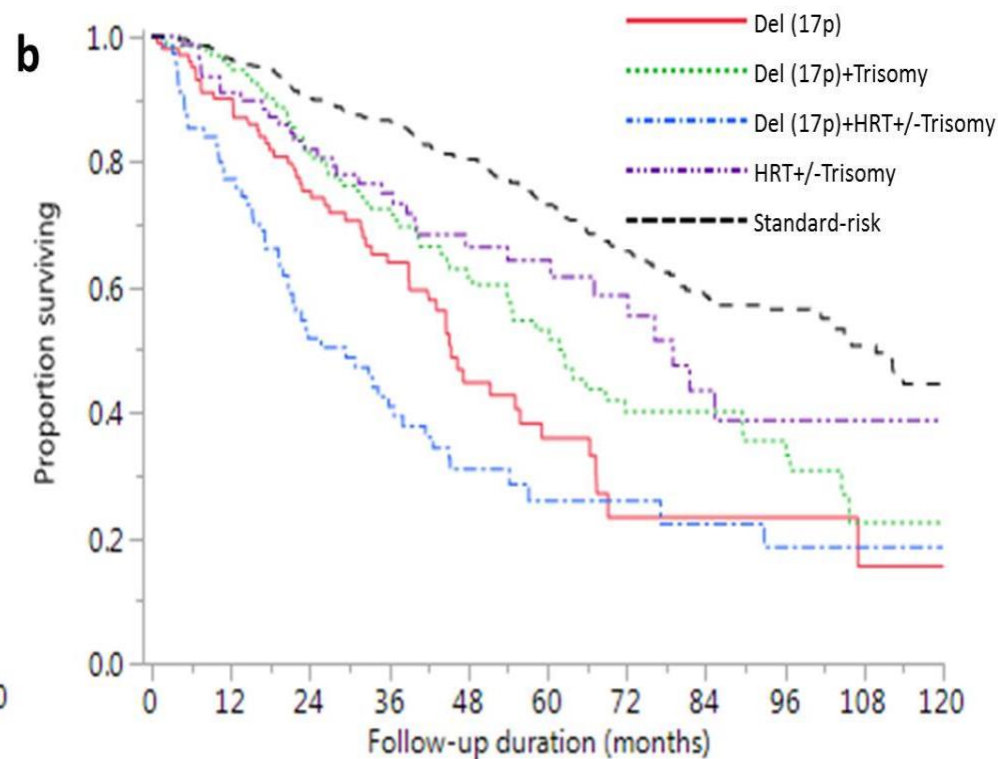
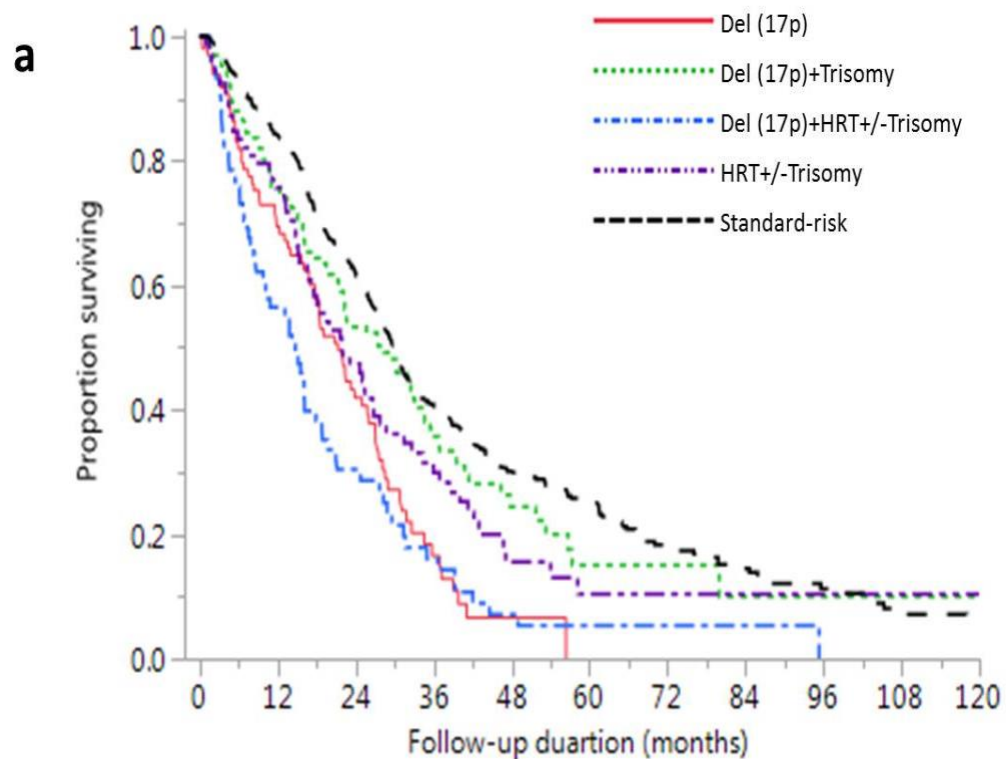
Del(17p)	310	185	103	49	19	6	5	3	1	1	1
HRT	79	58	34	19	6	4	4	2	1	1	1
SR	541	425	292	162	103	68	39	24	15	8	4

Del(17p)	310	272	200	147	88	59	35	30	23	8	3
HRT	79	71	62	48	33	24	18	9	6	5	4
SR	541	514	456	397	298	214	164	117	86	54	23

### 3.3.4.7 Impact of FISH abnormalities(Fig 24)

To elucidate the impact of combinations of FISH abnormalities, we divided the entire patient cohort (cases and controls) into the following groups: cases were divided into del(17p) alone (n = 135), del(17p) with hyper- diploidy (n = 100), del(17p) with HRT (irrespective of presence of hyperdiploidy) (n = 75), and controls were divided into HRT (irrespective of presence of hyperdiploidy) (n = 79) and SR patients (n = 541). The median PFS in the above five groups were 22.4 months (95% CI, 17.8–27.0), 27.3 months (95% CI, 19.6–34.5), 14.7 months (95% CI, 9.8–17.9), 22.0 months (95% CI, 16.7–26.8), and 30.1 months (95% CI, 27.5–31.5), respectively, ( $P < 0.001$ )(Fig 24a). The median OS in the above five groups were 51.4 months (95% CI, 42.1–62.8), 60.3 months (95% CI, 47.8–89.6), 29.5 months (95% CI, 20.0–38.1), 79.1 months (95% CI, 60.5-not reached), and 109.8 months (95% CI, 99.9–125.6), respectively, ( $P < 0.001$ ) (Fig. 24b). Presence of hyperdiploidy was associated with longer PFS ( $P = 0.007$ ) and only a trend toward longer OS ( $P = 0.272$ ) in del(17p) patients. Coexistent HRT worsened the OS ( $P = 0.004$ ).

**Fig 24: a)PFS :b) OS of MM patients subdivided into subgroup identified by unique cytogenetically defined outcomes**



Del (17p)	102	60	32	9	2	0	0	0	0	0	0
Del (17p)+Trisomy	133	87	53	31	13	5	4	2	1	1	1
Del (17p)+HRT+/-Trisomy	75	38	18	9	4	1	1	1	0	0	0
HRT+/-Trisomy	79	58	34	20	6	4	4	2	1	1	1
Standard-risk	541	425	292	162	103	68	39	24	15	8	4

Del (17p)	102	89	67	47	22	15	5	4	3	2	1
Del (17p)+Trisomy	133	126	97	74	50	34	22	20	15	4	1
Del (17p)+HRT+/-Trisomy	75	57	36	26	18	10	8	6	5	2	1
HRT+/-Trisomy	79	71	62	48	33	24	18	9	6	5	4
Standard-risk	541	514	456	397	298	214	164	117	86	54	23

### **3.3.4.8 Sub-group analysis for survival outcomes(table 10)**

We stratified patients according to age (using 65 years as cut-off), ISS stage, LDH, and PC proliferative rate at diagnosis and the type of induction therapy. The results are shown in Table . There was no difference in PFS between patients with del(17p) and HRT across all the subgroups analyzed. The OS was shorter in del(17p) group compared to the HRT group in patients with age <65 years, ISS I/II stage and those patients who received PI-containing induction regimen or early SCT. However, the difference was abolished and both groups had similar OS in presence of adverse factors, such as advanced age, ISS III stage or when they received non-PI containing induction regimens or delayed or no SCT. This loss of difference in OS was primarily due to a marked reduction in OS in the HRT group in presence of additional risk factors as shown in Table 10. For example, in the del(17p) group, ISS I/II and ISS III stages were associated with median OS of 58.3 and 33.3 months, respectively, while in the HRT group, the OS decreased from 81.6 months in ISS I/II stages to 38.7 months in ISS III stage. Similarly, a non-PI-containing induction was associated with reduction of median OS from 54.3 months in the del(17p) group to 45.2 months, while it decreased from not reached to 67.1 months in the HRT group. Across all subgroups, the SR group showed consistently better out- comes when compared to the del(17p) group.

<b>Table 10. Sub-group analysis for survival outcomes in patients based on prognostic factors and therapy</b>				
<b>Survival outcomes and subgroups</b>	<b>De novo del(17p)</b>	<b>High-risk translocation</b>	<b>Standard-risk</b>	<b>P*</b>
Progression free survival				
Age <65 (n=487)	21.0 (16.8-27.0)	25.4 (17.5-32.6)	32.3 (28.7-37.7)	0.176; <b>&lt;0.001</b>
Age ≥65 (n=443)	21.3 (16.0-25.8)	16.6 (11.2-25.5)	27.4 (25.3-30.6)	0.520; <b>0.002</b>
ISS I/II (n=541)	27.0 (21.1-30.3)	24.6 (17.1-31.5)	30.9 (29.1-34.8)	0.699; <b>0.002</b>
ISS III (n=265)	14.3 (9.6-16.9)	16.7 (5.1-34.4)	24.8 (19.7-28.5)	0.212; <b>&lt;0.001</b>
PI-containing induction (n=557)	22.6 (18.4-27.5)	25.0 (18.1-32.6)	29.5 (26.3-31.8)	0.336; <b>&lt;0.001</b>
Others (n=371)	16.1 (13.8-22.0)	13.3 (5.1-25.5)	30.6 (27.1-33.8)	0.840; <b>&lt;0.001</b>
Normal LDH (n=556)	22.5 (18.4-28.2)	18.4 (15.1-25.4)	30.8 (28.0-33.1)	0.241; <b>&lt;0.001</b>
High LDH (n=115)	16.1 (8.3-17.9)	6.7 (1.5-21.9)	27.1 (18.3-32.3)	0.245; <b>&lt;0.001</b>
High PC proliferative rate (n= 101)	10.4 (5.1-18.6)	6.7 (2.3-17.1)	25.0 (17.9-31.3)	0.264; <b>&lt;0.001</b>
Low PC proliferative rate (n=405)	22.3 (17.8-28.8)	16.6 (13.3-22.0)	29.8 (26.3-32.1)	0.106; <b>0.044</b>
Overall survival				
Age <65 (n=487)	<b>55.2 (42.0-67.4)</b>	<b>81.6 (60.5-NR)</b>	<b>130.6 (112.6-NR)</b>	<b>0.030; &lt;0.001</b>
Age ≥65 (n=443)	44.7 (37.5-54.6)	67.1 (25.2-NR)	78.6 (70.4-103.6)	0.201; <b>&lt;0.001</b>
ISS I/II (n=541)	<b>58.3 (45.3-71.8)</b>	<b>81.6 (60.5-NR)</b>	<b>112.3 (103.6-NR)</b>	<b>0.039; &lt;0.001</b>
ISS III (n=265)	33.3 (23.2-44.7)	38.7 (21.1-NR)	64.7 (59.6-124.4)	0.179; <b>&lt;0.001</b>
Normal LDH (n=556)	53.9 (43.9-65.9)	67.1 (38.7-81.6)	105.0 (83.9-125.6)	0.322; <b>&lt;0.001</b>
High LDH (n=115)	26.8 (18.6-46.4)	NR (7.3-NR)	106.1 (71.7-NR)	0.295; <b>&lt;0.001</b>
High PC proliferative rate (n= 101)	32.9 (15.2-54.9)	21.5 (7.6-28.0)	62.5 (42.7-85.9)	0.217; <b>0.008</b>
Low PC proliferative rate (n=405)	47.8 (41.6-67.4)	72.3 (38.7-NR)	103.6 (80.4-130.6)	0.144; <b>&lt;0.001</b>
PI-containing induction (n=557)	54.3 (40.7-62.6)	NR (54.0-NR)	124.4 (79.9-NR)	0.007; <b>&lt;0.001</b>
Others (n=371)	45.2 (36.5-58.3)	67.1 (27.5-85.4)	106.1 (84.8-125.6)	0.405; <b>&lt;0.001</b>



Early SCT (n=462)	54.9 (45.3-66.5)	85.4 (72.3-NR)	130.6 (106.1-NR)	<b>0.038; &lt;0.001</b>
Delayed or no SCT (n=468)	37.5 (25.7-45.4)	60.5 (27.5-NR)	83.9 (74.0-112.2)	0.098; <b>&lt;0.001</b>
*P-value for log-rank test in Kaplan Meier analysis. The first value represents comparison between del(17p) and high-risk translocation groups and the second value represents comparison between del(17p) and standard-risk groups. ISS-International staging system, LDH- lactate dehydrogenase, and PI- proteasome inhibitor.				

### **3.3.4.9 Predictors of outcome in patients with de novo del(17p)(Table 11)**

We performed univariable analysis with age  $\geq 65$  vs.  $< 65$  years, serum creatinine  $> 2$  vs.  $\leq 2$  mg/dL, bone marrow PC percentage  $\geq 50$  vs.  $< 50\%$ , ISS III vs. I/II stage, elevated vs. normal LDH, presence vs. absence of an HRT, presence vs. absence of monosomy 13, presence vs. absence of hyperdiploidy, high vs. low PC proliferation rate and PI-containing vs. other induction therapy as independent variables to determine their association with PFS and OS. Variables with a p-value  $< 0.1$  in univariable analysis were included as potential predictors in multivariable Cox proportional hazards model and we arrived at a final model using stepwise backward elimination. To assess the impact of percentage of PCs with del(17p), we included each cut-point (viz. 20, 30, 40, 50, and 60%) with the above predictors. The results of the analysis are shown in Table 11. ISS stage III disease, elevated LDH and coexistent HRTs were associated with reduced OS, while percentage of PCs with del(17p) was not a significant predictor in the multivariable model.

<b>Table 11. Effect of baseline characteristics on survival measures in patients with de novo del(17p) (n=310)</b>						
Independent variable	<b>Progression-free survival (PFS)</b>			<b>Overall survival (OS)</b>		
	p-value for univariable analysis	HR (95% CI) for multivariable analysis	p-value for multivariable analysis	p-value for univariable analysis	HR (95% CI) for multivariable analysis	p-value for multivariable analysis
Age ≥65 vs <65 years (147 vs. 163)	0.714	NI	-	0.010	1.40 (0.96-2.04)	0.079
Serum creatinine >2 vs ≤2 mg/dL (52 vs. 231)	0.027	1.18 (0.66-2.00)	0.561	0.072	0.84 (0.48-1.43)	0.534
Bone marrow PCs ≥50% (164 vs. 140)	0.110	NI	-	0.536	NI	-
ISS III vs I/II stage (93 vs. 154)	<0.001	1.92 (1.34-2.73)	<b>&lt;0.001</b>	<0.001	2.08 (1.42-3.04)	<b>&lt;0.001</b>
Elevated vs normal LDH (49 vs. 157)	0.001	1.71 (1.04-2.53)	<b>0.011</b>	0.003	1.83 (1.16-2.80)	<b>0.009</b>
High-risk translocation vs. no high-risk translocation (75 vs. 235)	0.001	1.44 (0.97-2.10)	0.071	<0.001	1.53 (1.01-2.27)	<b>0.044</b>
Monosomy 13 vs no monosomy 13 (163 vs. 147)	0.020	1.08 (0.75-1.57)	0.672	0.024	1.04 (0.63-1.74)	0.883
HRD vs. no HRD (154 vs. 156)	<0.001	0.72 (0.51-1.03)	0.070	0.010	0.75 (0.51-1.11)	0.155
High PC proliferative rate vs. low proliferative rate (42 vs. 98)	<0.001	1.56 (0.93-2.60)	0.090	0.042	0.87 (0.48-1.54)	0.630
PI-containing induction vs other induction therapy (219 vs. 89)	0.935	NI	-	0.472	NI	-

Percentage of plasma cells with del(17p)						
• ≥20% vs. <20% (224 vs. 36)	0.074	0.99 (0.35-4.16)	0.990	<0.001	1.15 (0.41-4.85)	0.812
• ≥30% vs. <30% (207 vs. 53)	0.057	1.93 (1.14-3.53)	<b>0.013</b>	<0.001	1.44 (0.80-2.86)	0.241
• ≥40% vs. <40% (193 vs. 67)	0.012	2.04 (1.28-3.44)	<b>0.002</b>	0.001	1.29 (0.66-2.77)	0.473
• ≥50% vs. <50% (170 vs. 90)	0.025	1.60 (1.06-2.50)	<b>0.023</b>	0.011	1.23 (0.68-2.32)	0.495
• ≥60% vs. <60% (153 vs. 107)	0.050	1.10 (0.65-1.89)	0.724	<0.001	1.36 (0.89-2.13)	0.1531
HR-Hazard ratio, HRD- Hyperdiploidy, LDH-Lactate dehydrogenase, NI- Not included in analysis, PC- plasma cell, and PI- proteasome inhibitor. The final multivariable model included 174 patients for PFS and 191 patients for OS for whom the parameters were available.						

## **3.4 Aim IIb: Natural History of 17p in MM : Acquired del17p**

### **3.4.1 Patients**

#### **Patient identification and timing of FISH evaluation**

We reviewed the Dysproteinaemia database at Mayo Clinic, MN to identify 80 patients with MM and acquired del(17p), defined as first FISH test negative for del(17p) with detection of del(17p) on a follow-up FISH test. 76 patients were included in the analysis. All patients had FISH testing between 2004 and August 2016. Fifty seven (75%) patients had their first FISH within 6 months of diagnosis of MM; 19 (25%) patients had their first FISH more than 6 months after diagnosis of MM.

#### **Exclusion criteria**

Of these, 76 patients had RRMM, while in 2 patients, del(17p) was detected prior to stem cell transplant (SCT) after having attained a response to previous line of therapy. In two patients, del(17p) was detected during assessment for stem cell harvest after a partial response (PR) was attained after induction therapy. These 4 patients were excluded from evaluation.

#### **Control selection and FISH timing**

Del(17p) included interstitial deletion involving the short arm of chromosome 17 [del(17p13.1)] and/or monosomy 17. We identified 2 control patients for each case, who were diagnosed during the same time period, but did not demonstrate del(17p) at any time during follow-up. The cases and controls were not matched for potential predictors of acquisition of del(17p) including age, cytogenetic abnormalities or initial treatment. Control patients (n=152) had at least two FISH tests negative for del(17p), the second test being at a comparable time point or later relative to timing of detection of del(17p) in the respective case. The second FISH test was done at relapse. We also

compared survival outcomes in the acquired del(17p) group with a cohort of 310 patients with del(17p) detected at diagnosis.

### **3.4.2 Response criteria**

We defined responses to treatment using the 2016 Revised International Myeloma Working Group Criteria[356]. Progression free survival (PFS) was defined as the duration between initiation of therapy and progression or death, and OS was defined as the duration from diagnosis of MM or detection of del(17p) to death due to any cause.[357] Number of lines of therapy were defined according to accepted guidelines.[358] Early SCT was defined as SCT within one year of starting treatment for MM.

### **3.4.3 Methods**

#### **3.4.3.1 FISH probes and testing**

Bone marrow aspirate samples enriched for mononuclear cells by the Ficoll method were used for preparing cytospin slides. Cytoplasmic immunoglobulin staining was used to identify PCs and the FISH analysis was performed as described previously from our institution using the following probes: 3cen (D3Z1), 7cen (D7Z1), 9cen (D9Z1), 15cen (D15Z4), 11q13 (CCND1-XT), 13q14 (RB1), 13q34 (LAMP1), 17p13.1 (p53), 17cen (D17Z1), 14q32 (IGH-XT), 14q32 (3'IGH,5'IGH), 4p16.3 (FGFR3), 16q23 (c-MAF), 6p21 (CCND3), 20q12 (MAFB), 1p32 (p73), and (1q22).[101] The cut-points for a positive test were 7% and 9% for del(17p13.1) and monosomy 17 respectively. Relative loss of del(17p) was defined as del(17p) with trisomy/tetrasomy involving chromosome 17. High-risk translocations (HRTs) included t(4;14), t(14;16) and t(14;20). Hyperdiploidy was defined as presence of trisomy and/or tetrasomy of 2 or more odd-numbered chromosomes.

### **3.4.3.2 Estimation of plasma cell proliferative rate**

PC proliferative rate was defined as the proportion of clonal PCs actively proliferating in the S-phase of cell cycle. Slide technique using the deoxyuridine method (called PC labeling index-PCLI) was used for this till May 2012.[359] After that, PCLI was supplanted by flow cytometric technique. Briefly, this involves identification of immunophenotypically atypical PCs and estimating the DNA content in PCs. By appropriate gating, proportion of atypical PCs in the S-phase is estimated. The details of the technique are summarized in a recent publication.[360] High PC proliferative rate was defined by a PCLI of  $\geq 1.5\%$  or a PC- S-phase fraction of  $\geq 3\%$  in the flow cytometric technique.[361, 362]

### **3.4.3.3 Statistical analysis**

Categorical variables were summarized as proportions and compared between groups using Fisher's exact test. Continuous variables were summarized as median and compared between groups using non-parametric Mann-Whitney U test. We estimated PFS and OS using the Kaplan-Meier method and used the log-rank test to compare them between groups. We used Cox proportional hazards model to identify factors at detection of del(17p) affecting PFS and OS. Odds ratio (OR) was calculated to identify factors at diagnosis of MM associated with later detection of del(17p) by FISH. A two-tailed  $P < 0.05$  was considered significant for all statistical tests. We used JMP® Pro 14.0 software package (SAS Institute Inc., Cary, NC, USA) for statistical analysis.

### 3.4.4 Results

#### 3.4.4.1 Baseline characteristics(table 12)

The characteristics of 76 patients with acquired del(17p) at initial diagnosis of MM, 152 control patients at diagnosis and the acquired del(17p) cohort at detection of del(17p) are shown in **Table 12**. Patients with acquired del(17p) and controls were similar with respect to baseline characteristics, and initial treatment, except relatively low hemoglobin at diagnosis in cases (median- 10.8 g/dL vs. 11.3 g/dL; P=0.035), higher occurrence of t(4;14) among cases (15.8% vs. 6.6%; P=0.033) and higher proportion of patients with elevated lactate dehydrogenase (LDH) among evaluable cases [13.7% (7/51) vs. 4.1% (5/121); P=0.043] relative to controls. The characteristics of de novo del(17p) group have been published elsewhere[363]. Briefly, 47.4% patients were 65 years or older; 36.7% patients had international staging system (ISS) III stage and 23.8% patients had elevated LDH; and 24.4% patients had concurrent HRTs. More than 95% patients in this cohort were treated initially with novel agents (PI+IMiD- 38.6%; PI- 32.5%; and IMiD- 25.6). 56% patients received SCT during their treatment.



<i>Characteristic</i>	<i>Acquired del(17p) at diagnosis (n=76)</i>	<i>Control patients at diagnosis (n=152)</i>	<i>Acquired del(17p) at detection of del(17p) (n=76)</i>	<i>P* (Acquired del(17p) vs. control at diagnosis)</i>
Age, years, median (range)	61.9 (42.4-80.9)	61.4 (28.8-84.7)	65.6 (44.6-82.2)	0.972
Age ≥65 years, n (%)	28 (36.8)	51 (33.5)	39 (51.3)	0.659
Female gender, n (%)	30 (39.5)	63 (41.4)	30 (39.5)	0.886
<b>Haemoglobin, g/L, median (range), (n=69/150/75)</b>	<b>10.8 (5.4-14.5)</b>	<b>11.3 (7.1-16.7)</b>	<b>10.4 (7.2-14.7)</b>	<b>0.035</b>
Calcium, mg/dL, median (range), (n=60/146/73)	9.6 (7.2-15.4)	9.6 (7.9-15)	9.5 (7.1-11.5)	0.598
Creatinine >2 mg/dL, n(%), (n=64/145/74)	8 (12.5)	10 (6.9)	4 (5.4)	0.191
BMPC percentage, median (range), (n=70/150/75)	40 (4-93)	50 (3-98)	40 (2-100)	0.384
High plasma cell proliferative rate*, n (%) (n=45/106/65)	14 (31.1)	22 (20.7)	32 (49.2)	0.211
Percentage of plasma cells with del(17p), median (range) (n=-/-71)	-	-	89 (9-100)	
M-protein level, g/dL, median (range), (n=65/146/74)	2.8 (0-12.3)	2.7 (0-6.8)	1.8 (0-6.4)	0.999
M-protein isotype, n (%), (n=76/152/76)				
IgG	46 (60.5)	88 (57.9)	46 (60.5)	0.097
IgA	21 (27.6)	36 (23.7)	21 (27.6)	
Light chain	5 (6.6)	25 (16.4)	5 (6.6)	
Others	4 (5.3)	3 (2.0)	4 (5.3)	
ISS I/II vs. III, n (%), (n=56/136/56)	42 (75.0)/ 14 (25.0)	110 (80.9)/ 26 (19.1)	43 (76.8)/ 13 (23.2)	0.434
<b>Elevated LDH, n (%), (n=51/121/61)</b>	<b>7 (13.7)</b>	<b>5 (4.1)</b>	<b>21 (33.4)</b>	<b>0.043</b>
Prior lines of therapy, median (range)			2 (1-10)	
Proteasome inhibitor exposed/ refractory, n (%)			57 (67.1)/33 (43.4)	

Immunomodulatory drug exposed/ refractory, n (%)			70 (92.1)/57 (75.0)	
Alkylating agent exposed/ refractory, n (%)			60 (78.9)/25 (32.9)	
Proteasome inhibitor and immunomodulatory drug refractory			30 (39.5)	

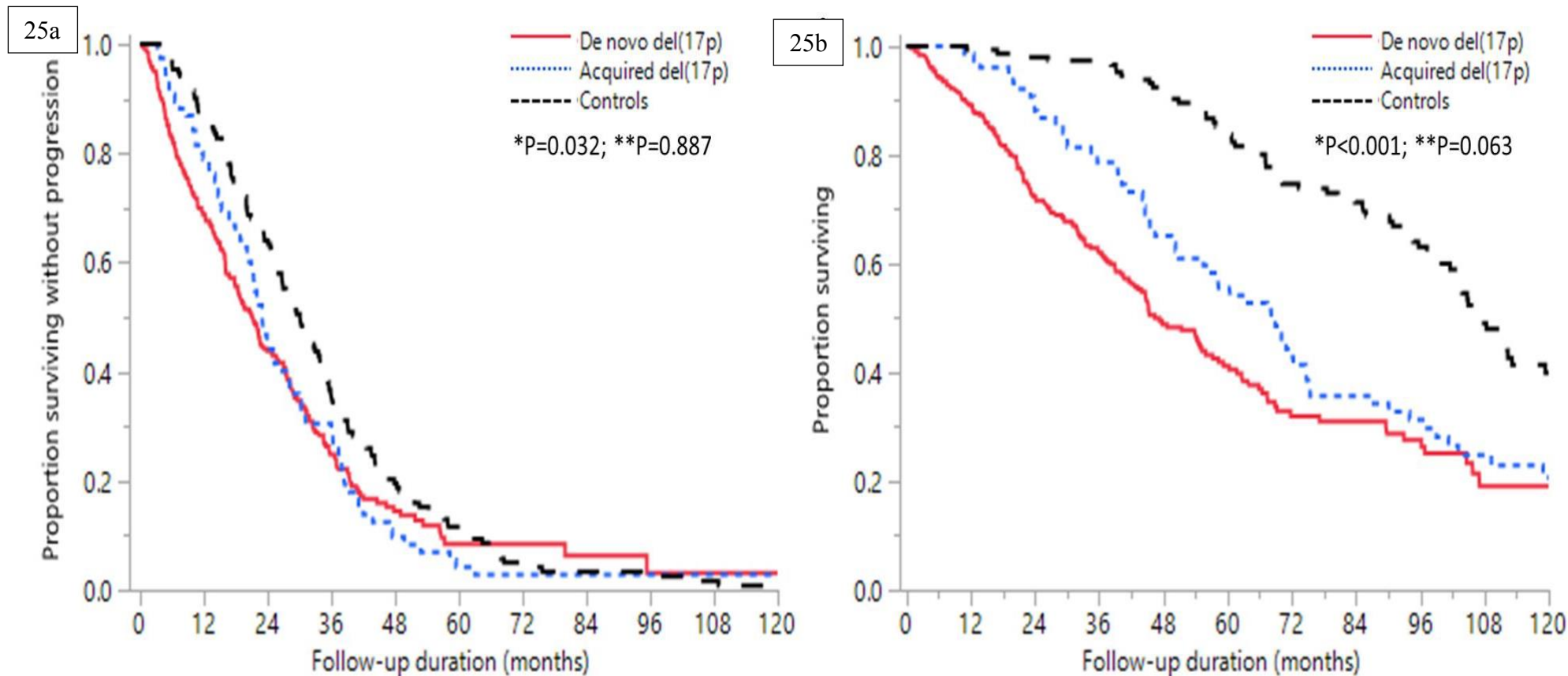
\*P for Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables. †Calculation is limited to cases and controls who had FISH after May 2009 [n=37 and n=69 for cases and controls at diagnosis and n=68 for cases at detection of del(17p)] when probes for these abnormalities were introduced. ‡Calculation limited to cases and controls who had FISH after August 2014 [n= 4 and n=8 for cases and controls at diagnosis, and n=14 for cases at detection of del(17p)] when probe for 1q gain and del (1p) were introduced. BMPC indicates bone marrow plasma cell; FISH, Interphase fluorescence in situ hybridization; ISS, International Staging System; and LDH, Lactate dehydrogenase.

### 3.4.4.2 Comparison of survival outcomes(table 13)

Comparing survival outcomes from initial diagnosis, PFS in the acquired del (17p) and control groups were 23.0 months (95% CI, 20.2-27.8) and 30.1 months (95% CI, 26.0-33.9) respectively (P=0.032). The OS in the two groups were 68.2 months (95% CI, 50.8-74.8) and 106.1 months (95% CI, 101.6-119.4) respectively (P<0.001). The results were also valid in subgroups based on prognostic factors except HRTs (**Table 13**). PFS in patients with de novo del(17p) was similar [21.2 months (95% CI, 17.8-23.9); P=0.887] and OS trended towards being shorter [47.3 months (95% CI, 42.7-55.9); P=0.063] when compared to the acquired del(17p) group. The comparison of PFS and OS for the three groups is shown in **Fig 25**. OS landmarked from detection of del(17p) for cases and a corresponding time point for controls were 18.1 months (95% CI, 11.9-25) and 56.2 months (95% CI, 44.4-79.7) respectively (P<0.001).

<b>Table 13. Sub-group analysis for overall survival from diagnosis in patients with acquired del(17p) and controls based on prognostic factors at diagnosis and therapy</b>			
<i>Subgroups</i>	<i>Acquired del(17p) (n=76)</i>	<i>Control patients (n=152)</i>	<i>P*</i>
Age <65 (48 vs. 101)	72.3 (49.6-99.0)	106.1 (98.5-127.8)	0.002
Age ≥65 (28 vs. 51)	58.2 (44.1-71.3)	105.9 (68.6-138.8)	<0.001
High-risk translocation (14 vs. 14)	56.8 (24.0-119.0)	98.3 (60.5-111.8)	0.419
No high-risk translocation (62 vs. 138)	68.9 (50.8-74.8)	111.7 (103.4-129.3)	<0.001
ISS I/II (42 vs. 110)	71.3 (50.8-75.4)	106.1 (101.6-129.3)	<0.001
ISS III (14 vs. 26)	44.4 (13.8-103.1)	104.8 (60.7-111.8)	0.036
Low LDH (44 vs. 116)	63.8 (44.6-99.0)	105.9 (98.5-119.4)	0.004
High LDH (7 vs. 5)	55.5(23.2-86.4)	100.9 (80.6-NR)	0.011
Low PC proliferative rate (31 vs. 84)	74.6 (58.3-99.0)	106.1 (98.3-137.8)	0.025
High PC proliferative rate (14 vs. 24)	42.2 (24.0-103.1)	103.4 (67.1-NR)	0.031
PI-containing induction (21 vs. 37)	34.5 (24.0-50.8)	104.8 (70.2-137.8)	<0.001
Others (55 vs. 115)	74.6 (63.8-96.5)	106.1 (98.5-127.8)	0.003
*P for log-rank test for Kaplan Meier analysis; ISS indicates international staging system; LDH, Lactate dehydrogenase; PC, Plasma cell; and PI, Proteasome inhibitor.			

Fig 25: a) PFS and b) OS are shorter in patients with del17p loss. De novo MM patients have shorter median OS(Fig 25b)



De novo del(17p)	310	185	103	49	19	6	5	3	1	1	1	310	272	200	147	88	59	35	30	23	8	3
Acquired del(17p)	76	59	34	22	7	3	2	2	1	1	1	76	75	66	58	48	40	30	25	21	13	9
Controls	152	131	95	51	27	16	6	4	4	2	1	152	151	147	140	128	115	91	83	63	44	28

#### **3.4.4.3 Patient characteristics at detection of del(17p)**

The median time from diagnosis of MM to detection of del(17p) in the acquired del(17p) cohort was 35.6 months (range, 4.8-116.1). Patients had received a median of 2 (range, 1-10) prior lines of therapy before detection of del(17p). Del(17p) was detected in median of 89% (range, 9-100) of PCs tested by FISH. Fifty seven (67.1%) and 33 (43.4%) patients respectively were exposed to and refractory to a PI; 70 (92.1%) and 57 (75.0%) patients respectively were exposed to and refractory to an IMiD (majority being lenalidomide and/or pomalidomide); and 60 (78.9%) and 25 (32.9%) patients respectively were exposed to and refractory to an alkylating agent. Thirty (39.5%) patients were PI and IMiD-refractory.

#### **3.4.4.4 Cytogenetics at acquisition of del17p(table 14)**

Seventy (86.8%) patients had del(17p13.1). Five (6.6%) patients had monosomy 17. One (1.3%) patient demonstrated both del(17p13.1) and monosomy 17. Four (5.3%) patients had relative loss of 17p. HRTs were present in 14 (18.4%) patients. Compared to initial FISH, proportion of patients with hyperdiploidy increased at detection of del(17p) (42.1% to 59.2%;  $P<0.001$ ). A similar change was seen with monosomy 13 (35.5% to 53.9%;  $P<0.001$ ) while no change was seen with del(13q) (3.9% and 6.6%;  $P=0.187$ ). Even though 4 (28.6%) patients at follow-up had 1q22 gain, none of these patients were tested for it initially. So, we cannot ascertain if this was newly acquired.

High-risk chromosomal translocation	14 (18.4)	14 (9.2)	14 (18.4)	0.055
<b>t(4;14)</b>	<b>12 (15.8)</b>	<b>10 (6.6)</b>	<b>12 (15.8)</b>	<b>0.033</b>
t(6;14) <sup>†</sup>	0 (0)	0 (0)	0 (0)	-
t(11;14)	15 (19.7)	37 (24.3)	15 (19.7)	0.505
t(14;16)	2 (2.6)	3 (2.0)	2 (2.6)	1.000
t(14;20) <sup>†</sup>	0 (0)	1 (1.4)	0 (0)	1.000
Hyperdiploidy (trisomy or tetrasomy involving ≥2 odd-numbered chromosomes)	32 (42.1)	69 (45.4)	41 (53.9)	0.673
Del (13q) and/or monosomy 13	30 (39.5)	67 (44.1)	45 (59.2)	0.570
• Del (13q)	3 (3.9)	8 (5.3)	5 (6.6)	0.756
• Monosomy 13	27 (35.5)	59 (38.8)	41 (53.9)	0.666
(1q22) gain <sup>‡</sup>	0 (0)	1 (12.5)	4 (28.6)	1.000
Del (1p32) <sup>‡</sup>	0 (0)	0 (0)	0 (0)	-

\*P for Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables. <sup>†</sup>Calculation is limited to cases and controls who had FISH after May 2009 [n=37 and n=69 for cases and controls at diagnosis and n=68 for cases at detection of del(17p)] when probes for these abnormalities were introduced. <sup>‡</sup>Calculation limited to cases and controls who had FISH after August 2014 [n= 4 and n=8 for cases and controls at diagnosis, and n=14 for cases at detection of del(17p)] when probe for 1q gain and del (1p) were introduced. BMPC indicates bone marrow plasma cell; FISH, Interphase fluorescence in situ hybridization; ISS, International Staging System; and LDH, Lactate dehydrogenase.

#### **3.4.4.5 Treatment after detection of del(17p)**

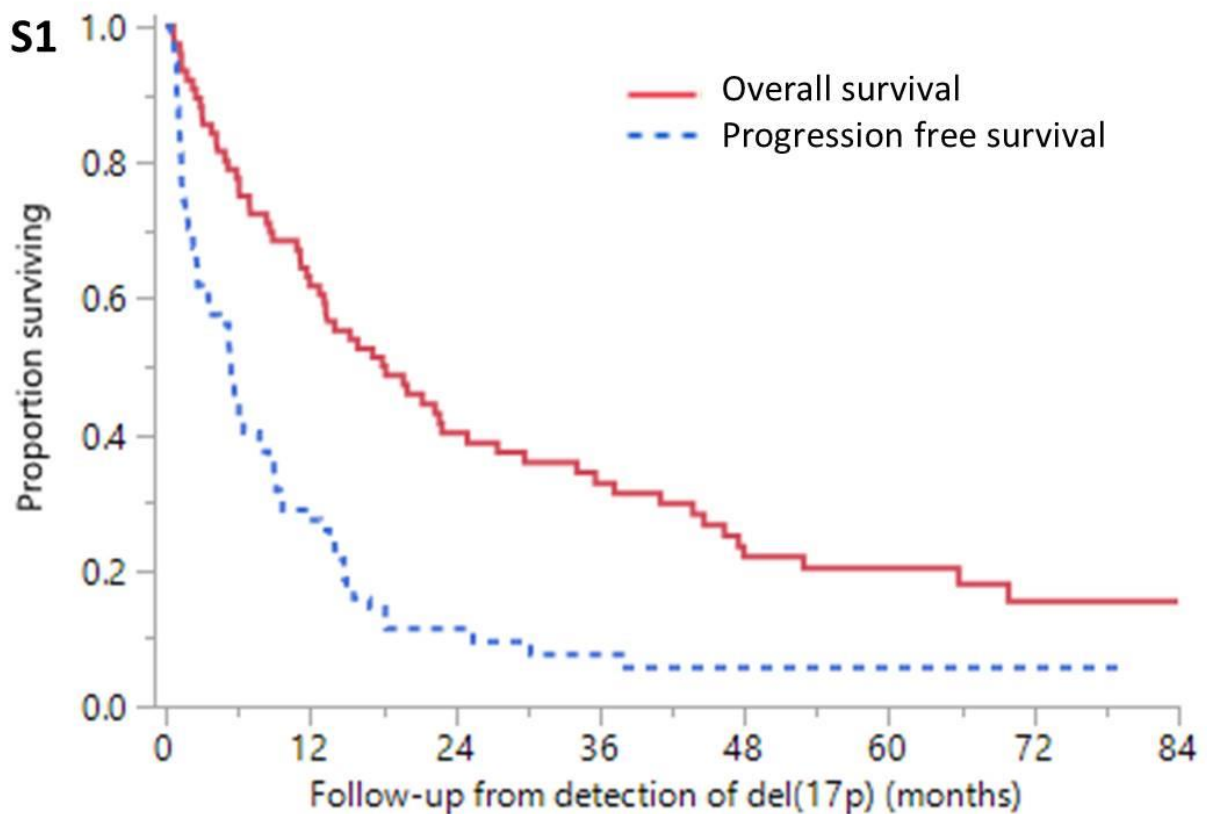
All patients received therapy after detection of del(17p). IMiD-based and PI-based regimens were used in 22 (28.9%) patients each and PI+IMiD-based regimen was used in 15 (19.7%) patients. Monoclonal antibodies were used in 4 (5.3%) patients. Seven (9.2%) patients proceeded directly to SCT without additional therapy and 6 (7.9%) patients received other therapies. Overall, SCT was part of the next line of treatment in 14 (18.4%) patients (one of them underwent allogeneic SCT).

#### **3.4.4.6 Response to therapy after detection of del(17p)**

Among 67 patients who were evaluable for response, stringent complete response (sCR), CR, very good partial response (VGPR) and PR were attained in 3 (4.5%), 3 (4.5%), 16 (23.9%) and 9 (13.4%) patients respectively. A minimal response was seen in 3 (4.5%) patients. Twenty (29.8%) patients showed stable disease while 13 (19.4%) developed progressive disease.

### 3.4.4.7 Survival after detection of del(17p)(Fig 26)

The median PFS from start of next line of therapy was 5.4 months (95% CI, 2.7-7.7).  
 The median OS from detection of del(17p) was 18.1months (95% CI, 11.9-25.0)



<b>OS</b>	<b>76</b>	<b>47</b>	<b>28</b>	<b>22</b>	<b>14</b>	<b>11</b>	<b>5</b>	<b>3</b>
<b>PFS</b>	<b>76</b>	<b>19</b>	<b>7</b>	<b>4</b>	<b>2</b>	<b>1</b>	<b>1</b>	<b>-</b>

Fig 26: Survival outcomes in patients with acquired del(17p) - The median OS was 18.1 months (95% CI, 11.9-25.0) and progression to next treatment line was 5.4 months(95% CI 2.7-7.7)

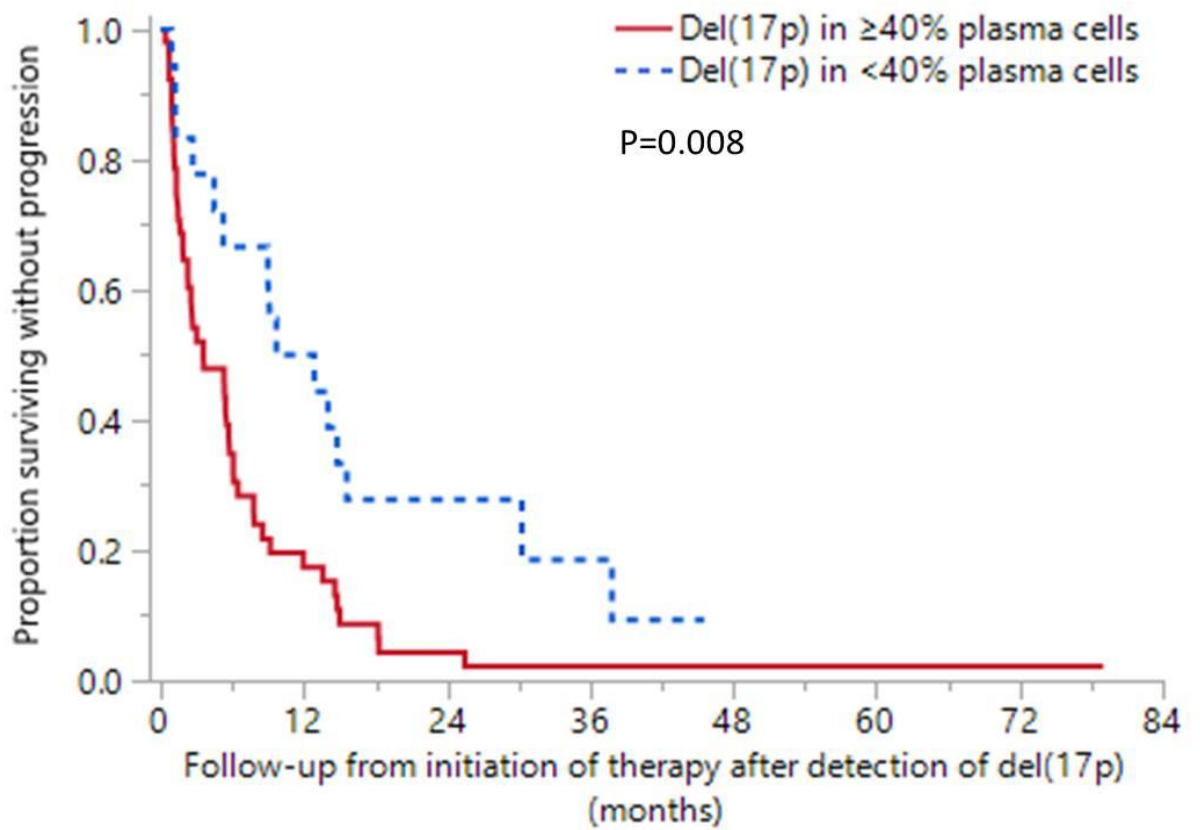


### 3.4.5 Predictors of survival after detection of del(17p)

#### 3.4.5.1 Factors affecting PFS after detection of del(17p)(Fig 27)

To identify factors affecting PFS and OS from detection of del(17p), we used age  $\geq 65$  vs.  $<65$  years, serum creatinine  $>2$  vs.  $\leq 2$  mg/dL, bone marrow PC percentage  $\geq 50$  vs.  $<50$ , ISS stage III vs. I or II, elevated vs. normal LDH, presence vs. absence of an HRT, presence vs. absence of monosomy 13, presence vs. absence of hyperdiploidy, high vs. low PC proliferative rate, prior PI and IMiD refractoriness and different proportions of PCs with del(17p) (20%, 30%, 40%, 50% and 60%) in the Cox proportional hazards model (**Table 13**).

After univariable analysis, ISS stage III disease, high PC proliferative rate, PI and IMiD-refractoriness and presence del(17p) in  $\geq 30\%$ ,  $\geq 40\%$  and  $>50\%$  PCs, had  $P < 0.1$  for predicting shorter PFS and were included in multivariable analysis. Using multivariable Cox proportional model with step-wise backward elimination, presence of del(17p) in  $\geq 40\%$  PCs predicted shorter PFS with hazard ratio (HR) of 2.21 (95% CI, 1.21-4.01) ( $P=0.009$ ). The median PFS in patients with del(17p) involving  $\geq 40\%$  PCs and  $<40\%$  PCs were 3.5 months (95% CI, 1.9-5.7) and 11.3 months (95% CI, 4.4-15.6) respectively ( $P= 0.008$ )

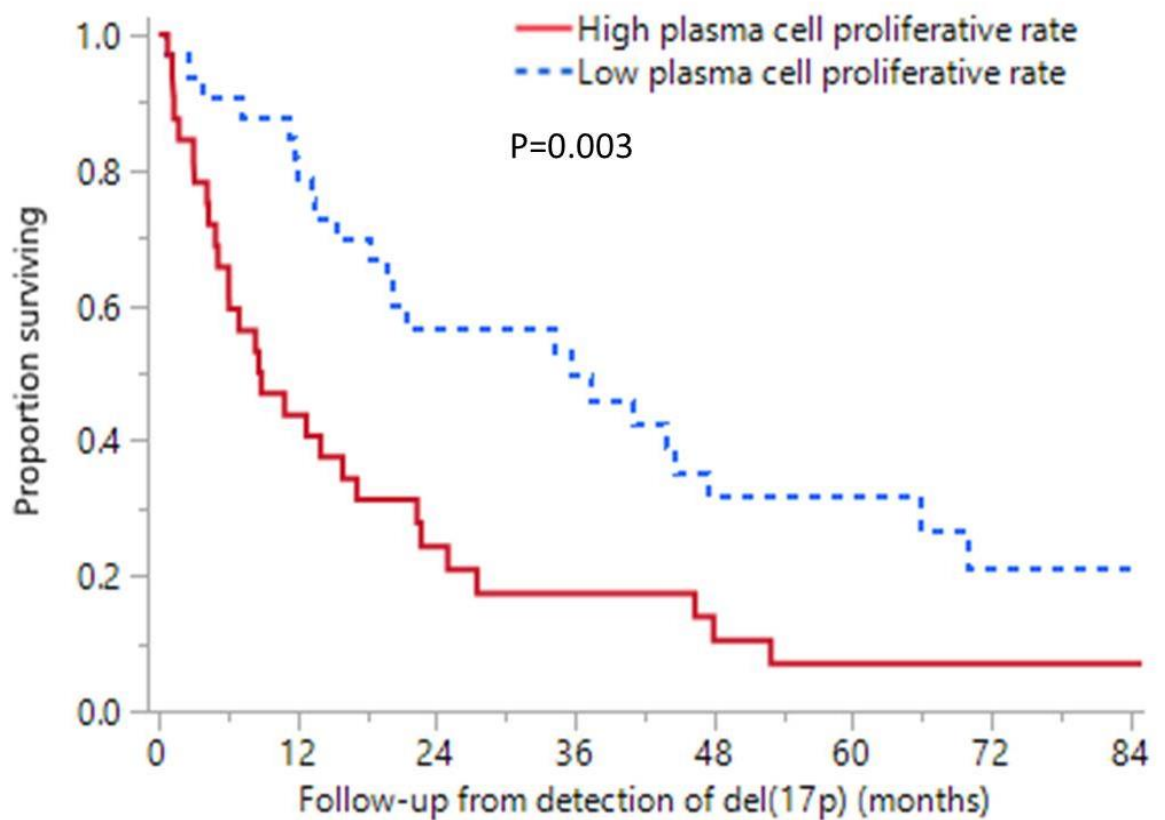


Del(17p) in $\geq 40\%$ PCs	53	8	2	1	1	1	1	-
Del(17p) in $< 40\%$ PCs	18	9	4	2	-	-	-	-

Fig. 27: Shorter PFS in patients with  $\geq 40\%$  plasma cells

### 3.4.5.2 Factors affecting OS after detection of del(17p)(Fig 28)

In a similar analysis, ISS III stage, elevated LDH, high PC proliferative rate and PI and IMiD-refractoriness were included in the multivariable model to determine predictors of shorter OS. A higher PC proliferative rate alone predicted shorter OS with HR of 2.28 (95% CI, 1.31-3.96) (P=0.004). The median OS in patients with high PC proliferative rate at detection of del(17p) was 8.9 months (95% CI, 4.9-17.1) vs. 35.6 months (95% CI, 18.2-47.5) in those with a low PC proliferative rate (P=0.003)



<b>High PC proliferative rate</b>	<b>32</b>	<b>14</b>	<b>7</b>	<b>5</b>	<b>3</b>	<b>2</b>	<b>2</b>	<b>2</b>
<b>Low PC proliferative rate</b>	<b>33</b>	<b>26</b>	<b>17</b>	<b>14</b>	<b>9</b>	<b>8</b>	<b>3</b>	<b>1</b>

Fig. 28: Shorter OS in patients with high PC proliferation rate vs low proliferation

### 3.4.5.3 Factors Predictive of acquisition of del17p (Table 15)

To identify predictors at diagnosis of acquisition of del(17p), we compared patients with acquired del(17p) with controls in a case-control fashion and calculated OR. High LDH at baseline [OR- 3.69 (95% CI, 1.11-12.24)], presence of t(4;14) [OR- 2.66 (95% CI, 1.09-6.48)] and presence of an HRT [OR- 2.23 (95% CI, 1.00-4.95)] predicted acquisition of del(17p). Age  $\geq$ 65 years, ISS III stage, t(11;14), any trisomy/tetrasomy, hyperdiploidy, monosomy 13, bone marrow PC%, initial therapy, and exposure to high-dose melphalan in first year and before acquiring del(17p) were not predictive

<b>Table15. Comparative analysis between patients with acquired del(17p) and controls for putative risk factors at diagnosis for acquiring del(17p).</b>	
<i>Presumed risk factor for acquiring del (17p)</i>	<i>Odds ratio (95% CI)</i>
Age $\geq$ 65 years (28/76 vs. 51/152)	0.1.16 (0.65-2.05)
ISS III stage (14/56 vs. 26/136)	1.41 (0.67-2.96)
<b>Elevated LDH (7/51 vs. 5/121)</b>	<b>3.69 (1.11-12.24)</b>
Bone marrow plasma cell % $\geq$ 50% (21/70 vs. 62/150)	0.61 (0.33-1.11)
High plasma cell proliferative rate (14/45 vs. 22/106)	0.72 (0.78-3.79)
<b>High-risk translocations (14/76 vs. 14/152)</b>	<b>2.23 (1.00-4.95)</b>
<b>t(4;14) (12/76 vs. 10/152)</b>	<b>2.66 (1.09-6.48)</b>
t(11;14) (15/76 vs. 37/152)	0.76 (0.39-1.50)
Monosomy 13 (27/76 vs. 59/152)	0.86 (0.49-1.54)
Any trisomy/tetrasomy (38/76 vs. 90/152)	0.69 (0.40-1.20)
Hyperdiploidy (trisomy/tetrasomy of $\geq$ 2 odd-numbered chromosomes) (32/76 vs. 69/152)	0.87 (0.50-1.53)
PI-containing induction (21/76 vs. 37/152)	1.19 (0.64-2.22)
Alkylating agent in induction (17/76 vs. 36/160)	1.00 (0.52-1.94)
Autologous SCT with high dose melphalan within 1 year of diagnosis (and before detection of del(17p) for cases) (33/76 vs. 82/152)	0.65 (0.38-1.14)
ISS indicates international staging system; LDH, Lactate dehydrogenase; PI, Proteasome inhibitor; and SCT, stem cell transplantation.	

## Discussion

Increasing evidence supports a significant impact of cytogenetic abnormalities affecting the treatment response and survival outcomes for MM patients. These abnormalities are classified as primary abnormalities which are common in MGUS stage and secondary abnormalities, that increase in frequency with more active disease. The disease has 50% of patients with IgH translocations (non-hyperdiploid group), and the non-hyperdiploid abnormalities unique to the subclonal population of MMPC makes the disease biology complex and a clinical challenge for treatment. OS of patients with high-risk cytogenetic aberrations in MM has been shorter as previously noted.

Several initial studies identified the additive prognostic significance of t(4;14) and del17p to conventional MM ISS staging system which is computed on patient's  $\beta 2$  microglobulin and albumin levels. These chromosomal aberrations favourably risk stratified MM patients into low[no del17p or t(4;14)], intermediate (neither low or high) and high(either or both of t(4;14), del17p) risk-groups[364, 365]. Palumbo et al. confirmed these findings in their report studying 3060 patient pooled data from 11 trials with available ISS, LDH and cytogenetic abnormalities related data. Here, adding t(4;14) and del17p to standard ISS predicted for PFS and OS, in MM patients with five year OS rates of 82%, 62% and 40% in R-ISS(revised ISS with cytogenetic aberrations) stage I, II and III groups respectively. Importantly this prognostication system was valid irrespective of ASCT, novel agent or age of the patient[107].

Therapies targeting high-risk cytogenetic abnormalities are currently needed. t(4;14) occurs in about 15% of MM patients. Chng et al. showed that MM patients and cell lines with t(4;14) have a high centrosome index (CI) and overexpress aurora kinases and further patients with high CI have shorter PFS despite treatment with Bortezomib[366]. We studied the role of A1014907 in MM cell lines and patient cells to identify its role in Aurora kinase inhibition. The cell cycle inhibition secondary to Aurora kinase inhibition was validated by our; experiments however, we observed a dramatic difference in the sensitivity of t(4;14) containing MM cell lines when compared with the non-t(4;14) cell lines. This is unlikely to be explained merely by

aurora kinase inhibition. Chang H et al. previously demonstrated 75% FGFR3 expression in t(4;14) MM patients[367]. We hypothesized a role of simultaneous inhibition of FGFR3 expression and aurora kinases in t(4;14) cell lines. Our results show that A1014907 inhibits aurora kinases and FGFR3. In cells lacking FGFR3 expression, A1014907 caused cell cycle arrest in a majority of them but failed to induce marked apoptosis. In cells expressing wild type FGFR3 or those with activating mutations in FGFR3 (Y373C or G382D), A1014907 caused potent cell cycle arrest besides pronounced apoptosis.

Furthermore, we dissected the FGFR3 mediated downstream pathways and showed clear evidence that FGFR3 inhibition by A1014907 resulted in down-regulation of both the PLC $\gamma$ /PKC and Stat3 signalling pathways leading to apoptosis induction in these cell lines. Upregulation of pErk and pAkt in MM1S reflects upon the possible cause of drug resistance in the absence of FGFR3 expression. Importantly, A1014907 effectively induced cytotoxicity in FGFR3 expressing CLL cell lines, highlighting its broader application in other malignancies with high CI and FGFR3 expression. Combination with Stat3 and Akt inhibitor would be useful in non-FGFR3 expressing tumour systems.

We describe the outcomes of 310 del17p MM patients treated at Mayo Clinic, Rochester. This subset of patients lacks a definitive description of disease characteristics and associated features. A lack of specific studies with large patient cohorts accurately assessing patients with del17p has predominantly directed efforts to extrapolate results from more extensive MM trials or small cohort-based studies. Majority of patients presenting with high risk features received a PI-containing induction. Over 50% had an SCT. PR was Seventy-six percent although lower in comparison to SR and high-risk translocation groups. In the del17p group, the median PFS and OS were 21 months and 47 months, respectively. The ISS stage, LDH level and simultaneous high-risk translocations predicted for OS.

Low Hb, high LDH level and PC proliferation rate, were noted in the del17p cohort. Similar observations were made by Fonseca et al. in a smaller patient cohort. Compared to a previous report of 110 patients, we observe lesser del17p with ISS III disease in our patient sample(our cohort: 36% vs 45% in the study cohort) [368].

There is a discrepancy noted in selection criteria for cut-off's defining acceptable del17p %. The GMMG-HD4/HOVON65 trial used a 10% threshold for del17p and demonstrated PFS and OS adverse events with the lesser cut off; the IFM group used a higher 60% cut off for del17p assessment however they had lesser number of purified PC's in many samples (median % of 6% plasma cells)[141, 369]. t(4;14), trisomies, t(11;14) and chromosome 13 abnormalities are common in del17p patients as previously described[368].

In keeping with bortezomib's role in improving outcomes of high-risk cytogenetics, majorly all patients with del17p and high-risk translocations in our study received a PI regimen based induction therapy[140, 141]. Combinations of PI and IMiD were favoured in Del(17p) patients when compared to HRT-patients (39 vs 24%). Interestingly, PI-based induction did not predict for an improved PFS or OS in patients with del(17p) in our analysis. SCT within a year was more likely pursued in del17p cohort although this approach did not ascertain an OS benefit in these patients.

Neben et al. showed an OS of 62% in MM patients treated with velcade doxorubicin and dexamethasone induction followed by HDT/ASCT and bortezomib maintenance. Otherwise, the inferior PFS and OS in the del17p patient has been previously reported[101, 125]. While the nature of the studies make direct comparisons difficult as simultaneous high-risk abnormalities might not be considered, it would be interesting to note the role of PI/IMiD based maintenance.

Similar PFS was seen in HRT and del17p patients. This could be due to similar induction therapy related effects in both subgroups. Shorter median OS after del17p acquisition suggests an increased relapse risk or disease becoming treatment refractory. There is a significant influence of concurrent genetic abnormalities for OS outcomes as noted in our study. The presence of simultaneous high-risk abnormalities with del17p portends a poor OS at 29.5 months compared to 51.4 months and 79 months for Del17p alone and controls with high-risk translocation respectively. The PFS for Del17p cohort with simultaneous high-risk translocations in our cohort was 14.7 months which is comparable to the PFS of 17 months seen by Merz et al. in a similar del17p cohort of patients with gain 1q21[368].

Concurrent hyperdiploidy in del 17p cohort trended towards improved OS. Several previous studies demonstrate a similar adverse effect of cumulative high-risk cytogenetics with ameliorating role of trisomies[67, 111, 370]. Del17p is either R-ISS stage II or III based on other features[107]. It is noteworthy to consider restratification of R-ISS classification given the adverse outcomes of simultaneous del17p and high-risk translocations as an independent category irrespective of other factors.

Importantly, 21 patients with relative loss of 17p, have unclear significance in previous reports. Our results show similar poor survival for this cohort as compared to the del17p group and hence should be considered an adverse prognostic marker

Our studies highlighted a lack of significant role PC clone with del17p when taken together with other adverse factors. Hence, If del17p is detectable beyond background detection rate, It should be considered significant for del17p and considered high risk.

We next studied the natural history of 76 MM patients who acquired del17p after diagnosis while on treatment. The OS of this group was naturally shorter which we estimated at 18 months. These patients have been shown to do poorly in several studies with median PFS( ranging between 3.4-7.6 months)[297, 371]. Comparing the median OS, some The median OS in clinical trial patients of RRRM treated with bortezomib-based regimen was 11.5 months, which is shorter than the OS in our patients from the detection of del(17p)[372]. The eloquent trial showed favourable improvement of PFS in relapsed and/or refractory MM(RRMM) in patients with del17p and t(4;14) with a median survival of 26 months in elotuzumab, lenalidomide and dexamethasone group[373]. Although they considered del17p if any MMPC was positive for the same, the results underlie the poor prognosis in this subset of patients with RRMM. However, we are unable to ascertain, the timing of del17p in the trials. Interestingly, in a recent trial of ixazomib or placebo, with lenalidomide and dexamethasone in RRMM, among patients with del(17p) in the two arms, PFS was 21.4 and 9.7 months respectively, the PFS in patients with del(17p) being similar to those with standard-risk cytogenetics[374]. These results were in patients with del(17p) defined using  $\geq 5\%$  PCs with del(17p). When using cut-offs of 20% and 60%,



21.4 vs 6.7 months, and 15.7 vs 5.1 months respectively were obtained in the two groups. In our series, using cut-offs from 20 to 60%, PFS ranged from 5.3 to 3.5 months, suggesting progressively decreasing PFS with an increase in the size of the PC clone. A cut-off of 40% predicted shorter PFS in multivariable analysis. However, the size of the PC clone with del(17p) was not a predictor of OS. This is similar to the results we observed in a series of patients with de novo del(17p).

High PC proliferative rate at the detection of del(17p) predicted a shorter OS. PC proliferative rate is a prognostic factor in patients with PC disorders including MM and light chain amyloidosis.[375-378] PC proliferative rate detects cells in S-phase and is a marker of active DNA synthesis by malignant PCs. Presence of high PC proliferative rate may represent further deregulation of cell cycle control in patients with del(17p).

An important finding of our study is a high OR for acquired del17p when certain specific parameters including, high LDH at diagnosis and presence of HRTs, especially, t(4;14). High LDH is usually a marker of aggressivity in the context of cancer biology. In solid tumours including renal cell carcinoma, melanoma and prostate carcinoma, high LDH portends poor OS and is a marker of metastatic disease[379]. High LDH levels are usually found at diagnosis and are predictive of disease relapse in haematological malignancies[380]. In MM increased LDH predicts for aggressive disease forms and reduced survival post chemotherapy[381, 382]. Hence a high LDH at diagnosis is likely a marker of advancing disease. A previous sequential analysis of patients has shown that presence of HRTs at baseline is associated with detection of more copy number abnormalities on follow-up and this was postulated to be due to higher genomic instability in these patients.[383]. Also, the use of high-dose melphalan with autologous SCT was not associated with the acquisition of del(17p). This is contrary to a previous report which associated high-dose melphalan and autologous SCT with the acquisition of high-risk abnormalities including del(17p) at relapse.[384] However, the risk factors we identified were present at baseline in only a few patients in the acquired del(17p) and control groups, and our results need confirmation in larger prospective datasets such as CoMMpass.[385]

The results of our study would likely require validation in prospective studies with all available data. Baseline LDH, anticipated FISH testing at specific time intervals, all treatment-related data including maintenance therapy, are some of the shortcomings of our study. Further given the retrospective nature of the data, patients lacked homogeneity of therapy within the same subgroups. Subclonal analysis of TP53 which is predictive of poor outcome was not assessed[386]. Data on the 1q gain were not complete due to a lack of availability of a FISH probe for the same before 2014.

## Conclusion

Targeted treatment approach has significant advantages in the treatment of cancers as compared to conventional chemotherapy. A1014907 showed significant FGFR3 inhibition activity which could have a significant role in high-risk MM disease patients with this inherently activated pathway. There is a broader scope for A1014907 in cancer therapeutics as seen with our data on CLL patients with active FGFR3 signalling. This has significant implications for an individualized therapeutic approach using molecular diagnostic tools such as gene expression profiling to identify patients with activated specific signalling pathways.

Del17p is a poor prognosis chromosomal aberration in MM with poor OS outcomes. ISS III stage, high LDH and high-risk translocations predict for shorter OS in patients with del(17p).

del17p patients showed increased association with t(4;14) and high-risk translocations, highlighting an important association of secondary translocations in MM. This would significantly impact response to treatment as noted in our study; hence the need for newer therapeutic targets with the ability to overcome the poor prognostic impact of high-risk translocations and del17p

Some of our unpublished work using gene expression profiling(GEP) showed an increased association of del17p with Aurora kinase A expression. This is plausible given increased PCL1 and hence replication rate as shown in our study. Exploiting multi-targeted agents impacting dysregulated cellular mechanisms are likely to induce more profound disease response with lesser generalized cytotoxicity.

While we establish an increased association of t(4;14) and del17p, it would be noteworthy to see whether the subset of these patients who have an activated FGFR3 pathway tend to do better with targeted treatment such as A1014909 or it can perhaps ameliorate the impact of simultaneous del17p.

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