

Genetics of High-Risk Myeloma

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This thesis is submitted for the degree of PhD

1 Declaration

I, Vallari Shah, confirm that the work presented in this thesis is my own. Where work has been performed by others, this has been indicated in the thesis.

Portions of this work have been published, in manuscripts and conference abstracts, but have not been previously submitted for a degree at this, or any other, institution.

2 Acknowledgements

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3 Abstract

Despite significant progress in therapy in the last two decades, the outlook for multiple myeloma (MM) patients is still poor as the disease remains incurable and only 33% of patients survive for up to 10 years after diagnosis. Reasons that underlie poor survival rate include genomic instability, which results in relapse and progression. As a result, there is interest in investigation of genomic aberrations and mutations that drive disease progression.

I aimed to determine the genetic events that are associated at presentation with high-risk myeloma. I sought to ascertain whether there was an association between poor survival and low incidence genetic markers through performance of meta-analyses of large patient numbers. Additionally, I sought to answer specific questions regarding deletion and mutation of the tumour protein p53 (*TP53*) gene in terms of clonal heterogeneity at presentation. I investigated whether extreme numbers of copies at the chromosomal arm 1q were associated with survival. Furthermore, I investigated gene expression signatures in myeloma.

I addressed these aims by evaluating data from two large randomised controlled trials of newly diagnosed myeloma patients. Through the study of genetic high-risk lesions and clinical annotation, I was able to evaluate these cytogenetic lesions in thousands of uniformly treated patients.

The most striking findings were that patients with subclonal *TP53* deletion had a similarly poor prognostic association to those with clonal *TP53* deletion, and that mono-allelic *TP53* alterations had independent prognostic association to those with bi-allelic alterations. We also determined that gain(1q21) was associated with survival rates that were independent of amplification (1q21) and other high-risk lesions.

My work provides critical analysis of the methods that are used to determine risk in newly diagnosed myeloma and supports the call for uniform and comprehensive reporting of risk through standardisation of trial report requirements in terms of risk calling.

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7 List of abbreviations

ADAR1	RNA-specific adenosine deaminase 1
amp	Amplification
ANP32E	Acidic nuclear phosphoprotein 32 family member E Alipoprotein B mRNA editing enzyme, catalytic polypeptide like
APOBEC	like
ARF	ADP-ribosylation factor
ASCT	Autologous stem cell transplant
ASPM	Assembly factor for spindle microtubules
ATM	Ataxia telangectasia mutated
ATR	Ataxia telangectasia and rad3-related protein
AUC	Area under the curve
AUCi	Integrated area under the curve (over time)
BAX	B-cell lymphoma 2 associated X
BCL2	B-cell lymphoma 2
BCL6	B-cell lymphoma 6
BCL9	B-cell lymphoma 9
BCMA	B-cell maturation antigen
BD	Bortezomib, dexamethasone
BIM	BCL2 interacting mediator of cell death (also called BCL2L11)
BIRC2	Baculoviral IAP repeat-containing protein 2
BIRC3	Baculoviral IAP repeat-containing protein 3
BIRC5	Baculoviral IAP repeat-containing protein 5
BLIMP1	B lymphocyte maturation protein 1
BMP6	Bone morphogenic protein 6
BRAF	B-RAF proto-oncogene, serine/threonine kinase
BRCA2	Breast cancer 2 gene
BTG2	B-cell translocation gene antiproliferation factor 2
CAN	Copy number aberration
CAR-T	Chimeric antigen receptor T cell therapy
CASP4	Caspase 4
CAV1	Caveolin 1
CCND1	Cyclin D1
CCND2	Cyclin D2
CCND3	Cyclin D3 Carfilzomib, cyclophosphamide, lenalidomide and dexamethasone
CCRD	dexamethasone
CD	Cluster of differentiation
CDK2	Cyclin-dependent kinase 2
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2C	Cyclin-dependent kinase inhibitor 2C
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2

CKS1B	CDC28 protein kinase regulatory subunit 1B
CLN3	Ceroid-lipofuscinosis neuronal 3
CNA	Copy number aberration
CNS	Central nervous system
CR	Complete response
CRBN	Cereblon
CRD	Cyclophosphamide, Revlimid (lenalidomide), dexamethasone
CRL4	Cullin RING ligase 4
CRP	C-reactive protein
CT	Computer tomography
CTD	Cyclophosphamide, thalidomide, dexamethasone
CUL4	Cullin-4A
CVAD	Cyclophosphamide, vincristine, doxorubicin, dexamethasone
CX3CR1	C-X3-C Motif chemokine receptor 1
CYLD	Cylindromatosis
D	Diversity
DDB1	DNA-binding protein 1
del	Deletion
	homologue, exosome endoribonuclease and 3'-5'
DIS3	exoribonuclease
DKK1	Dickkopf WNT signalling pathway inhibitor 1
DNA	Deoxyribonucleic acid
DPd	Daratumumab, pomalidomide, dexamethasone
DRd	Daratumumab, Revlimid, dexamethasone
	Daratumumab, Velcade (one brand name of bortezomib),
DVd	dexamethasone
E2F	E2F transcription factor
E2F7	E2F transcription factor 7
EBF1	Early B cell factor 1
EBPL	Emopamil-binding protein like
EBPL	Emopamil-binding related protein like
EFS	Event-free survival
eGFR	Estimated glomerular filtration rate
ERCC5	Excision repair 5, endonuclease
ERK	Extracellular signal-regulated kinases
ESPL1	Extra spindle pole bodies like 1, separase
EV15	Ectopic viral integration site 5
FAF1	FAS associated factor 1
FAM46C	Family with associated similarity 46 member C
FAM49A	Family with associated similarity 49 member A
FDR	False discovery rate
FGFR3	Fibroblast growth factor receptor 3
FOXO3	Forkhead box O3
GADD45	Growth arrest and DNA damage inducible alpha
GEP	Gene expression profiling
HDAC	Histone deacetylase
HDM-Auto	High-dose melphalan and autologous stem cell transplantation
Hetero-del	Heterozygous deletion

Homo-del	Homozygous deletion
HRD	Hyperdiploid myeloma
HUWE1	HECT, UBA and WWE domain-containing E3 ubiquitin protein ligase 1
IDH2	Isocitrate dehydrogenase 2
iFISH	Interphase fluorescent in-situ hybridisation
IgA	Immunoglobulin isotype A
IgD	Immunoglobulin isotype D
IgE	Immunoglobulin isotype E
IgG	Immunoglobulin isotype G
IgH	Immunoglobulin heavy chain
IgK	Immunoglobulin light chain kappa
IgL	Immunoglobulin light chain lambda
IgM	Immunoglobulin isotype M
IKZF1	Ikaros family zinc finger protein 1
IKZF3	Ikaros family zinc finger protein 3, also known as Aiolos
IL-2	Interleukin 2
IL-6	Interleukin 6
IL6R	Interleukin 6 receptor
ILF2	Interleukin enhancer binding factor 2
IMiD	Immuno-modulatory drugs
IMW	International myeloma workshop
IMWG	International Myeloma Working Group
IRd	Ixazomib, Revlimid, dexamethasone
IRF4	Interferon regulatory factor 4
ISS	International scoring system
ITGB7	Integrin subunit beta 7
J	Joining
KCNN3	Potassium calcium-activated channel subfamily N member 3
KRAS	Kirsten rat sarcoma virus proto-oncogene
KRD	Carfilzomib, Revlimid, dexamethasone
LDH	Lactate dehydrogenase
LTB	Lymphotoxin B
MAF	MAF BZIP transcription factor
MAFB	MAF BZIP transcription factor B
MCL1	Myeloid cell leukaemia 1, BCL2 family apoptosis regulatory
MCM6	Minichromosome maintenance complex component 6
MDM2	Mouse double minute 2
MDM4	Mouse double minute 4
MEK	Mitogen-activated protein kinase kinase
MGM2	2-methyleneglutarate mutase
MGUS	Monoclonal gammopathy of unknown significance
MLPA	Multiplex ligand probe amplification
MM	Multiple myeloma
MMSET	Multiple myeloma SET-domain protein
MP	Melphalan and prednisolone
MR	Minimal response
MRD	Minimal residual disease

mRNA	Messenger RNA
MTF2	Metal response element binding transcription factor 2
MYC	MYC proto-oncogene
NCAPG	Non-SMC condensin I complex subunit G
NDMM	Newly diagnosed multiple myeloma
NF-kB	Nuclear factor kappa light-chain-enhancer of activated B cells
NFKB1	Nuclear factor kappa B subunit 1
NFKB2	Nuclear factor kappa B subunit 2
NHS	National Health Service
NOXA	Latin for damage, member of BCL2 protein family
NRAS	Neuroblastoma rat sarcoma virus oncogene homologue
OS	Overall survival
P	Phosphorylation
PAD	Velcade, adriamycin, dexamethasone
PAX5	Paired box 5
PCL	Plasma cell leukaemia
PCR	Polymerase chain reaction
PD	Progressive disease
PDZK1	PDZ domain containing 1
PET	Positron emission tomography
PFS	Progression-free survival
PI	Proteasome inhibitor
PML	Progressive multifocal leukoencephalopathy nuclear body scaffold
POLK	DNA polymerase kappa
PR	Partial response
PRDK2	PRDK2
PRDM1	Positive regulatory domain I-binding factor
PSMD4	Proteasome 26S subunit, non-adenosine triphosphatase 4
PTPN11	Protein tyrosine phosphatase non-receptor type 11
PUMA	p53 upregulated modulator of apoptosis
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma virus
RAG	Recombinase activating gene
RB1	Retinoblastoma 1
RCBTB2	Regulator of chromosome condensation 1 and BTB domain-containing protein 2
Rd	Revlimid, dexamethasone
R-ISS	Revised international staging system
RNASEH2B	Ribonuclease H2 subunit B
ROC	Receiver operating characteristics
ROC1	Regulatory of cullins
RPL5	Ribosomal protein L5
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SCL16fA1	Basigin and solute carrier family 16 member 1
SD	Stable disease
SEER	Surveillance, epidemiology and end results programme database

SNP	Single nucleotide polymorphism
SPAG5	Sperm-associated antigen 5
TMED5	Transmembrane emp24 protein transport domain-containing 5
TP53	Tumour protein P53
TRAF2	Tumour necrosis factor receptor-associated factor 2
TRAF3	Tumour necrosis factor receptor-associated factor 3
TXNDC5	Thioredoxin domain-containing 5
Ub	Ubiquitin
UBAP2L	Ubiquitin-associated protein 2 like
UBE2Q1	Ubiquitin conjugating enzyme E2 Q1
UPR	Unfolded protein response
V	Variable
VAD	Vincristine, adriamycin, dexamethasone
VAF	Variant allele fraction
VCD	Velcade (bortezomib), cyclophosphamide, dexamethasone
VEGF	Vascular endothelial growth factor
VGPR	Very good partial response
VMP	Velcade, melphalan, prednisolone
VRd	Velcade, Revlimid, dexamethasone
VTd	Velcade, thalidomide, dexamethasone
WES	Whole exome sequencing
WHO	World Health Organization
WWOX	WW domain-containing oxidoreductase
XBP1	X-bx protein 1
β2M	β2-microglobulin

1 Introduction

1.1 Background

Multiple myeloma (MM) is a haematological malignancy that is characterised by malignant transformation of terminally differentiated plasma cells (1). In most cases, the proliferation of these plasma cells leads to the production of a detectable antibody or light chain, which is termed a paraprotein which can be detected in serum. This proliferation leads to pathology and end organ damage in the later stages (2). It is an area of intense ongoing research into its pathophysiology, to develop novel therapeutic strategies and to improve patient outcomes.

1.2 Epidemiology and causation

Myeloma accounts for approximately 10% of haematological malignancies and 2% of all cancers. It has an overall incidence of 60-70 per million of the population (3). Most patients with this disease are elderly, with a median age of 69 years at presentation. Approximately 45% of patients are over the age of 75 at diagnosis. Myeloma has a variable ethnic distribution; it is twice as common among patients with African ancestry as it is among Caucasians. It has a lower incidence in Asians (4). Additionally, it is twice as prevalent in men as it is in women. In the UK, approximately 4500 individuals are diagnosed with MM annually, and incidence is increasing due to the ageing population (5). Patient survival rates have improved from three years to eight years during the last 15 years due to improvements in therapy and supportive care (3, 6). However, despite this, myeloma remains the sixteenth most common cause of death in the UK and is deemed incurable (7).

Risk factors for the development of myeloma are poorly understood. Some potential risk factors include obesity and occupation (8). The observation that families of patients with MM have a two- to four-fold increased risk of monoclonal gammopathy of unknown significance (MGUS) suggests that there is a genetic inherited predisposition to the development of MGUS/MM (9-15). This is also suggested by its ethnic distribution (16).

1.3 Normal plasma cell differentiation

To understand myeloma pathology, it is important to understand normal plasma cell biology. Plasma cells are terminally differentiated B cells that are responsible for the production and secretion of immunoglobulin, which forms a major part of the adaptive immune system and the development of which is summarised in Figure 1.

B cells develop in the bone marrow from pluripotent haematopoietic stem cells. During initial development, from early pro-B to the large pre-B cell state, a pre-B receptor is generated. Under normal circumstances, an immature B lymphocyte differentiates in the bone marrow after rearrangement of heavy and light chain immunoglobulin genes prior to antigen encounter. The production of the B-cell receptor requires the mutation-based linkage of gene segments that are called the variable (V), diversity (D) and joining (J) segments in a sequence of events termed V(D)J recombination. This occurs in the immunoglobulin heavy chain on chromosome 14 first, and then in the light chains on chromosomes 2 and 22. Binding of the constant part of the immunoglobulin determines its isotype, which is initially encoded by the C μ domains of immunoglobulin isotype M (IgM) at the immature B-cell stage, although immunoglobulin isotype D (IgD) is also expressed on the immature B cell (17). Recombination is mediated by the enzymes that are encoded by recombinase activation genes 1 and 2 (*RAG1* and *RAG2*), which catalyse DNA breaks and enable end joining. The process is regulated by transcription factors paired box 5 (PAX5) and early B-cell factor 1 (EBF1) (18-21).

After expression of functional IgM at the cell surface and further maturation, the cell migrates as a naïve B lymphocyte to secondary lymphatic tissue, where stimulation with antigen leads to proliferation and differentiation of the B cells to form plasmablasts. These mature to become short-lived plasma cells, which generally produce IgM. Antigen-stimulated B cells also enter the follicle centres of lymph nodes, where they undergo active somatic hypermutation of heavy chain immunoglobulin (IgH) and light chain immunoglobulin (IgL) gene sequences during iterative cycles of proliferation, somatic hypermutation and apoptosis. This results in selection of a clone that expresses immunoglobulin that has high affinity for the cognate antigen. In addition, class switching takes place. This results in switching of immunoglobulin isotypes from IgM to IgG, IgA and more rarely IgE, which are associated with the

constant region of the antibody. Isotype-switched B cells migrate to the bone marrow and are terminally differentiated to form long-lived plasma cells that secrete antibodies and therefore provide serological memory (22-24).

Both somatic hypermutation and isotype switching are conducted by the enzyme known as activation-induced deaminase (AID). The processes involve double-stranded DNA breaks and DNA repair through non-homologous end joining (25, 26). This process is thought to be responsible for the major translocation abnormalities that are implicated in myeloma pathogenesis. These are detailed later in this chapter (27-29). The production of three characteristic transcription factors, B-lymphocyte induced maturation protein 1 (Blimp-1), interferon regulatory factor 4 (IRF4) and X-box binding protein (XBP1), is increased at the plasma-cell differentiation stage. Concurrently, there is downregulation of transcription factors that were previously required for earlier steps in B-cell development, including PAX5 and B-cell lymphoma 6 (BCL6) (30-32).

In plasma cells that have undergone isotype switching, ongoing V/D/J somatic (S) mutations become more frequent during disease progression in many patients, particularly within the S μ intronic enhancer region, which plays an important role in the control of Ig production. The mechanism by which mutations continue is unclear, but switch-recombinant mechanisms of DNA repair have been implicated (33).

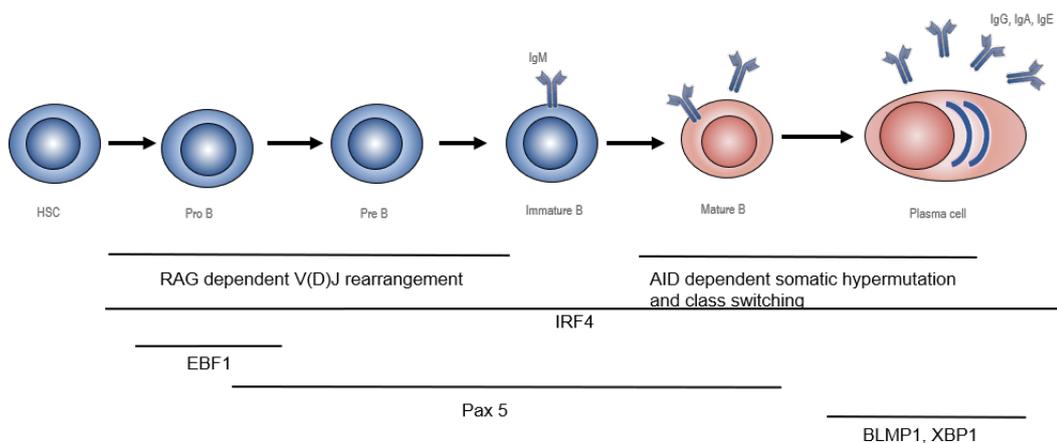


Figure 1: B-cell development in bone marrow from haematopoietic stem cell (HSC) to Pro-B cell, pre-B cell, immature B cell and finally mature B cell maturation in lymphoid organs (25, 26, 30-32)

1.4 Plasma-cell dyscrasia

Myeloma usually evolves from a MGUS asymptomatic phase, which is typically detected due to the presence of a paraprotein or light-chain protein in the blood stream and progresses over time to asymptomatic (smouldering) myeloma. This then progresses to symptomatic myeloma in a substantial number of cases ((34-36). These forms of the disease are described in the section 1.5.

The evolution from MGUS to symptomatic myeloma involves the interplay of genetics, micro-environmental factors and the failure of immunological checkpoints leading to the progression of its precursor state (MGUS). A number of genetic and epigenetic ‘hits’ in tumour suppressor genes and oncogenes that lead to transformation. These constitute an initiating event such as hyperdiploidy or chromosomal translocations that lead to MGUS and are followed by secondary events described in the myeloma biology section of this thesis leading to further progression to symptomatic MM. These are described further in section 1.6.

These genetic events are thought to contribute to cancer initiation and maintenance through deregulation of a small number of oncogenic pathways. They include disruption of the DNA damage repair pathways, the RAS-RAF-MEK-ERK signalling pathway (37), the NF- κ B pathway which has been shown to regulate DNA transcription, cell survival and immune signalling, the PI3K pathway, the JAK/STAT pathway (38).

As a result of changes to plasma cell signalling pathways, the bone marrow micro-environment undergoes several changes. The equilibrium between osteoblasts and osteoclasts is skewed towards bone loss. Osteoclasts release growth factors to promote tumour growth. Other bone marrow niche cells including mesenchymal stem cells, adipocytes and immune cells are also likely to effect MM progression. Changes to immune cells lead to a failure of immunological check points (38). Together, these changes lead to cancer progression.

1.4.1 MGUS and smouldering myeloma

The first pathogenetic step in the development of MM is thought to be the emergence of a limited number of clonal plasma cells that are known clinically as MGUS. Patients with MGUS have no MM-defining events, which are listed in Table 1. It is prevalent in 3-4% of the over-50-years-old population and has a 1% annual risk of progression to MM or related malignant conditions (35). Asymptomatic myeloma is defined as outlined in Table 1. Asymptomatic myeloma is a heterogeneous disease from which a fraction of patients' progress to symptomatic myeloma at different rates but the average rate of progression is 10% per year.

1.4.2 Myeloma, plasma cell leukaemia and extramedullary disease

Progression from the asymptomatic stage is marked by the appearance of symptoms that are caused by end-organ damage, although the recent introduction of criteria such as a high light-chain ratio or a high plasma-cell burden in the bone marrow now enable earlier treatment of this disease (this is summarised in Table 1). While most patients present with disease in the bone marrow, myeloma can also present in the extramedullary regions and/or as plasma cell leukaemia in circulating blood. As myeloma is incurable, inevitable relapse can involve a higher incidence of extramedullary disease and secondary plasma-cell leukaemia.

1.4.3 Clinical presentation and diagnosis

As the disease progresses from MGUS to the symptomatic myeloma stage, clonal expansion of plasma cells suppresses the normal function of the plasma-cell population and leads to immunosuppression and recurrent infections, impaired haematopoiesis, lytic bone lesions through complex cell-cell interactions and impaired renal function through a variety of mechanisms. Most patients present at this symptomatic stage and because few patients are seen at an earlier stage and screening for paraproteins is not routine around the world. (4)

Plasma cells in myeloma typically excrete an IgG monoclonal antibody (called a paraprotein) in 50% of cases. The IgA subtype accounts for 30% of patients and 15% of patients who present with myeloma carry the light chain of the antibody only. In rare cases, patients have an IgD (<1%) or IgM (0.2%) subtype of MM. Even rarer are

the IgE or non-secretory MM subtypes. The presence of antibodies as well as light-chain secretion itself can cause symptoms in addition to neoplastic plasma-cell growth.

In symptomatic myeloma, patients present with the 'CRAB' criteria, namely hypercalcaemia, renal impairment, anaemia and bone disease. Other presenting symptoms include fatigue, recurrent bacterial infections and spinal-cord compression (4). The criteria for diagnosis of symptomatic myeloma were extended in 2014 to include uninvolved serum free light-chain ratio ≥ 100 (involved free light-chain level must be $>100\text{mg/l}$), bone-marrow infiltration $\geq 60\%$ and ≥ 1 focal lesion that is detectable on MRI. The extension was made due to the recognition that a substantial proportion of these patients progress within two years to the symptomatic stage (2).

The change in criteria for diagnosis of symptomatic myeloma has relevance as it effectively changed the timepoint at which treatment for patients with myeloma could begin. Whereas previously, patients were only treated when they showed existing organ damage, the new criteria allow treatment at an earlier stage. The redefinition of the disease and indications for treatment are also likely to affect interpretation of the trials that have been studied as part of the work that has been performed for this thesis (Myeloma IX and Myeloma XI trials are described in more detail later in the methods section) but may also change responses to therapy as well as incidence of genetic abnormalities that may be found among newly diagnosed patients.

Additionally, it has implications for the epidemiology of myeloma, in which some of the improvements that are seen in survival rates after diagnosis or treatment may be related to the earlier classification of the disease as symptomatic. Although the change makes retrospective comparisons more challenging, it has undoubtedly reduced morbidity that has previously been caused by patients having to wait for end-organ damage before they could start treatment for myeloma.

Table 1: Defining events in the development of multiple myeloma. (Adapted from revised International Myeloma Working Group definition of MGUS and symptomatic MM (2)). MRI=magnetic resonance imaging, CT=computer tomography, PET=positron emission tomography

Type	Definition
Non-IgM monoclonal gammopathy	Non IgM monoclonal paraprotein <30g/l Clonal bone marrow plasma cells <10% Absence of myeloma-defining events or amyloidosis attributable to plasma-cell proliferation
IgM monoclonal gammopathy	IgM monoclonal paraprotein <30g/l Clonal bone-marrow plasma cells <10% Absence of myeloma-defining events or amyloidosis attributable to plasma-cell proliferation No evidence of hyperviscosity, constitutional symptoms, lymphadenopathy, hepatosplenomegaly or other end-organ damage that can be attributed to an underlying lymphoproliferative disorder Abnormal free light-chain ratio
Light-chain monoclonal gammopathy	Abnormal free light-chain ratio (<0.26 or >1.65) No immunoglobulin heavy-chain expression on immunofixation Clonal bone-marrow plasma cells <10% Absence of myeloma-defining events or amyloidosis attributable to plasma cell proliferation Urinary monoclonal protein <500mg/24 hours
Asymptomatic myeloma	Serum M protein (IgG or IgA) \geq 30g/l or urinary M protein \geq 500mg/24 hours and/or clonal bone-marrow plasma cells between 10% and 60% Absence of myeloma-defining events or amyloidosis
Symptomatic myeloma	Clonal bone-marrow plasma cells >10% or biopsy-proven extramedullary plasmacytomas and one of the following myeloma-defining events Evidence of end-organ damage attributable to the underlying plasma-cell proliferative disorder <ul style="list-style-type: none"> - Hypercalcaemia >0.25 mmol/l above upper limit of normal or >2.75mmol/l. - Renal insufficiency: creatinine clearance <40ml/min or serum creatinine >177 μmol/l - Anaemia: haemoglobin >20g/l below lower limit of normal or <100g/l. - Bone lesions: \geq1 osteolytic lesion on radiograph/CT/PET-CT Additional features – one or more of the following <ul style="list-style-type: none"> - Clonal plasma cells >60% - Involved/uninvolved serum free light-chain ratio \geq100 - >1 focal MRI lesion

1.5 Myeloma biology

The genetic events that lead to defective function of plasma cells are detectable at the MGUS stage. These defects remain throughout the disease course and are known as initiating events. Secondary genetic events are thought to lead to disease progression as described above. These secondary ‘hits’ result in enhanced survival of subclones and enable clonal evolution of the disease and resistance to treatment. Primary and secondary genetic events are summarised in the figure below and are detailed in this section (39, 40).

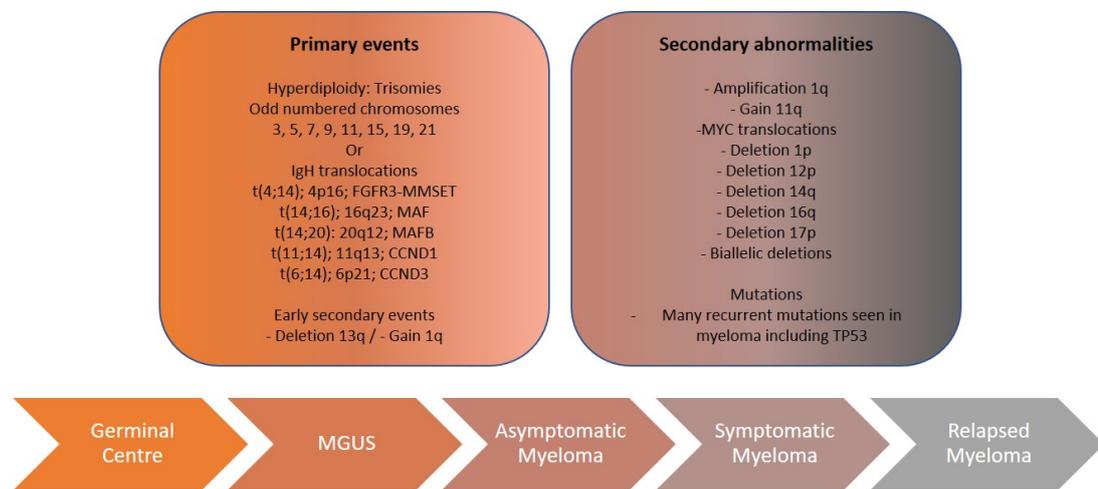


Figure 2: Primary and secondary genetic abnormalities in MM (41)

1.5.1 Primary ‘initiating’ genetic events

Primary genetic events that lead to the development of MGUS and smouldering myeloma either involve hyperdiploidy (HRD), which is defined as the production of multiple copies of odd-numbered chromosomes (particularly chromosomes 3, 5, 7, 9, 11, 15, 19, and 21), or involve translocations of the IgH locus at chromosome 14 (14q32.33). The translocations are seen in 35-50% of cases and are also called non-hyperdiploid (HRD) sub-types. These two categories of subtype appear to be mostly mutually exclusive (42).

Hyperdiploid subtypes

Hyperdiploid MM is characterised by a gain of chromosomes 3, 5, 7, 9, 11, 15, 19 and/or 21, which leads to the production of genomes that consist of between 48 and

74 chromosomes (43). The pathogenesis of this increase in the number of chromosomes is not completely defined but is theorised to happen during failed mitosis. Hyperdiploidy has been known to co-occur with IgH translocation in around 10% of cases (40).

In terms of signalling pathways, HRD tumours display heterogeneity. In addition to the above changes in copy numbers, specific recurrent mutations that are associated with this subtype including rapidly accelerated fibrosarcoma/mitogen-activated protein kinase (RAF/MEK) pathways. This suggests that cancer progression depends on use of this pathway. Similarly, progression of MGUS/asymptomatic tumours to HRD tumours has been associated with translocations of the MYC proto-oncogene (*MYC*), which suggests that they may drive progression in these tumours. Other pathways that have been found to be overexpressed in HRD tumours include those that are associated with proliferation: nuclear factor kappa light-chain-enhancer of activated B cells (NF- κ B), hepatocyte growth factor (HGF) and interleukin 6 (IL-6) (44).

Overall, hyperdiploid MM is associated with a standard or neutral survival. Some studies demonstrate that trisomies 3 and 5 are associated with a good prognosis but that trisomy 21 is associated with a poorer outcome (45). Additionally, there is debate about whether hyperdiploidy can abrogate the poor prognosis that is associated with certain secondary cytogenetic abnormalities; some studies suggest that the prognosis is abrogated, while others have demonstrated that secondary changes in copy numbers that are of poor risk levels still carry an adverse prognosis in this subgroup of patients but they are present at lower frequencies compared with the figures for adverse-risk IgH translocations in that subgroup (46).

Initiation of IgH translocations

More than 90% of primary translocations, which occur in the non-HRD subtype of MM, involve the immunoglobulin heavy chain (IgH) gene locus at chromosome 14q32.33. They are the result of defective class-switch recombination or somatic hypermutation. Both processes require AID to generate double-stranded DNA breaks in the loci that encode for Ig. This process results in creation of a fusion product in

which the fusion gene partner is under the control of the IgH enhancer and oncogene expression is enhanced (43, 47). These translocation events are thought to lead to dysregulation of cyclin D expression (48). Although the translocations are important in pathogenesis of myeloma, they are insufficient in isolation to cause pathology. Secondary genomic aberrations are therefore needed for the disease to progress from the MGUS stage. The primary IgH translocations remain important throughout the progress of the disease and provide prognostic information. They are described in more detail below.

The five IgH partner chromosomes and genes that have been most commonly identified in patients with MM are: 11q (the cyclin D1 gene (*CCND1*)), 4p16.3 (the fibroblast growth factor receptor 3 (*FGFR3*) gene and the multiple myeloma SET-domain protein (*MMSET*) gene) (15%) (49-51); 6p21 (the cyclin D3 gene (*CCND3*)) (1%) (52), 4p16 (*MMSET/FGFR3*), 16q23 (the MAF-BZIP transcription factor gene (*MAF*)) and 20q12 (the MAF-BZIP transcription factor B gene (*MAFB*)). Their frequency, fusion gene partners, and prognostic associations are listed in Table 2.

Table 2: Primary IgH translocations in myeloma. CCND1=cyclin D1, CCND3=cyclin D3

Primary translocations	Fusion genes	Prognostic association	Frequency
t(11;14)	<i>CCND1</i>	Neutral, possibly poorer when associated with <i>CCND1</i> mutations	15-20%
t(4;14)	<i>MMSET</i>	Poor risk	~15%
t(14;16)	<i>MAF</i>	Poor risk	5%
t(14;20)	<i>MAFB</i>	Poor risk	1%
t(6;14)	<i>CCND3</i>	Neutral	1-2%

1.5.1.1.1 Translocation t(11;14)

Translocation t(11;14) is the most frequent primary translocation with prevalence of 15-20%. The translocation results in fusion of cyclin D1 (*CCND1*) and IgH and leads to upregulation of *CCND1* expression (49-51). The prognostic relevance of t(11;14) is considered neutral, although some studies suggest that the co-occurrence of this

translocation with an activating mutation in *CCND1* in 10% of patients is associated with poor prognosis (53). In addition, it has been reported that a large proportion of plasma cell leukaemias have t(11;14), which suggests heterogeneity in disease outcomes in some patients (54).

1.5.1.1.2 Translocation t(4;14)

The t(4;14) chromosome rearrangement is observed in 15% of patients and has been associated with adverse prognosis. The translocation leads to deregulation of *MMSET* and *FGFR3* with the breakpoint between *MMSET* and *FGFR3*, and the result is overexpression of *MMSET* in all patients and *FGFR3* in 70% of patients.

MMSET is a methyltransferase, the action of which leads to increased methylation of histone H3K36, which modulates expression of several genes. *MMSET* also regulates methylation of histone H4K20 and subsequently affects recruitment of p53 binding protein 1 (53BP1) at the site of DNA damage. *FGFR3* is deleted in approximately 30% of cases (55) and is mutated in 17% of patients who carry t(4;14). Mutations are likely to be a result of somatic hypermutation on der (14) and have an uncertain clinical significance (53). The high proportion of cases that involve *FGFR3* deletion suggests that this gene may not be critical to the pathogenesis of this subtype.

The translocation t(4;14) has been associated with a poor prognosis in myeloma (56-59). However, trials that have studied therapies that contained bortezomib have been shown to abrogate that risk (60, 61) although this has not been a consistent finding in all studies (62).

1.5.1.1.3 Translocation (14;16)

The t(14;16) subtype of myeloma accounts for around 5% of patients with MM. It results in overexpression of *MAF* oncogenic splice variant c-*MAF* (63), a transcription factor that upregulates expression of a number of genes including *CCND2*, chemokine receptor 1 (CCR1) and integrin B7 by binding directly to promoter regions (63, 64). Additionally, the translocation breakpoints are located downstream of *MAF*, in the last exon of the tumour suppressor WW-domain-containing oxidoreductase (*WWOX*) gene. Mutations of *MAF* have been found in 13% patients with MM that harboured t(14;16), which may again be associated with translocation events. Studies suggest

that *MAF* upregulates the DNA editing enzymes that are known as alipoprotein B mRNA editing enzymes, catalytic polypeptide like (APOBEC3A and 3B). This upregulation is associated with a predominantly APOBEC mutational signature in this MM subgroup and which is characterised by high mutation rate (65). Studies suggest that the t(14;16) translocation is associated with poor prognosis (66, 67), although its low frequency of occurrence has resulted in a failure to discover a prognostic impact of this translocation in other meta-analyses (68). Although it is included in the revised international staging system (R-ISS) for MM, it is frequently not included in the high-risk criteria reported in several commercial clinical trials.

1.5.1.1.4 Translocation (14;20)

The t(14;20) translocation is detected in 1% of myeloma patients and is thought to behave similarly to t(14;16) due to its similarities in gene upregulation (56). The translocation results in direct upregulation of *MAF* gene paralogue *MAFB*. *MAFB* has been found to be mutated in 25% patients with t(14;20) and, as is the case with t(14;16), the t(14;20) translocated tumours have an APOBEC mutation signature that is thought to be caused by APOBEC4 expression (65). Some studies suggest that this translocation is associated with poor prognosis (67). However, due to its rarity, many studies have not reached statistical significance in terms of its association with prognosis and so it is not included in the R-ISS or the more recent International Myeloma Working Group (IMWG) staging system for myeloma (69, 70).

1.5.1.1.5 Translocation (6;14)

The t(6;14) translocation is also rare and occurs in 1-2% patients with myeloma. It results in an IgH-*CCND3* fusion, which results in the overexpression of *CCND3*. The overall prognostic association of this translocation is thought to be neutral although it has not been studied in detail due to its rarity.

1.5.2 Secondary genetic events

The accumulation of secondary genetic events, including additional translocations and copy number alterations, somatic mutations and epigenetic modifications, is responsible for progression from MGUS to myeloma as well as further disease relapse and progression (71-73).

1.5.2.1. Secondary translocations

Secondary translocations commonly involve the *MYC* locus (8q24) and are seen in around 3-4% of cases in the MGUS or asymptomatic myeloma stages, in around 15-20% of patients at the symptomatic stage and in a higher proportion of patients at relapse (74). *MYC* translocations are largely found in the HRD subgroup (64%) as well as in a proportion of patients with no detectable hyperdiploidy or classic primary translocation (65, 75). The most common partners for *MYC* translocations are: IgH (16.5%), IgL (16.5%), IgK (6%), the family with associated similarity 46 member C gene (*FAM46C*) (9.5%), the forkhead box 03 gene (*FOXO3*) (6%) and the bone morphogenic protein 6 gene (*BMP6*) (3.5%). Other translocation partners include the genes for retinoblastoma protein 1 (*RBI*), X-box protein 1 (*XBPI*), thioredoxine domain containing 5 (*TXNDC5*), *CCND3* and *CCND1* (65, 75). These fusions lead to overexpression of the *MYC* gene due to its position next to super-enhancers on the translocation site. There are numerous other translocations in myeloma that occur at low frequencies (65).

1.5.2.2 Secondary copy number aberrations

There are many secondary copy number aberrations that are found in myeloma. These aberrations are of different sizes that vary from focal to chromosomal arm-size deletions, gains or amplifications (76). Secondary copy number gains and losses occur frequently in myeloma and are mostly subclonal events (77). The most frequent deletions of chromosomal regions include del(13q), del(14q), del(8p), del(6q), del(12p), del(1p) and del(17p). Secondary gains and amplifications also occur in myeloma. The most common of these include gain(1q), gain(8q24) and gain(11q) (76, 78, 79).

Although the aberrations described above have been classified as primary events by most researchers, it should be noted that gain(1q) and del(13q) are frequently observed at the MGUS stage and may be involved in the early oncogenesis of the disease (80). The next section describes specific secondary events that are associated with prognosis in myeloma.

Table 3: Frequent secondary chromosomal losses and gains with associated genes and frequencies. Adapted from (40, 43)

Chromosomal deletion	Possible associated tumour suppressor gene	Frequency in myeloma (%)
13	<i>RB1, EBPL, RNASEH2B, RCBTB2, DIS3, BRCA2</i>	45-50
11q	<i>BIRC2 and BIRC3, ATM</i>	7
8p	Unknown	25
1p	<i>CDKN2C, FAF1, FAM46C</i>	30
14q	<i>TRAF3</i>	30
16q	<i>CYLD and WWOX</i>	30
17p	<i>TP53</i>	10
Chromosomal gain		
1q	<i>CKSB1, ANP32E, BCL9, PDZK1, MCL1</i>	35-40
8q	<i>MYC</i>	15
11q	<i>CCND1</i>	15

1.5.2.1.1 Gain of chromosome 1q

Gain(1q) (including amplification) is one of the most common cytogenetic abnormalities in MM and has been identified in 30-50% of newly diagnosed multiple myeloma (NDMM) (81-86). The gain of 1q is thought to be an early event in MM genesis; it is detectable at the MGUS stage (72, 87) incidence increases at the asymptomatic stage (88) A proportion of patients who have no detectable HRD karyotype or IgH karyotype have gain(1q) as their predominant genetic lesion. Amplification of 1q only (>3 copies) has been reported in approximately 5-18% of newly diagnosed patients (86, 88, 89). A frequently minimally amplified region that has been identified between 1q21-q23.3 has been found to contain 679 genes (76).

An increase in gain(1q) cases has been detected in up to 70% of relapsed cases in both matched and unmatched cases (90, 91), which suggests that it has a role in myeloma progression. Additionally, frequency of amplification (amp) of 1q has been reported to increase with disease progression from 18% to 44% in one study (88), which again suggests that this lesion may be an ongoing driver of disease progression (86).

There is conflicting data with regards to the role of gain(1q) as an independent prognostic factor (67, 92-96). Some studies have demonstrated an association between gain(1q) and shortened survival (84, 85, 88, 97-99). However, other studies have provided evidence that gain(1q) does not have a negative prognostic impact (84, 86). Additional controversy exists with regards to the number of additional chromosome 1q copies are needed for the association with prognosis with some studies suggesting that only amplification 1q has an association with survival (100).

I discuss this lesion in further detail within the Gain (1q) results chapter, in which I report on this lesion in the context of the Myeloma IX and the Myeloma XI trial. Through the results presented in this chapter, I aim to add to the evidence to help to resolve these debates.

1.5.2.1.2 Deletion of chromosome 13q

Deletion of chromosome 13 is the most common recurrent structural abnormality in myeloma and is present in 45-59% patients with newly diagnosed symptomatic myeloma (76, 79). Although del(13q) is associated with disease progression, it is also a feature of MGUS, which suggests that it plays a role in early oncogenesis (80, 101). SNP array-mapping studies have found that more than 85% of these deletions involve the whole long arm of chromosome 13 (76, 101-103).

Several genes are implicated as drivers of the deletion of 13q including *RBI*, the emopamil binding-related protein-like gene (*EBPL*), ribonuclease H2 subunit B (*RNASEH2B*), regulator of chromosome condensation 1 (*RCC1*) and BTB domain-containing protein 2 (*RCBTB2*), and microRNAs mir16-1 and mir-15a (76). It is most frequently associated with non-hyperdiploid tumours. It may play a role in providing t(4;14) tumours with a survival advantage, given that these two lesions co-segregate in >80% of t(4;14) cases (104).

Although initial studies suggested that there was an association of del(13) with prognosis (105, 106), subsequent studies that performed multivariable analyses with co-segregated lesions such as t(4;14) and del(17p) found that del(13) was not independently associated with poor prognosis (107). An extensive study into the role of del(13q) found that, when this lesion was analysed by metaphase cytogenetics, it

was found to have a prognostic role, but it did not have independent prognostic significance when it was detected by interphase fluorescent in-situ hybridisation (iFISH). This result suggested that proliferation played a role in the assessment of survival when the results were assessed by standard cytogenetic results (108). It is of note, however, that bi-allelic inactivation of *RBI* has been associated with poor prognosis in MM that is independent of other known genetic risk markers (109).

1.5.2.1.3 Deletion of 1p

Deletions of chromosome 1p (del(1p)) are frequent events in MM patients (18-38%) (37, 110-112). Unlike gain 1q in which the whole chromosome arm is frequently involved, several regions on 1p have been identified that are minimally altered by deletion, including 1p32.3, 1p31.3, 1p22.1-1p22.3 and 1p12 (37, 76). Only 26% of del(1p) was found to involve deletion of all these regions (37).

Del(1p) is thought to be a secondary event as it is rarely found at the MGUS stage and increases in incidence through disease progression (113). Deletion of both cyclin-dependent kinase inhibitor 2C (*CDKN2C*) and *FAM46C* has been shown to increase at relapse in matched sequential samples (91, 114), which suggests a potential involvement of del(1p) in disease progression or treatment resistance. The frequency of del(1p) has been shown to increase, with rates of 31% at relapse and 62% in secondary plasma-cell leukaemia (PCL) (113). Bi-allelic inactivation of *CDKN2C* was also found to increase at relapse as it showed a frequency of 6.5% compared with 4.6% in NDMM patients (109).

Deletion 1p has been associated with higher levels of beta 2 microglobulin (B2M) and consequently a higher grade on the ISS (110). It has also been associated with a higher lactate dehydrogenase (LDH) and plasma-cell percentage in the bone marrow (113). Deletion 1p22 and del(1p32) are associated with t(4;14), del(17p) and del(13q) at diagnosis. Additionally, there is a strong association between both del(1p22) and del(1p32) with gain(1q) (37, 110, 113, 115). There is some evidence that del(1p12) may be mutually exclusive with del(17p) (116), but other studies have not borne this out (37). Bi-allelic loss of *CDKN2C* has been shown to be associated with a high risk GEP70 signature and pathogenesis-related classification by gene expression, both of which denote a worsened prognosis.

Some studies suggest that there is a negative association of del(1p) with survival at diagnosis while others have found no significant association (82, 110, 117-119). This is also the case in relapse (91, 120). Most studies consistently report an overall survival difference but less commonly a progression-free survival difference (121).

Several areas of debate remain regarding deletion 1p. Firstly, the specific region or regions that are associated with survival within the region of chromosome 1p are debated. Most researchers who have performed studies agree that the region of 1p32 is associated with survival (110, 113, 118, 119). However, it is not yet clear whether the region of 1p21-22 is prognostic; some studies have found an association with shortened survival (110, 113, 115) while others have found no association on multivariate survival analysis (118, 119). The association between deletion 1p12 and survival is also uncertain. Some studies suggest that there is a negative association with survival (91, 113) while others do not (53, 118, 122). One study has suggested that increasing numbers of areas of deletion with del(1p) confer a worse prognosis (113). Most of the studies to date that have looked at the region of 1p have not included all relevant areas of 1p deletion and all high-risk genetics into the same multivariate analysis, so interpretation of these results is difficult due to co-association of different 1p deletions in particular as well as associations between 1q gain and 1p deletion.

Controversy also exists as to whether del(1p) is associated with shortened survival in all patient subgroups. Most studies of this group have been carried out on intensively treated patients, in whom a prognostic impact has been detected (110, 111, 115). However, some studies suggest that patients who are treated less intensively do not show a negative association between survival and del(1p) (118). Additionally, few studies have evaluated the effect of deletion 1p in the context of different therapy in MM besides defining treatment differences between intensively treated patients. One study has suggested that deletion 1p22 confers sensitivity to bortezomib treatment (123), but in this study, the results were achieved through gene expression of a surrogate variable.

1.5.2.1.4 Deletion of 17p

The short arm of chromosome 17 is frequently deleted in myeloma (45). Deletion of chromosome 17p is thought to be one of the most important genetic factors that are associated with poor outcome in myeloma (124). SNP array-based methods have revealed a minimally deleted and a bi-allelically deleted region at 17p13.1. This region is associated with poor prognosis in myeloma (53, 76, 125-127). It harbours the tumour suppressor gene *TP53*, which has been found to be altered in more than half of all human cancers (128). *TP53* is also frequently found to be mutated by the time of diagnosis in myeloma. This finding provides further evidence that this is a key gene of interest at this site.

Deletion 17p is not often found at the MGUS stage. The incidence of deletion rises between the asymptomatic and symptomatic myeloma stages to reach an incidence rate of 10% at diagnosis, which increases further by the time of relapse. Additionally, bi-allelic deletions and mutations occur at increased rates at *TP53* relapse (90, 91, 109, 114, 129-133). Incidence rates for this alteration of up to 50-75% have been reported at the plasma-cell leukaemia stage. Increasing prevalence of the deletion through disease progression suggests del(17p) has a role in the progression of the disease.

Several studies have demonstrated the poor prognostic impact of del(17p) (55, 67, 98, 107, 124, 125, 134-141). For example, patients with del(17p13) demonstrate a reduced response to IMiDs such as thalidomide (125) and lenalidomide (141, 142) compared with patients who do not show this deletion. It also retains its poor prognostic association despite autologous transplantation (124, 143-145). There is some debate about whether treatment with bortezomib may be beneficial in the context of del(17p). Some studies show improvement in survival rates with bortezomib therapy (146), while others do not (62). However, even in cases of multi-modal, intensive treatment regimens such as total therapy 3 to total therapy 5 (TT3-TT5), which involve a combination of proteasome inhibitors, immune modulators and autologous stem-cell transplantation, del(17p) retains its poor prognostic association (136). Therefore, based on this large amount of evidence that 17p deletion is associated with poor prognosis, this deletion has been included in the R-ISS prognostic score for MM (69).

Despite the strong evidence of its poor prognostic association, clinical trials do not report the risk that is associated with this lesion in isolation and often combine it with studies of t(4;14). Therefore, evaluation of this single high-risk lesion is difficult in the context of recent therapies (147). As a result of this scarcity of evidence, adaptation of therapy to MM according to cytogenetics has therefore not been widely adopted into standard clinical practice (148, 149).

In addition, there are several other areas of debate with regards to this lesion in MM. I discuss this lesion in further detail within the *TP53* results chapter, in which I report on this lesion in the context of the Myeloma XI trial. Through the results presented in that chapter, I aim to add to the evidence to help to resolve these debates.

1.5.3 Single nucleotide variants and indels

In addition to the structural chromosomal changes that have been described, the progression to myeloma and plasma-cell leukaemia is associated with the accumulation of single nucleotide variants and of insertions or deletions in the genome (indels) in tumour suppressor genes, oncogenes and other genes that are involved in cell survival (53, 100, 127, 131). Results of large studies on the whole exome sequencing, whole genome sequencing and mutational panels have revealed that there is a spectrum of mutational frequency in MM (37, 53, 65, 116, 127, 131, 150, 151). The most frequent somatic mutations that were reported in these studies are summarised in Table 4. A heterogeneous mutational landscape was observed in all the studies. In addition to individual mutations within genes, the number of mutations that were found per patient appeared to have prognostic association. The numbers of mutations were found to be greater in subtypes of myeloma that showed distinct mutational signatures such as the APOBEC signature (131). The clinical significance of most recurrent mutations are yet to be robustly established, with the exception of *TP53* mutations, which has been shown to be consistently associated with poor prognosis in MM.

The genes in myeloma that are the most frequently mutated are those that occur within the pathway that consists of a series of kinases called the rat sarcoma virus oncogene homologue - rapidly accelerated fibrosarcoma - mitogen-activated protein kinase

kinase - extracellular signal-regulated kinase cascade, which is known as the RAS-RAF-MEK-ERK pathway. These include the Kirsten rat sarcoma virus proto-oncogene homologue (*KRAS*) and neuroblastoma rat sarcoma virus oncogene homologue (*NRAS*), which are mutated in around 40% of myelomas; *BRAF*, one of a family of RAF genes, is mutated in approximately 8% of patients. These genes show mutual exclusivity. Overall, about 50% of MM patients have mutations in the genes of this pathway. *NRAS* mutations are found at a higher frequency in hyperdiploid tumours than in non-HRD tumours (37).

About 14% of recurrent mutations occur within genes that act in the NF- κ B pathway, such as tumour necrosis factor receptor-associated factor 3 (*TRAF3*), lymphotoxin beta (*LTB*), cylindromatosis (*CYLD*), nuclear factor kappa B subunit 1 (*NFKB1*) and subunit 2 (*NFKB2*). The NF- κ B pathway has been shown to regulate DNA transcription, cell survival and immune signalling. Genes within the cell cycle transition pathways, which include *CCND1*, *RBI*, *CDKN2C* and *CDKN1B* were mutated in around 5% of cases. The exosome exo- and endoribonuclease gene (*DIS3*) is mutated in approximately 10% of NDMM patients. It is involved in the human exome complex and in the quality check of RNA and its degradation. *FAM46C* is also mutated in about 10% of myeloma cases.

Table 4: Most common recurrent somatic mutations in multiple myeloma (53, 100, 127, 131)

Gene Mutation	Chromosome	Frequency (%)	Association with other genetic abnormalities
<i>KRAS</i>	12p	20	
<i>NRAS</i>	1p13.2	20	Hyperdiploidy, t(11;14)
<i>FAM46C</i>	1p12	10	Hyperdiploidy
<i>DIS3</i>	13q	10	Del(13q)
<i>BRAF</i>	7q34	8	
<i>HUWE1</i>	Xp11.22	6	t(11;14)
<i>TP53</i>	17p	3-8%	Del(17p)
<i>TRAF3</i>	14q32.32	6	Del(14q)
<i>FGFR3</i>	4p16.3	4	t(4;14)
<i>HISTH1E</i>	6p22.2	4	
<i>PTPN11</i>	12q24.13	3	Hyperdiploidy
<i>PRDK2</i>		4	t(4;14)
<i>CYLD</i>	16q	3	Del(16q)
<i>PRDM1</i>	6q21	2-3%	
<i>RB1</i>	13q	2	
<i>IRF4</i>	6p25.3	2	t(11;14)

1.5.4 Gene expression profiling in myeloma

The result of all the genetic changes that are described above effect messenger RNA (mRNA), which is subsequently translated into proteins. Study of gene expression therefore enables the consideration of other unknown genetic factors as well as those that affect transcriptional regulation. In order to study this, gene expression arrays have been developed and used as a research tool in myeloma.

Initially, supervised and unsupervised cluster-based methods were used to identify subsets of myeloma in several centres across the world. Zhan et al. (2006) initially

performed this analysis, which resulted in the characterisation of seven gene clusters by distinct gene expression profiling (GEP) (56). These clusters roughly correlated with, but did not completely overlap with, the primary genetic events in MM that have been described above. Overlapping subgroups included one that was named the MS (largely a t(4;14)) subgroup, which was characterised by overexpression of *MMSET* and *FGFR3* in 75% of samples; and another that was dubbed the MF group (largely made up of t(14;16) and t(14;20) subgroups), which was characterised by overexpression of *MAF* or *MAFB*, *CCND2*, the C-X3-C motif chemokine receptor 1 gene (*CX3CR1*) and integrin subunit beta 7 (*ITGB7*) with reduced expression of Dickkopf WNT signalling pathway inhibitor 1 (*DKK1*). Further, the CD1 and CD2 clusters were characterised by overexpression of *CCND1* and *CCND3* (largely t(11;14) and t(6;14) subgroups). The HY cluster was overrepresented by the HRD karyotype. Patients who exhibit multiple primary cytogenetic abnormalities were classified into additional clusters that included the proliferation (PR) cluster, which was characterised by increased numbers of cell cycle, proliferation, and cancer testis genes. The low bone (LB) cluster was largely made up of HRD cases that were found to have a lower incidence of bony lesions.

Subsequent to the above analysis, Broyl et al. (152) repeated unsupervised clustering analysis and found three further clusters. These included the NF-κB cluster, in which patients showed increased expression of NF-κB pathway genes. The myeloid cluster was characterised by increased expression of myeloid gene. The third additional cluster was named the PRL3 cluster due to the overexpression of this gene. In both studies, the so called proliferation (PR), MS (overrepresented by t(4;14)) and MF (overrepresented by *MAF* translocations) subgroups had shorter overall survival rates (48, 56, 152).

Subsequent to the unsupervised classification studies described above, several gene expression prognostic and predictive signatures have been developed for risk prediction in MM. These are explored in more detail in the chapter that explores previously developed gene expression signatures within the results part of this thesis.

1.6 Current treatment options for myeloma

There is currently insufficient evidence to suggest that treatment of MGUS or asymptomatic MM is beneficial. Further studies are underway to determine whether treatment of smouldering multiple myeloma (SMM) is beneficial (153). Patient survival rates have quadrupled in the past 40 years in the UK due to significant advances in treatment and supportive care. An approach to the treatment of newly diagnosed symptomatic MM is illustrated in Figure 3. Treatment regimens differ worldwide, depending on availability and affordability of anti-myeloma drugs. It is generally accepted that combination therapy is synergistic in MM. This section describes the sequencing of therapies and gives details of individual therapies that are available for myeloma. Each section describes the mechanisms of action of the therapies and discusses them in relation to genetic risk in myeloma, as this was the focus of the work that was performed for this thesis.

1.6.1 Induction

In the UK, the standard induction therapy involves the use of a combination therapy. The potential combinations used comprise an immunomodulatory agent (IMiD), steroids and either conventional chemotherapy or a proteasome inhibitor (PI) and steroid therapy. There is now a large amount of evidence that triplet-based therapies that include a PI and an IMiD are superior to doublet therapy in both upfront (154-160) and relapsed (161-163) patients. Triplet therapy is now recommended by the IMWG in the cytogenetic high-risk setting (147). In addition, there is growing evidence of the efficacy of quadruplet therapy, which also combines daratumumab to IMiD, proteasome inhibitor and steroid therapy. Older, less fit patients, who are unlikely to tolerate intensive regimes due to side effects, are usually offered either an IMiD-based or PI-based therapy with steroids and conventional chemotherapy such as cyclophosphamide or melphalan.

1.6.2 Consolidation

Consolidation therapy is designed to remove residual disease. In the UK this normally involves therapy with high-dose melphalan alongside autologous stem-cell transplantation (HDM-ASCT) in patients who are fit enough to tolerate this procedure.

HDM-ASCT treatment demonstrated improved overall survival rates (164-168) and has been established as standard up-front consolidation therapy for those who are fit enough to tolerate this (4). Even in the era of triplet induction therapy with carfilzomib, lenalidomide (Tradename: Revlimid among other brands) and dexamethasone (a combination known as KRd), there is evidence that transplantation leads to a higher proportion of patients in minimal residual disease (MRD) negativity compared with KRd consolidation alone in the cytogenetic high-risk group (169). There is some evidence that tandem ASCT is beneficial in the high cytogenetic risk setting (170) although this is yet to be robustly established.

1.6.3 Maintenance

Maintenance therapy involves the use of ongoing treatment after an autologous stem-cell transplant or induction therapy. Maintenance lenalidomide has recently been approved as for transplant eligible patients in the UK. Across the world, several maintenance regimens are offered that include lenalidomide maintenance, PI maintenance or a combination of the two.

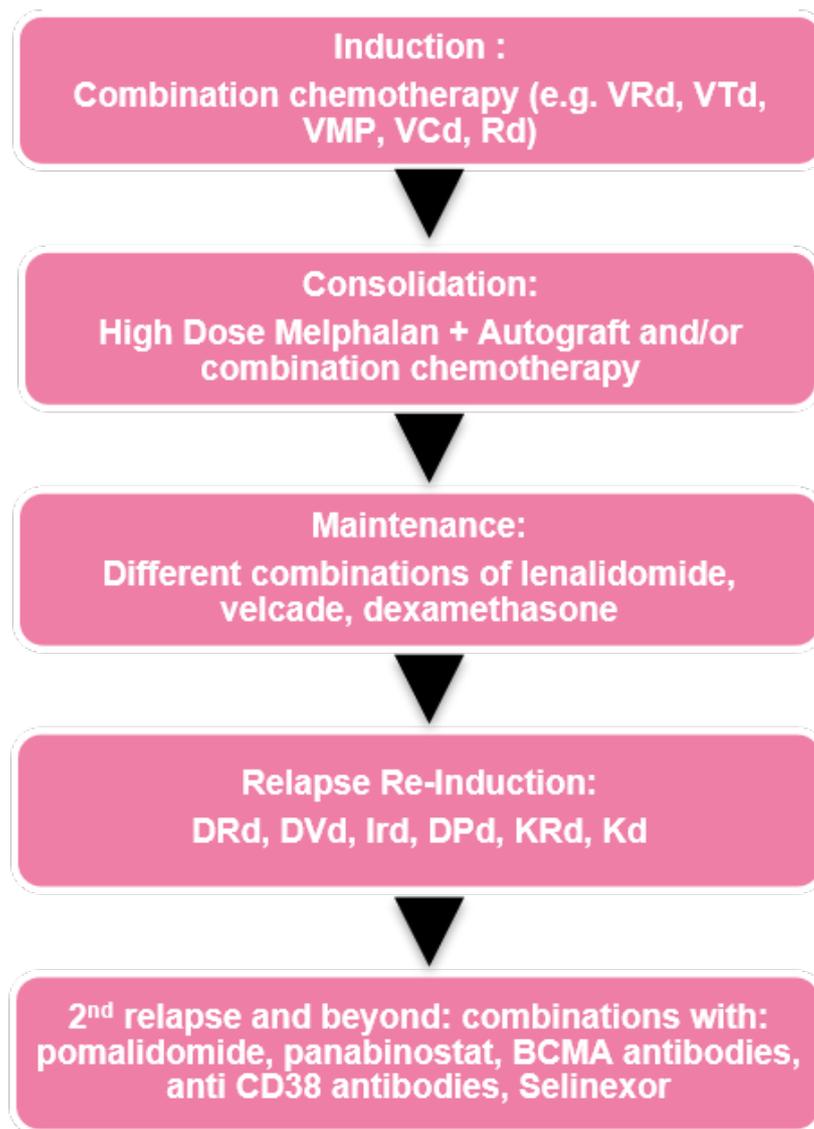


Figure 3: Current therapeutic strategy for the treatment of myeloma

Treatments vary widely across centres and countries. Diagram provides rough guide to therapies. V= bortezomib (brand name Velcade among others), R= lenalidomide (brand name Revlimid among others), d=dexamethasone, D = daratumumab, K=carfilzomib

1.6.4 Conventional chemotherapy

Prior to the use of alkylating agents, the overall survival period from diagnosis of myeloma was less than one year (171). The initial breakthrough came in 1969 with the introduction of melphalan for the treatment of myeloma (172). Further evidence for the use of steroids was gathered in the form of improved survival rates when steroids and melphalan were given in combination (173, 174). The combination of melphalan and prednisolone was used for several decades with no further improvement in survival rates until novel agents were developed (175).

Agents such as melphalan and cyclophosphamide are still used routinely in combination with more novel agents during induction, but they are gradually being phased out as they add toxicity to highly effective alternative agents in myeloma. Steroids continue to be used routinely with immunomodulatory agents as they act synergistically.

As melphalan demonstrated significant anti-MM activity, groups started to use higher doses in those patients who were resistant to the standard doses in conjunction with ASCT (164-167). This therapy combination demonstrated improved overall survival rates (168). Based on these results, HDM-ASCT has been established as standard up-front consolidation therapy (4).

1.6.5 Immunomodulatory drugs

Since the 1990s, significant progress has been made in the development of drugs for myeloma. This progress began with the discovery of the anti-myeloma activity of the IMiD thalidomide. Thalidomide derivatives such lenalidomide and pomalidomide have been subsequently developed. IMiD agents have broad anti-myeloma mechanisms of action, which include the following:

1. Changes to tumour suppressor genes and oncogenes, in particular IRF4, which lead to arrest of the cancer cell cycle and apoptosis (176-178);
2. Alterations to tumour microenvironments through downregulation of interleukin 6 (IL6) and vascular endothelial growth factor (VEGF). Role in changes to osteoclastic differentiation (179).

3. Immunomodulation through increased expression of interleukin 2 and TNF-alpha, which leads to T-cell mediated immune response, direct plasma-cell death and increased immune response that is mediated by natural killer cells (180).

Thalidomide was found to exert its mechanism of action through interaction with cereblon (CRBN) that is encoded by the *CRBN* gene (181). CRBN complexes with several proteins: the ubiquitin ligase protein Cul4A, damaged DNA binding protein 1 (DDB1), regulator of Cullins 1 (ROC1) and a substrate receptor to form an E3 ubiquitin ligase complex known as Cullin RING ligase 4 (CRL4). When this complex is activated, it targets molecules for degradation in the proteasome, which leads to downregulation of growth-factor genes. Further studies have demonstrated that binding of IMiDs to CRBN induces ubiquitination and degradation of two lymphoid transcription factors, Ikaros (IKZF1) and Aiolos (IKZF3), which are known to be responsible for B- and T-cell maturation (182, 183). IKZF3 has been shown to play a critical role in plasma-cell development (184). Later studies have demonstrated an association between IMiD resistance and persistently elevated levels of CRBN and IKZF1/IKZF3 in vitro and in vivo (185, 186). Other research groups also went on to find that the presence of IMiDs and CRBN suppressed production of the transcription factor interferon regulatory factor 4 (IRF4) (183). Studies have shown that IRF4 is a critical mediator of myeloma-cell survival. *MYC* was also identified as a target of IRF4 (176-178).

An additional mechanism of action of IMiDs includes interference with the glutamine synthetase pathway. CRBN appears to ubiquitinate glutamine synthetase and thereby to target it for degradation (187). IMiDs may also operate through ubiquitin-independent mechanisms of action. IMiDs have been shown to bind basigin and solute carrier family 16 member 1 (SLC16A1) to form a complex, which binds to CRBN to promote cell proliferation, invasion and angiogenesis. IMiDs compete to bind to the basigin and SCL16A1 complex, and this process leads to impaired cancer-cell growth (188, 189). Expression of the mRNA of Ikaros, Aiolos and basigin may provide prognostic information regarding IMiD sensitivities (190).

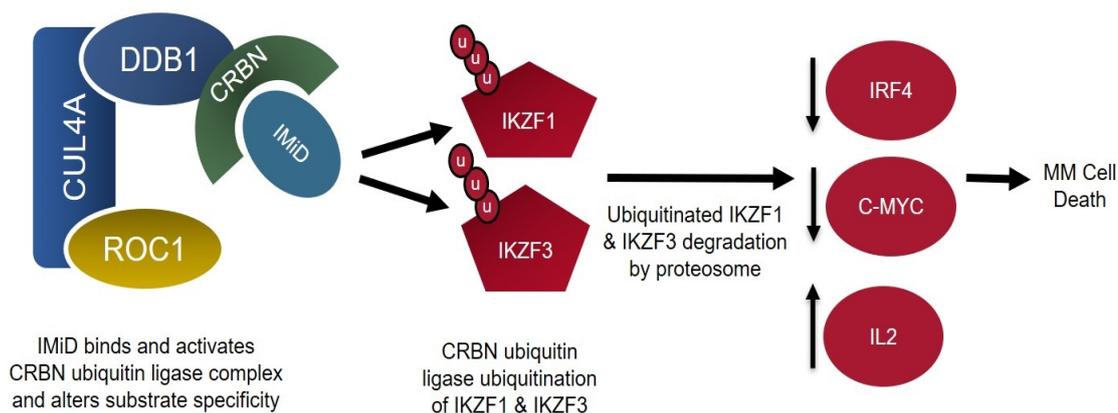


Figure 4: Schematic representation of the mechanisms of action of immunomodulatory drugs in MM (181-183)

Thalidomide was the first immunomodulatory drug to be explored in the treatment of myeloma. It was investigated in 1999 and showed significant anti-myeloma activity (191). Following this finding, groups explored use of thalidomide in combination with dexamethasone (192) and cyclophosphamide (193). However, thalidomide has significant side effects that lead to a high discontinuation rate due to high rates of peripheral neuropathy, venous thrombosis and fatigue (194).

Lenalidomide was developed as a derivative of thalidomide. This demonstrated anti-myeloma activity in combination with dexamethasone in newly diagnosed patients (195). It has also been trialled in combination with cyclophosphamide and dexamethasone (196, 197). This combination has been found to reduce rates of neuropathy but increase rates of myelosuppression compared with use of thalidomide.

Pomalidomide was also developed as a derivative of thalidomide. It was marketed as useful treatment for relapsed/refractory patients. This agent demonstrated activity in patients who showed resistance to lenalidomide and proteasome inhibitor (198, 199). It is currently used in the relapsed/refractory setting in myeloma. There are currently several other IMiDs at various stages of development.

There is conflicting data on the efficacy of lenalidomide in patients with high-risk cytogenetic abnormalities. Among relapsed patients, it has been shown that outcomes

for patients with t(4;14) who were treated with lenalidomide had similar outcomes to those who did not have that mutation. This study did not show improvement in survival rates for patients with deletion 17p (142). The IFM group in contrast showed adverse outcomes for relapsed/refractory patients with t(4;14) who were treated with lenalidomide (200).

The impact of IMiD therapy varies across this class of drugs, as pomalidomide therapy in the relapsed/ refractory setting demonstrated improved survival in patients with del(17p) vs. t(4;14), although survival rates in this group were still shorter than those of other patients with other cytogenetic abnormalities (201). These results were confirmed in another study among relapsed/ refractory patients among whom pomalidomide treatment vs. dexamethasone alone for patients with del(17p) was shown to improve survival compared with patients with t(4;14) and the survival rate for patients with del(17p) was similar to that for standard-risk patients in the relapsed refractory setting (202).

1.6.5.1.4 Proteasome inhibitors

Protein synthesis and degradation is tightly regulated within all cells and the ubiquitin-proteasome system plays a key role in this process (203). This system targets misfolded proteins as well as proteins that are no longer needed. This pathway plays an important role in cell survival and proliferation. Proteins that are destined for degradation are ubiquitinated via covalent attachment of a c-terminal glycine residue of ubiquitin to lysine. Ubiquitinated proteins are subsequently degraded in the central part of the proteasome (204). This system particularly targets proteins such as cell-cycle regulators (cyclins), TP53 and NF- κ B pathway proteins.

Cancer cells, especially myeloma cells, are dependent on the proteasome system for clearance of abnormal proteins due to the large amounts of paraprotein that are produced by these cells (204, 205). Proteasome inhibitors target this susceptibility through inhibition of the 26S proteasome. Several mechanisms of cellular toxicity that proteasome inhibitors are thought to follow have been proposed. One consequence of proteasome inhibition is the accumulation of inhibitory kappa B (I κ B), which is an inhibitor of NF- κ B. Inhibition of NF- κ B leads to decreased expression of adhesion

molecules as well as their decreased growth and survival and increased anti-angiogenic factors. It also leads to decreased levels of pro-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2,) and this decrease triggers apoptosis of myeloma cells via cytochrome-c and caspase-9 activation (204). Additionally, activation of the c-Jun terminal kinase pathway (JNK) leads to caspase-dependent apoptosis. Increased levels of p53 are thought to provide another mechanism of apoptosis induction, even in the presence of p53 mutations. Additionally, the presence of PIs is thought to lead to decreased degradation of pro-apoptotic proteins Bim, Bid and Bik, leading to apoptosis (204).

1.6.5.1.5 Bortezomib is a specific inhibitor of the 26S proteasome and was one of the first proteasome inhibitors to demonstrate activity both in vitro and in vivo (206, 207). Several trials have now shown the efficacy of bortezomib. Subsequently carfilzomib (208-210) and ixazomib (211-213) have been granted approval to be used for treatment based on several clinical trials that have shown their efficacy.

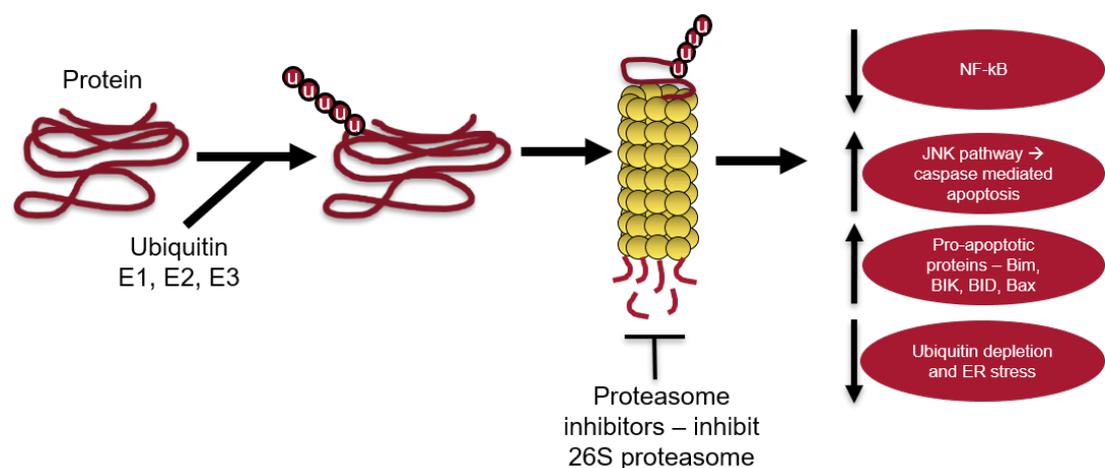


Figure 5: Schematic representation of mechanisms of action of proteasome inhibitors (204)

Bortezomib-based induction therapy has been found to be important in the treatment of high-risk disease. Studies that test bortezomib against standard therapy demonstrate improved response to t(4;14) (214), but long-term follow up of this short-term treatment demonstrated limited improvement in overall survival rates. The same study found no improvement in survival rates in patients with del (17p) (62). Longer term bortezomib induction therapy and maintenance studies by the Dutch-Belgian

Haemato-Oncology Group (HOVON) demonstrated improvement in survival rates of patients with t(4;14) as well as those with del (17p) (60, 89). The studies suggest that long-term exposure to a proteasome inhibitor in these two genetic lesions is important.

Carfilzomib treatment with lenalidomide and dexamethasone vs. lenalidomide and dexamethasone demonstrated improvement in survival rates among high-risk patients in the ASPIRE study (162). Similarly the ENDEAVOR study demonstrated improvement in survival rates in the high-risk setting with carfilzomib vs. bortezomib therapy (215).

1.6.5.1.6 Epigenetic modifiers

Histone deacetylases (HDACs) are enzymes that are known to play a role in the regulation of DNA through epigenetic regulation of histone proteins (216). These enzymes have also been shown to regulate non-histone proteins such as the tumour suppressor p53, the transcription factor BCL6 and the E2F family (185). Panobinostat is the first HDAC inhibitor that has demonstrated efficacy in myeloma patients (217-220).

1.6.5.1.7 Monoclonal antibodies

Immunotherapy in the form of monoclonal antibodies has generated great interest in the treatment of MM as monoclonal antibodies can target proteins that are stably expressed at high levels in MM cells, irrespective of disease stage and in spite of the genetic evolution of the disease. They act in a different way to the drugs mentioned above and have shown great efficacy in the treatment of MM recently.

Cyclic adenosine diphosphate ribose hydrolase, known as CD38, was initially described as a good target for antibody therapy in MM by Stevenson et al. in the 1990s (221). Daratumumab (anti-CD38) was developed commercially and demonstrated activity both on its own (222-224) and in combination with IMiD therapy (225) and proteasome inhibitors (226). Based on these results, daratumumab can be used in second- and fourth-line therapy to treat MM in the UK.

The addition of daratumumab has been shown to be beneficial for cytogenetic high-risk patients among both front-line, transplant eligible, newly diagnosed patients and those who are transplant ineligible. This has been shown with the following therapy combinations: bortezomib, thalidomide, dexamethasone (VTD) (227), bortezomib, lenalidomide, dexamethasone (VRD) (228), bortezomib, melphalan, prednisolone (VMP) (229), and lenalidomide with dexamethasone (RD) (230). However, all these studies demonstrate that patients with high-risk cytogenetics still show poorer responses than standard-risk patients. Additionally, high risk is defined as del(17p) and t(4;14) in most of these studies, and there is no or limited analysis of the effects of individual cytogenetic abnormalities. Similarly, in the relapsed setting, the addition of daratumumab has shown improved survival rates of cytogenetic high-risk patients but these rates remain below those of patients with standard-risk cytogenetics. These include combinations with RD (225), bortezomib and dexamethasone (VD) (226) and carfilzomib with dexamethasone (KD) (231).

Other antibodies such as elotuzumab that target the surface antigen CD319, known as SLAMF7 protein, have also been developed and demonstrate their efficacy in combination with lenalidomide and dexamethasone (232). In addition, a study that combined elotuzumab with lenalidomide and dexamethasone demonstrated improved survival rates in patients with del(17p), gain(1q) and t(4;14) (232). Anti-B cell maturation antigen (BCMA) targets such as belantamab and mafodotin are showing promise and have recently gained licence approval from the US Food and Drug Administration for the treatment of relapsed MM (233). Several other anti-BCMA antibodies are currently in the trial phases of development. Bi-specific antibodies that target both BCMA and CD3 are also in development.

1.6.5.1.8 Chimeric antigen receptor T cell therapy

In addition to monoclonal antibody therapy, T cells are also under development as a treatment against MM through the development of chimeric antigen-receptor T-cell therapy (CAR-T) against MM. Most CAR-T treatments in MM are in trial phases and are targeted towards BCMA. CAR-T to target CD38 and SLAMF7 are also in development (234).

1.6.5.1.9 Targeted therapies

Although focus is sharpening on personalised medicine in cancer therapy, until recently, there were no treatments that offered particular benefit in specific subgroups of myeloma. The development of the BCL2 inhibitor venetoclax has driven a search to find cancers that overexpress this transcription factor. The t(11;14) subtype of myeloma was found to be associated with BCL2 (235, 236) and treatment of this type with venetoclax has shown possible efficacy in the relapsed refractory setting (237). Evaluation of new drugs on different subtypes of MM in the future may point the way forward for other targeted therapy in MM, which can utilise dependencies of subtypes of MM.

1.7 High-risk myeloma

Risk is generally used to prognosticate the future for patients who have high-risk disease that is associated with a short progression-free survival (PFS) or short overall survival (OS) compared with standard-risk patients (238, 239). The work that has been performed for this thesis has mainly focused on risk at the time of diagnosis and so we concentrate on this within this section.

For symptomatic myeloma, the IMWG defines disease-specific mortality that is expected to be within two to three years at the time of diagnosis as high risk. This accounts for approximately 5-10% of patients as calculated through use of early mortality data from phase 3 trials, and 10-20% of patients as calculated through use of single institutional studies and data collected from the surveillance, epidemiology and end results programme in the US (70, 240). Risk markers can be broken down into host-related, tumour-related, and environment-related groupings, as summarised in Figure 6.

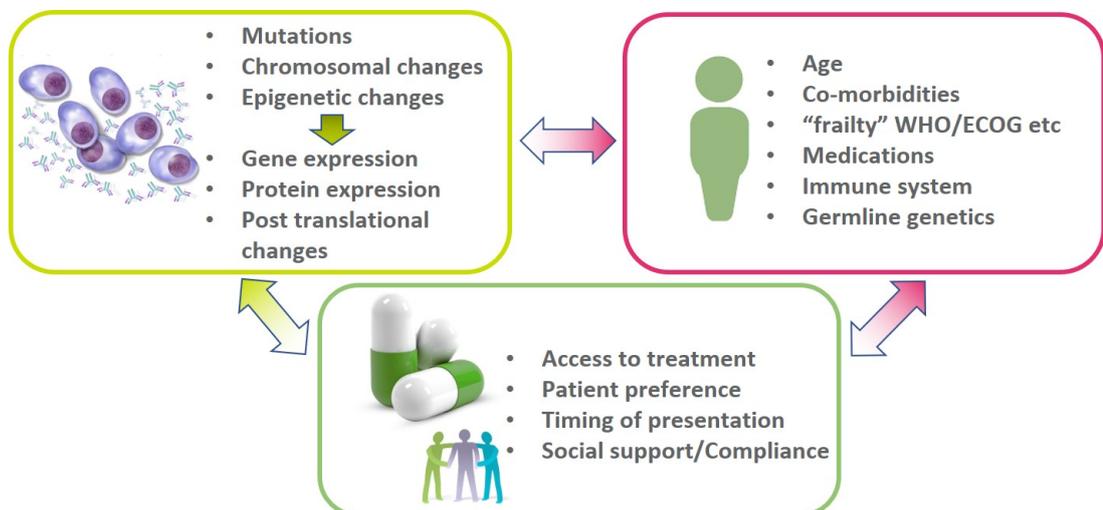


Figure 6: Factors associated with outcomes in myeloma

1.7.1 Host-related risk

The most important host-related factor is age. Survival rates with treatment by both conventional and novel agents have been found to shorten incrementally for every 10-year age band (98, 241). Co-morbidities have impacts on patients' abilities to tolerate

treatment and side effects. Interactions between medications can also lead to reduced bio-availability of anti-myeloma drugs. More recently, the recognition that age, co-morbidities and physical and mental functions all have a cumulative effect on outcomes has resulted in the creation of geriatric assessments in elderly patients (242). There are now trials underway to see whether alteration of treatment according to the results of these geriatric assessments is able to improve outcomes in elderly patients with myeloma.

As immune therapy in myeloma advances rapidly, alterations in immune-mediated tumour killing in response to treatment that is related to patients' immune systems may come into play. Susceptibility to infection due to a poorer immune system in ageing patients may also effect mortality and side effects of immunosuppressive chemotherapy. Germline genetics may also play a part in the treatment of subtypes of myeloma and in pharmacokinetics of treatment.

1.7.2 Environmental factors related to risk

Environmental factors such as access to treatment play a key role in the risk of and speed of relapse. The exorbitantly high cost of many new myeloma treatments has restricted access to these drugs for a substantial proportion of patients with myeloma. In addition, the availability of healthcare, screening programmes and routine health checks may enable detection of MM prior to organ damage, which would improve prognosis. Social support has an additional impact on compliance to treatment (243-245). Social and economic differences that lead to different outcomes are becoming increasingly important in the era of rapid advancement in therapies in myeloma, although at present they are less studied than are medical treatments.

1.7.3 Tumour biology and risk

There are several aspects of tumour biology and their associated risks that have been explored in order to enable tumour-related prognostication. Risk factors such as underlying cytogenetic changes, molecular changes in tumours and the associations of these changes with outcomes have been described in the sections above. Many factors such as high levels of serum LDH and B2M and low levels of albumin have been used as surrogate markers of disease burden. Clinical risk factors such as amounts of

circulating plasma cells, extramedullary disease and organ impairment can also be taken into account. In the next section, clinical risk is explored as well as biochemical surrogates that are used for prediction of risk, as genetic risk has been extensively described in the previous section.

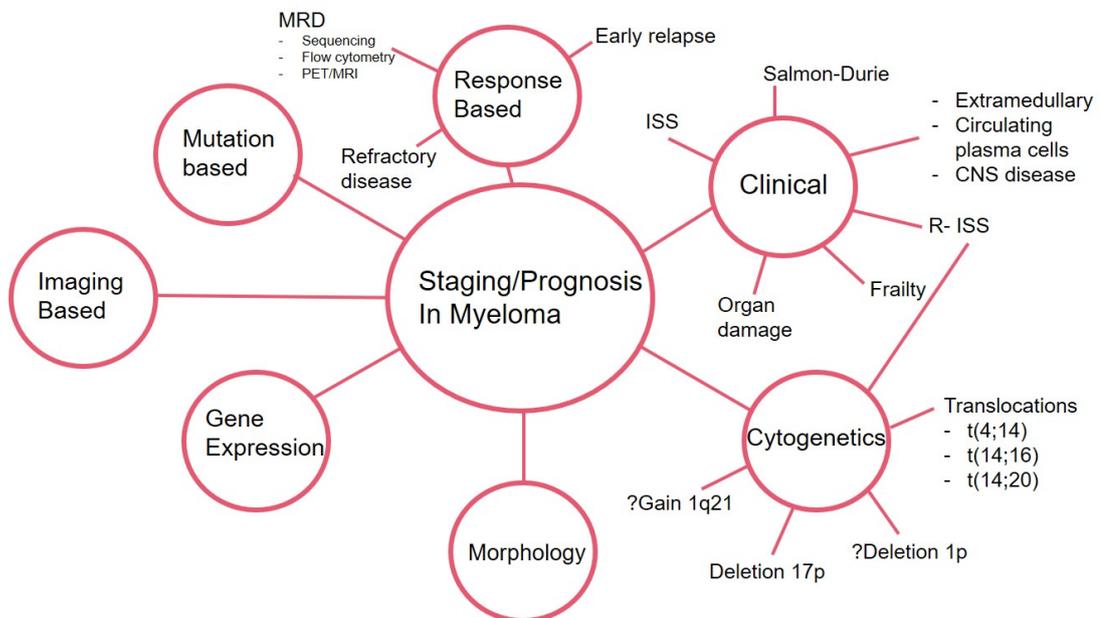


Figure 7: Risk scores and known associations with poor outcomes in myeloma

1.7.4 Clinical risk

Several clinical factors have been associated with the diagnosis of high-risk myeloma. These include the presence of extramedullary disease (246, 247), which is also found to be associated with higher levels of biochemical parameters such as LDH, anaemia and thrombocytopenia, high-risk gene expression signatures and high-risk cytogenetics such as *MAF* translocations. The presence of circulating tumour cells has also been found to be predictive of prognosis (248-251), even if levels of these circulating tumour cells do not meet the current diagnostic criteria for plasma-cell leukaemia, which is an established high-risk marker (252). The presence of CNS myeloma is also predictive of poor outcome (253, 254). Additionally, the extent of disease that is shown up by positron emission tomography (PET), computed tomography (CT) or magnetic resonance imaging (MRI) scans has also been shown to have prognostic value (255). The degree of organ impairment that is caused by the disease itself is also associated with outcomes, probably as a result of underlying

genetic risk but also due to the inability to deliver certain treatment due to organ impairment.

1.7.5 Biochemical and haematological parameters of risk

Surrogate biochemical and haematological markers have been used for risk prognostication. Initial risk scoring focused on tumour bulk as a measure of risk, along with parameters such as paraprotein quantification, haemoglobin concentration, bone lesions and urinary protein levels (256). Other biological parameters such as levels of C reactive protein (CRP) and of lactate dehydrogenase (LDH) were also identified as risk markers. The simplified ISS for myeloma was developed subsequently. It uses levels of B2M and albumin to prognosticate. Functional tests such as the proliferation index in plasma cells were also utilised for prognostication (257). In addition, plasma-cell morphology and in particular, blastic plasma-cell myeloma has been found to be associated with poor prognosis. The next section describes the attempt to stratify risk by application of different combinations of the above described risk factors.

1.7.6 Risk scores

One of the first attempts to stratify high-risk myeloma was based on clinical staging with readily available clinical parameters and was known as the Durie-Salmon staging system (256). As this system was affected by observer-related bias and was found to be inferior to the ISS, the ISS superseded this scoring system in 2005 (258). The ISS involved the use of two simple biochemical parameters, albumin and B2M levels, and was widely adopted by the worldwide myeloma community, which enabled comparison of trials and treatments. The ISS remains an independent prognostic variable, despite the subsequent inclusion of genetic characteristics in prognostic systems that were created subsequently (69).

As the amount of information about the disease has increased, particularly about chromosomal aberrations in myeloma, data that is taken from interphase fluorescent in-situ hybridisation (iFISH) studies on high-risk cytogenetic lesions has been combined with clinical information, including the ISS and LDH scores. The revised ISS (R-ISS) was developed in 2015 to take this growing knowledge into account to improve prognostication. The R-ISS takes into account the patient's status regarding

deletion 17p, t(4;14) and t(14;16) as well as LDH in order to assign a risk level (69, 259). At the same time, some groups also used signatures that combined both the presence of high-risk cytogenetic lesions with other parameters such as gene expression and a proliferation index, such as the mSMART 2.0 staging system (257). As more evidence has become available, the presence of additional cytogenetic abnormalities has been added to risk signatures by the IMWG; for instance, gain(1q) has been included in risk scoring by this group (70).

The risk-scoring systems that are explained above consider cytogenetic abnormalities in isolation. However, the UK-based Medical Research Council (MRC) Myeloma IX study has demonstrated that the presence of a combination of adverse-risk cytogenetic lesions brings an ultra-high risk. This study combined the number of adverse cytogenetic lesions that were present in a case with the ISS to create a risk-scoring system. This study has not subsequently been validated, although there is growing evidence that the presence of combinations of genetic lesions is associated with an ultra-high prognostic risk (260). However, there is ongoing debate regarding which specific genetic abnormalities constitute ‘double hit’ and ultra-high-risk myeloma. The mSMART 3.0 staging system now also includes double and triple hit myeloma as a poor prognostic marker.

More recently, next-generation sequencing studies have started to reveal higher levels of complexity in terms of risk prognostication with the inclusion in risk scores of ‘bi-allelic’ *TP53* alterations, which are discovered mainly through mutational analysis. A summary of the major prognostic scoring systems that are used in clinical practice is shown in Table 5.

1.7.7 Response to therapy

High-risk myeloma can be identified at the time of diagnosis as described in the section above; however, myeloma is a dynamic disease. Genetic subclones that are undetected at diagnosis can become dominant over time, or new genetic lesions can develop because of genetic instability. Additionally, patient and environment-related factors influence response to therapy. Disease risk can therefore also be stratified further at later timepoints such as at the end of induction, after transplant or during

maintenance therapy through assessment of disease response with use of recently introduced technologies such as flow cytometry or sequencing-based monitoring of MRD (261, 262). Assessment of disease risk at different stages of induction/consolidation and maintenance may therefore enable adaptation of therapy according to residual disease levels rather than at the time of florid relapse. Response can be assessed further through application of clinical indicators such as early disease relapse or aggressive relapse (263).

MRD assessment of myeloma is not currently standard practice in the UK as it does not alter management of disease. Several trials are currently being devised including the Myeloma XV trial which will use MRD to stratify therapy order to test whether it can be incorporated into the daily management of patients with MM.

Table 5: Clinical and iFISH-based prognostic scoring systems in myeloma

Staging system	Variables	Stages
Salmon-Durie (256)	Paraprotein level, type of monoclonal protein, haemoglobin, calcium, number of bone lesions, creatinine	I: Hb>10, Ca <10.5mg/dl, normal bone x-ray or solitary plasmacytoma, IgG <5 or IgA<3, urine M component <4g/24h. II: Neither I nor III III: Hb <8.5g/dl, Ca >12mg/dl, advanced lytic bone lesions, IgG >7 or IgA >5g/dl or urine light chain M component >12g/24h.
International staging system ISS (258)	Serum albumin and β 2-microglobulin (β 2M)	I: serum albumin \geq 3.5g/dl, β 2M <3.5mg/dl II: neither I or III III: β 2M>5.5mg/dl
Revised ISS (69)	ISS, LDH and iFISH	I: ISS stage I, normal LDH, standard risk iFISH II: neither I nor III III: ISS III and abnormal LDH or high-risk disease by iFISH (del(17p), t(4;14) or t(14;16)
MRC (67)	ISS and iFISH	ISS 1 with no adverse iFISH, ISS II/III with 1 adverse iFISH lesion or ISS III with >1 adverse iFISH (del(17p), gain(1q), t(4;14), t(14;16), t(14;20))
IMWG risk staging (70)	ISS, LDH, iFISH and age	Low risk: ISS stage I or II, absence of t(4;14), del(17p), gain(1q21) and age <55 Intermediate risk: all others High risk: ISS II or III and either t(4;14) or del(17p)
mSMART2 risk staging (257)	Serum albumin and β 2M, LDH and plasma cell iFISH and proliferation index	Standard risk: trisomies and/or t(11;14)/t(6;14) Intermediate risk: t(4;14) or amp(1q), complex karyotype, metaphase del(13q) or hypodiploidy, high PC S phase High risk: t(14;16), t(14;20) or del(17p) GEP high risk
mSMART3	Serum albumin and β 2M, LDH and plasma cell iFISH and proliferation index, mutations	Standard risk: as per mSMART2 High Risk: As per mSMART2 + gain(1q), p53 mutations, R-ISS III, high plasma cell S phase, Double/Triple-hit MM (2/3 high risk genetic abnormalities)

1.7.8 Risk adapted therapy and personalised medicine

There is increasing recognition that myeloma is a complex and heterogeneous disease with diverse outcomes. These are linked partly to tumour genetics but also to the patient characteristics themselves. The reason that scientists improve their knowledge of tumour biology and how it relates to patients and therapy response is to be able to adapt treatments so that the best therapeutic outcomes can be gained with the lowest toxicity.

The last two decades have led to great advances in the treatment of myeloma but also the recognition by the medical community of a subgroup of high-risk myeloma patients who benefit less from these therapies. Accurate diagnosis of this subgroup will help us to adapt early and treat these patients perhaps with intensification of their therapy. The targeting of molecular dependencies may also help us to improve outcomes. Recognition of patients who are at low risk is also important as this enables the possibility of de-escalating therapy in these patients to enable them to have an improved quality of life with reduced toxicity of treatment. The aim of this project is to improve understanding towards the aim of personalisation of care in myeloma through the assessment of disease risk.

This chapter has summarised the acquisition of knowledge of MM, which has been rapid. It also summarises the large and ever-changing armamentarium of possible treatment options that is available to patients for MM. However, despite rapid advances in both the above, we have not integrated knowledge from the two to reduce toxicity but maximise efficacy. The focus of the work that is described in this thesis was to try to resolve areas of debate within the MM community with regard to high-risk cytogenetic lesions so that we can move forward in the development of therapies and regimens which will benefit patients with high-risk MM.

The first results chapter of this thesis contains an analysis of established cytogenetic risk factors with an analysis of the Myeloma IX (with extended follow-up data) and XI trials with regard to the presence of known cytogenetic high-risk lesions. Next, *TP53* deletion and mutations are examined in depth to resolve debate around this topic in MM. In the third results chapter, I evaluate the gain and amplification of chromosome 1q with regard to both trials. In the final results chapter, gene expression

risk signatures are examined with regard to their association with prognosis as well as their associations with clinical and other genetic factors. I examine the additive value of gene expression signature-based prognostication to known chromosomal aberrations. By examining these factors in detail, with the use of large numbers of patients and detailed annotation in later chapters, I am able to examine the interrelationships between biochemical markers and genetics and to assess the additive benefit of the examination of these factors in combination to evaluate patient risk in the newly diagnosed myeloma setting.

2 Methods

The work that is described in this thesis used patient samples from two trials, MRC Myeloma IX (Myeloma IX) and the National Cancer Research Institute (NCRI) Myeloma XI (Myeloma XI) trials, which are described in more detail below. Both trials were for newly diagnosed myeloma patients and together comprised 6380 newly diagnosed patients who were recruited between 2003 and 2016 in more than 100 haematology centres in the UK.

2.1 The MRC Myeloma IX trial

2.1.1 Patient recruitment and treatment arms

Myeloma IX was a phase 3, multicentre, open label, randomised controlled trial of newly diagnosed MM patients. It employed a multifactorial design to answer several therapeutic questions with several steps of randomisation. It preceded the Myeloma XI trial and recruited 1960 patients between May 2003 and October 2007. It has completed recruitment and follow-up. The main trial outcomes have been published previously (193, 264-266). The study was undertaken with written informed consent from patients and ethical approval was obtained from the MRC Leukaemia Data Monitoring and Ethics Committee (MREC 02/08/95, ISRCTN68454111).

The criteria for entry into the trial were simply that patients must have had newly diagnosed myeloma and required treatment. The main exclusion criterion was renal failure that did not respond to intravenous rehydration. The trial was split into two pathways, the transplant-eligible pathway (intensive) and the non-transplant eligible pathway (non-intensive). The pathway to which patients were assigned was based on physician choice.

Patients on the intensive pathway were randomised to receive conventional chemotherapy regimens that consisted of cyclophosphamide, vincristine, doxorubicin and dexamethasone (CVAD) or cyclophosphamide, thalidomide and dexamethasone (CTD). Patients continued induction until they reached maximal response after which they were treated with high-dose melphalan and autologous stem-cell transplantation (HDM-ASCT). At 100 days post-transplant, patients were randomised to thalidomide maintenance vs. no maintenance.

Patients who entered the non intensive pathway were randomised to melphalan and prednisolone or to attenuated CTD (CTDa). This was continued until they reached maximal response and was followed by randomisation to thalidomide maintenance or no maintenance. Both intensively and non-intensively treated groups were also randomised to clodronic acid or zoledronic acid bisphosphonate treatment. Primary endpoints were progression-free survival and overall survival. Secondary endpoints were quality of life, skeletal-related events and toxicity. The Myeloma IX trial design is summarised in Figure 8 kindly provided by Leeds CTRU.

Table 6: Eligibility criteria for inclusion in Myeloma IX trial.

Inclusion criteria for initial randomisation
<ul style="list-style-type: none">● Aged 18 years or greater● Newly diagnosed as having symptomatic multiple myeloma or non secretory multiple myeloma based on:<ul style="list-style-type: none">○ Paraprotein (M-protein) in serum and/or urine○ Bone marrow clonal plasma cells or plasmacytoma● Related organ or tissue impairment● Written informed consent● Prepared to use contraception● Negative pregnancy test
Exclusion Criteria for initial randomisation
<ul style="list-style-type: none">● Asymptomatic myeloma● Solitary plasmacytoma of bone● Extramedullary plasmacytoma (without evidence of myeloma)● Previous or concurrent active malignancies, except surgically removed basal cell carcinoma of the skin or other in situ carcinomas● Previous treatment for myeloma, except the following<ul style="list-style-type: none">○ local radiotherapy to relieve bone pain or spinal cord compression○ prior bisphosphonate treatment○ low-dose corticosteroids (up to 12 mg/day dexamethasone or 80 mg/day prednisolone, for 14 days)○ up to four single doses of corticosteroids (total dose 1 g methylprednisolone, 200 mg dexamethasone, or 1.25 g prednisolone) Caution is advised in patients with a past history of ischaemic heart disease or psychiatric disorders, but exclusion is essentially to be at the discretion of the treating clinician.● Acute renal failure (unresponsive to up to 72 h of rehydration characterised by creatine >500 µmol/l or urine output <400 ml/day or requirement for dialysis). These patients are not eligible for this study but may be eligible for inclusion in MERIT (Myeloma Renal Impairment Trial). NB Patients with serum creatinine >2 x upper limit or normal (or creatinine clearance <20 ml/min) are eligible for Myeloma IX, but bisphosphonates should not be administered until serum creatinine has decreased to <2 x upper limit of normal (or creatinine clearance >30 ml/min)

Exclusion Criteria for maintenance thalidomide randomisation
<ul style="list-style-type: none">• Patients on intensive pathway who do not receive HDM/autograft• Patients on intensive pathway who received an allogeneic stem cell transplant• Patients on non-intensive pathway not reaching maximal response following induction therapy• Patients in both pathways showing progressive disease or relapse• Patients who are pregnant or unprepared to used contraception• Patients in both pathways with concurrent malignancy

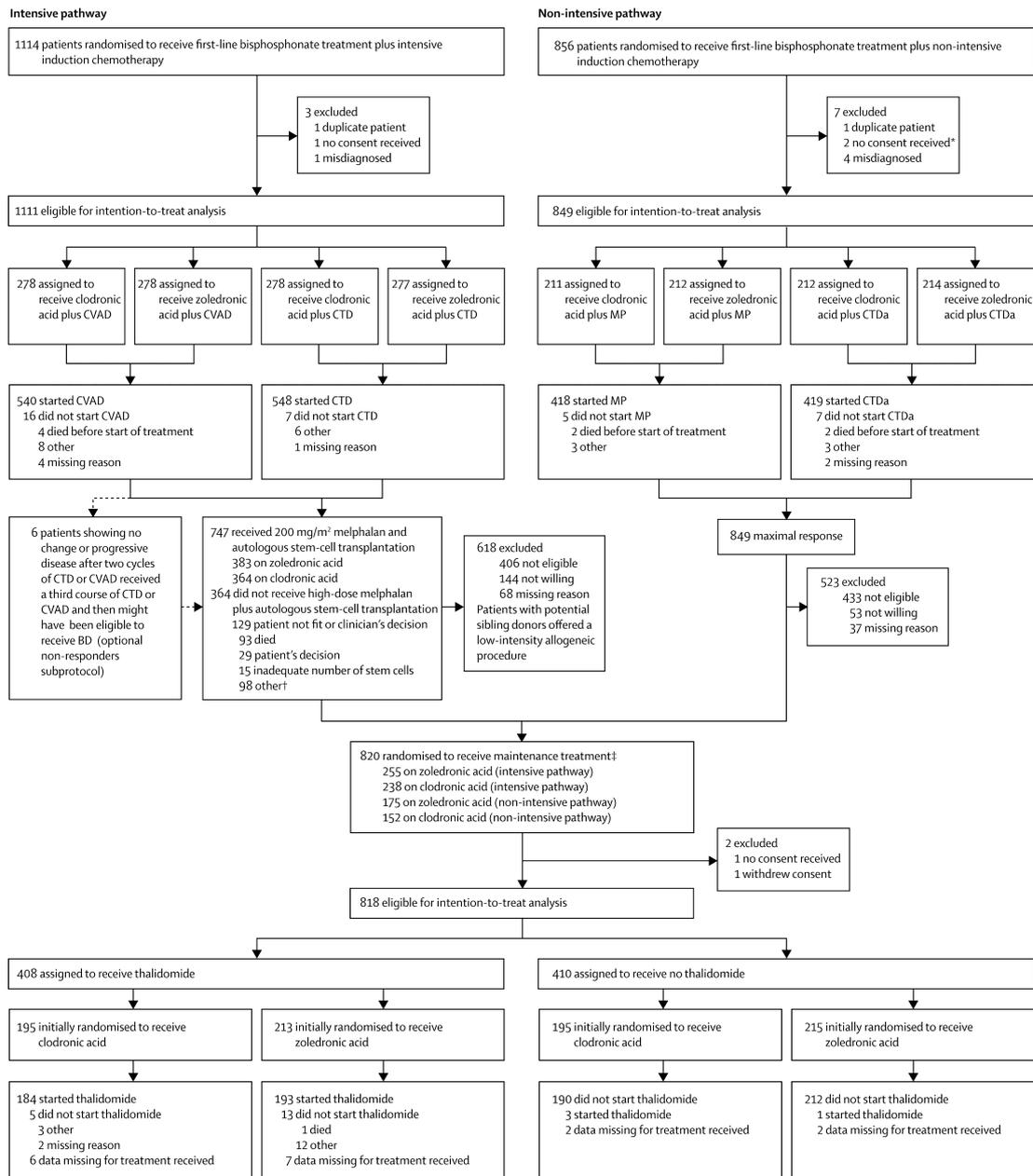


Figure 8: MRC Myeloma IX consort diagram. CVAD=cyclophosphamide, vincristine, doxorubicin and dexamethasone. CTD=cyclophosphamide, thalidomide and dexamethasone. MP=melphalan and prednisolone. CTDa=attenuated CTD. BD=bortezomib and dexamethasone

2.2 The NCRI Myeloma XI trial

The Myeloma XI clinical trial is a multicentre, open label, randomised controlled trial of newly diagnosed MM patients. It has a multifactorial design to answer several therapeutic questions with three stages of randomisation. It followed the Myeloma XI trial and recruited 4420 patients from January 2011 until June 2016. Some trial outcomes have been published separately. The study is closed to recruitment and still in follow up. The study was undertaken with written informed consent from patients and ethical approval that was obtained from the Oxfordshire Research Ethics Committee (MREC 17/09/09, ISRCTN49407852). Patients were eligible to join the trial if they were newly diagnosed myeloma patients who required treatment. The main exclusion criterion was renal failure that did not respond to intravenous rehydration (see full criteria in Table 7).

Patients were assigned to either transplant-eligible (intensive) or non-eligible (non-intensive) arms of the study by the treating physician based on each patient's age, comorbidities and frailty. Patients were randomised to either CTD or cyclophosphamide, lenalidomide, and dexamethasone (CRD) at induction. Drug doses were attenuated for the non-intensively treated patients (CTDa, CRDa). After induction, patients who showed suboptimal responses to treatment (minimal or partial response (MR or PR)) were randomised to receive intensification treatment through consolidation with cyclophosphamide, bortezomib (Velcade) and dexamethasone (VCD) or no consolidation. The eligibility criteria for this randomisation step is detailed in Table 8. Patients with progressive disease or stable disease received only VCD without randomisation. At the completion of induction and intensification, intensively treated patients received HDM-ASCT and then were randomised to lenalidomide maintenance or to observation. Non-intensively treated patients were randomised to maintenance lenalidomide or observation after induction and consolidation. Eligibility criteria for maintenance randomisation are detailed in **Table 9**. Treatment protocols are detailed in Figure .

Additional arms of the trial were added in June 2013. They included carfilzomib, Revlimid, cyclophosphamide and dexamethasone (CCRD) at induction. Data from this arm has not been analysed in this study as it had not been released by the clinical

trials unit at time of writing. Data from the lenalidomide and vorinostat maintenance arms have also been analysed within this thesis as data had not been released for these arms at the time of initial analysis.

The co-primary endpoints of the study were progression-free survival and overall survival from randomisation stages. Secondary endpoints included response rates following induction and intensification, toxicity, second progression-free survival and relevant biological endpoints.

Table 7: Eligibility criteria for inclusion in Myeloma XI trial induction. Adapted from Myeloma XI protocol version 9.0.

Inclusion criteria for initial randomisation
<ul style="list-style-type: none"> • Aged 18 or greater • Newly diagnosed symptomatic myeloma or non-secretory myeloma based on <ul style="list-style-type: none"> ○ Paraprotein in serum or urine ○ Bone marrow clonal plasma cells and organ impairment or symptoms considered by the clinician to be myeloma related. • Able to provide written informed consent
Exclusion Criteria for initial randomisation
<ul style="list-style-type: none"> • Asymptomatic myeloma • Solitary plasmacytoma of bone • Extramedullary plasmacytoma without evidence of myeloma • Previous or concurrent malignancy • Previous treatment of myeloma except for local radiotherapy for bone pain or spinal cord compression/previous bisphosphonate treatment and corticosteroids • Known history of allergy to boron or mannitol • Grade 2 or higher peripheral neuropathy • Acute renal injury unresponsive to up to 72 hours of rehydration, characterised by creatinine >500µmol/l or urine output <400mls/day or requirement for dialysis • Lactating or breastfeeding • Patients with active or prior hepatitis C

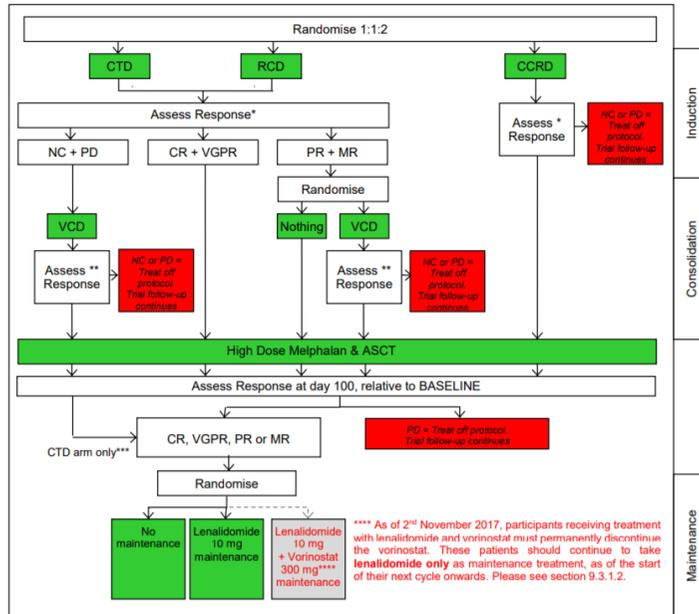
Table 8: Eligibility criteria for inclusion in Myeloma XI trial consolidation.
Adapted from Myeloma XI protocol version 9.0.

<p>Inclusion criteria for randomisation to bortezomib, cyclophosphamide and dexamethasone</p>
<ul style="list-style-type: none"> • Completed a minimum of 4 cycles of CTD or RCD as per their initial randomised treatment allocation in the intensive pathway or 6 cycles of CTDa or RCDA as per their initial randomisation allocation in the non-intensive pathway in accordance with Myeloma XI protocol. • At maximal response, showing a partial response or minimal response at the end of their randomised induction treatment
<p>Exclusion Criteria for randomisation to bortezomib, cyclophosphamide and dexamethasone</p>
<ul style="list-style-type: none"> • Received any other anti-myeloma treatment, apart from their initial randomised treatment allocation in Myeloma XI. (Participants who have received local radiotherapy to relieve bone pain or spinal cord compression are eligible). • Participants in the intensive pathway randomised to receive CCRD at induction. • Showing complete response (CR), very good partial response (VGPR), no change (NC), progressive disease or relapse. • Pregnant, lactating or breastfeeding, or women of childbearing potential and male participants whose partner is a woman of childbearing potential unprepared to use contraception or commit to absolute and continuous abstinence during treatment and for 3 months afterwards. • Previous or concurrent active malignancies. - Documented diagnosis of Myelodysplastic Syndrome (MDS).

**Table 9: Eligibility criteria for inclusion in Myeloma XI trial maintenance.
Adapted from Myeloma XI protocol version 9.0.**

Inclusion criteria for randomisation to maintenance randomisation
<ul style="list-style-type: none"> • Completed randomised induction treatment (a minimum of 4 cycles of CTD, or CCRD, a minimum of 6 cycles of CTDa or RCDA and, if required according to response / Reached maximal response to randomised induction chemotherapy • Received at least 100 mg/m² high-dose melphalan if entered into the Intensive pathway randomisation allocation, VCD for a maximum of 8 cycles) in accordance with Myeloma XI protocol
Exclusion Criteria for maintenance randomisation
<ul style="list-style-type: none"> • Failed to respond (PD or NC) to lenalidomide (RCD(a) / CCRD) induction • Failed to respond (NC) to all protocol treatment (i.e. no response achieved since trial entry). • Received any other anti-myeloma treatment, apart from their randomised treatment allocations. • Progressive disease (PD) or relapse from CR. (Note: increase in size of lytic lesions on radiological investigation and/or development of hypercalcaemia automatically places participants in the progressive disease category). • Pregnant, lactating or breastfeeding, or women of childbearing potential and male participants whose partner is a woman of child bearing potential unprepared to use contraception in accordance with the Celgene approved process for lenalidomide Risk • Management and Pregnancy Prevention, or commit to absolute and continuous abstinence • Previous or concurrent active malignancies. • Documented diagnosis of Myelodysplastic Syndrome (MDS).

Transplant-eligible pathway



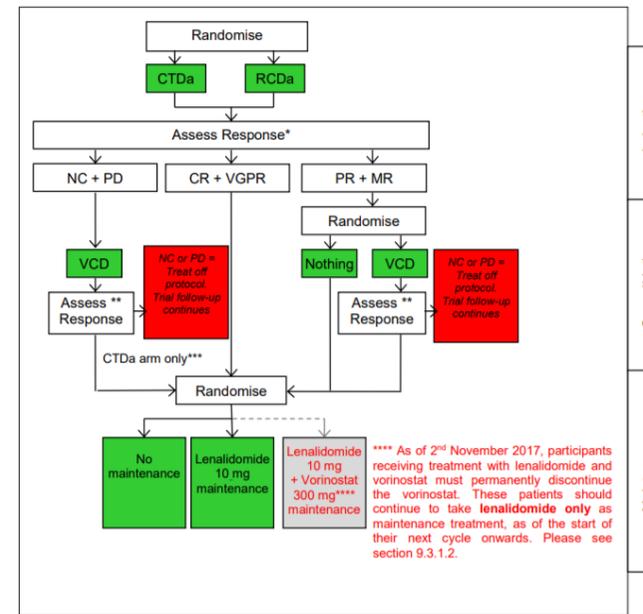
* In the absence of disease progression participants should receive a minimum of 4 cycles of induction chemotherapy (as long as they are responding) and should continue to maximum response or intolerance. Any participants receiving CTD or RCD showing NC after 4 cycles or progressive disease at any time during their induction chemotherapy should proceed to VCD. Participants receiving CCRD will not receive VCD.

** In the absence of disease progression participants should receive up to a maximum of 8 cycles of VCD and should continue to maximum response or intolerance.

*** Participants entered into the RCD or CCRD arm and assessed as NC or PD at the end of induction are not eligible for the maintenance randomisation.

**** Lenalidomide plus vorinostat maintenance is only available for those participants who were entered into the trial prior to PV6.0. NB: See Section 9.3.1.2 (page 50) for ongoing treatment details of participants randomised to the lenalidomide + vorinostat arm

Transplant non-eligible pathway



* In the absence of disease progression participants should receive a minimum of 6 cycles of induction chemotherapy (as long as they are responding) and should continue to maximum response or intolerance. Participants showing NC after 4 cycles or progressive disease at any time during their induction chemotherapy should proceed to VCD

** In the absence of disease progression participants should receive up to a maximum of 8 cycles of VCD and should continue to maximum response or intolerance.

*** Participants entered into the RCDa arm and assessed as NC or PD at the end of RCDa induction are not eligible for the maintenance randomisation

**** Lenalidomide plus vorinostat maintenance is only available for those participants who were entered into the trial prior to PV6.0. NB: See Section 9.3.1.2 (page 50) for ongoing treatment details of participants randomised to the lenalidomide + vorinostat arm

Figure 9: NCRI Myeloma XI protocol for transplant-eligible (intensively treated) and transplant non-eligible patients. Version 9.0, 2nd November 2017

2.2.1 Patient samples

Patients who were enrolled to the Myeloma IX and XI trials had bone marrow and blood samples taken at defined time periods of the treatment pathway including at presentation. Samples were split according to group and sent for central analysis. In Myeloma IX these were sent for cytogenetics testing to the Wessex Cytogenetics Centre and for Myeloma XI these were sent to the Institute of Cancer Research in the first instance.

2.2.2 White-cell and plasma-cell isolation

Bone-marrow samples were diluted with an equal volume of phosphate-buffered saline (PBS), layered into Ficol (GE Healthcare Life Sciences; 17-1440-02) at a 1:1 ratio and centrifuged at 1600rpm for 25 minutes. This enabled the separation of the buffy coat from red cells. The buffy coat was removed, washed in 10mls of PBS and centrifuged again for three minutes at 2000rpm. The process was repeated, and cells were re-suspended in 5mls PBS. Although I have performed this technique on a number of patient samples, the majority of these were carried out by scientific technicians who worked within the department.

2.2.3 CD138 Selection

Plasma cells were purified (>90%) using magnetic-activated cell sorting (MACS, Miltenyi Biotec, Bisley, UK). Anti-CD138 labelled magnetic beads (Product number 130-051-301, Milenyi Biotech) were incubated with white cells that were suspended in PBS for five minutes at room temperature prior to passage through magnetic columns. Although I have performed this technique on a number of patient samples, the majority of these were carried out by scientific technicians working within the department. Plasma-cell purity was confirmed by cytospin analysis.

2.2.4 DNA and RNA preparation

Following plasma-cell isolation, the number of cells in each sample was determined by a haemocytometer counting chamber. Isolated plasma cells were washed in PBS before being lysed in 350µl RLT lysis buffer (PN 1053393, Qiagen) and 100µl B-

mercaptoethanol. Samples were stored at -80°C until DNA and RNA purification could be performed.

The Qiagen all prep mini kit was used to purify DNA and RNA according to the manufacturer's instructions. The lysate was passed through an AllPrep DNA spin column. This enabled DNA binding. The column was washed and DNA was then eluted. Ethanol was then added to the flow-through from the AllPrep DNA spin column to provide RNA binding conditions. The sample was then run through a RNeasy spin column in which RNA bound to the membrane and contaminants were washed out. Nucleic acid concentration was determined by the measurement of light absorbance at a wavelength of 260nm using a nanodrop spectrophotometer (Thermo Fisher Scientific). DNA concentration was also measured by the picogreen assay (Thermo Fisher Scientific). Samples with an RNA integrity number (RIN) of <4 or RNA concentration of $<4\text{ ng}/\mu\text{l}$ were excluded from analysis. Samples with a DNA concentration of $<2\text{ ng}/\mu\text{l}$ were excluded from analysis.

2.2.5 In situ fluorescence hybridisation

All iFISH analysis that was performed for Myeloma IX as part of the work completed for this thesis was conducted at the Wessex Regional Genetics Laboratory by Dr Fiona Ross and team. After plasma-cell purification had been completed as detailed above, purified cells were suspended in a $400\mu\text{l}$ mixture of 3:1 methanol and acetic acid and stored at -20°C for at least 16 hours prior to resuspension in fresh fixative to make slides. Cells were suspended at a concentration of $2500\text{ cells}/\mu\text{l}$, and $0.2\mu\text{l}$ spots that contained 100-500 cells were placed on each slide. A minimum of 100 cells were scored for each probe. A selection of probes were used to identify IgH translocations and other copy number changes as detailed in Table 10. Clonal cut-off levels were set at 20% for numerical abnormalities and 10% for translocations (108) except in exploratory analyses, which are specified in this thesis.

All iFISH analysis was carried out on the Myeloma XI samples to validate the multiplex ligation-dependent probe amplification (MLPA) findings with regard to clonal and subclonal deletion of *TP53*. This work was carried out by myself at the Royal Marsden Hospital diagnostics laboratory following standard hybridisation

protocols, which have been detailed above. A second operator, Frances Aldridge, carried out a second verification of copy number deletional percentage. At least 200 cells were counted per operator and per sample to be studied for deletion 17p and an average deletion percentage was recorded after counting had been performed by two different operators.

Table 10: iFISH probes used in cytogenetic analysis of Myeloma IX and XI trials

Chromosome	Gene	Probe	Source
Myeloma IX			
5/9/15	Multiple	LS1 D5S23/D5S721/CEP 9/CEP15	Vysis (Abbott) 32-231021
14 Rearrangement	IgH break- apart probe	LSI IGH dual-colour break apart	Vysis (Abbott) 32-191019
t(4;14)	Translocation probe	LSI IgH/FGFR3 Dual Fusion	Vysis (Abbott)
t(11;14)	Translocation probe	LSI IGH/CCND1 XT Dual colour, Dual Fusion	Vysis (Abbott)
t(14;16)	Translocation probe	MAFA/IGH	Vysis (Abbott) 32-231014
17p11.1-q11.1		CEP17 (D17Z1)	Vysis (Abbott) 32-132017
17p13.1	<i>TP53</i>	LSI p53(17p13.1)	Vysis (Abbott) 32-190008
22q11.2	LSI 22	BCR	Vysis (Abbott) 32-192024
t(14;20) 20q12	MAFB	dJ54G6, dJ600E6, bA264H10, dJ1123D4, bA79G10	Sanger Institute (Hinxton, UK) RP11-79G10, RP11- 264H10, RP5-1123D4, RP1-54G6, RP4-600E6
t(6;14) 6p21	CCND3	bA298J23, dJ973N23, bA7K24, bA533O20	Sanger Institute (Hinxton, UK) RP11-298J23, RP5-973N23, RP11-533O20, RP11-7K24
1p12		bA418J17	In-house – Wessex
1p32.3	CDKN2C	bA278J17	In-house – Wessex
1q22	CKS1B	bA3217	In-house – Wessex
1q21.1	PDZK1	ba307C12	In-house – Wessex
13q14	RB1	bA305D1, bA174I10	In-house – Wessex, RMC, Bari, Italy.
Myeloma XI			
Del17p	<i>TP53</i>	LPS037	Cytocell (Cambridge , UK)
14 Rearrangement	IgH break- apart probe	LPH070	Cytocell (Cambridge , UK)
t(4;14)	Translocation probe	01N69-020	Vysis (Abbott)
t(11;14)	Translocation probe	08L58-020	Vysis (Abbott)
t(14;16)	Translocation probe	LPH073	Cytocell (Cambridge , UK)
t(14;20)	Translocation probe	LPH077	Cytocell (Cambridge , UK)

2.2.6 Multiplex ligation-dependent probe amplification

MLPA is a copy number assay, which determines the relative copy number changes of multiple DNA sequences that are produced in polymerase chain reaction (PCR). Two specifically designed probe mixes, the SALSA MLPA P425-B1 probemix and the X073-A1 probemix (MRC Holland, Amsterdam, The Netherlands) were compared with 11 reference regions for the chromosomal regions that are rarely altered in terms of myeloma copy number in MM. The contents of the probemix for P425-B1 and X073-A1 are listed in Table 12. The MLPA reaction uses dilution of 10-20ng/ μ l genomic DNA in 5 μ l volume. Samples were processed as per manufacturer's instructions by the laboratory technician within the team.

The assay used DNA that had been extracted as described above, which was eluted in tris-ethylenediaminetetraacetic acid (TE) buffer. Each MLPA probe consisted of two oligonucleotides that had to hybridise to adjacent DNA targets in order to be ligated by a thermostable ligase. The hybridisation sequence of each probe was 55-80 nucleotides (nt) long. After successful ligation, only complete fragments were amplified via 35 cycles of PCR and a fluorescence signal was produced. Unbound oligonucleotides were washed away. Only one pair of PCR primers was used to amplify all fragments. Only bound and ligated probes can amplify exponentially during a PCR reaction, so the amplification of a given probe depends on the presence and abundance of its target sequence in the sample DNA. Each probe generates an amplification product of unique length (130-490nt) which is separated and quantified by capillary electrophoresis. In each run, 13 reference germline male DNA samples are included. The assay uses 6-FAM as a probe fluorophore, and LIZ 500 as a size standard. MLPA analysis in tumour samples provides an average copy gain or loss within the tumour. In this study, PCR products were analysed through use of ABI 3730 DNA analyser (Life Technologies, Paisley, UK).

Samples were analysed by Coffalyser.net software (MRC-Holland). There are two steps to normalisation; the first is intra normalisation within reference probes that are relatively invariant in myeloma. The median of 10 reference probes is then calculated and a relative ratio is derived from the various probe values in the sample. A second normalisation step takes the intra-normalised ratio in a sample and divides this ratio with the sample probe in every reference sample. Differences in relative amounts of

probe-target sequences in the same sample result in different relative heights between patient sample and reference sample. The relative ratio reflects the copy number of target sequences (267). Interpretation of relative peaks is detailed in Table 11, based on previously published analyses (268, 269). An example of the output for one patient, which is displayed in a graphical format, is shown in Figure 9.

For the analysis that is described in the *TP53* results chapters of subclonal deletional data, alternate cut-offs were analysed and defined within the results with iFISH validation. Similarly, gain(1q) was also analysed in terms of sub-clonal gains and amplification using different MLPA cut-off values for exploratory research.

Table 11: Cut-off thresholds for MLPA copy number calling. These are used in the results chapter unless otherwise stated

Relative peak (sample vs. reference)	Interpretation of copy number
>1.6	Amplification (>3 copies)
1.2-1.6	Gain (3 copies)
0.75-1.19	Normal
0.25-0.74	Heterozygous loss
<0.25	Homozygous loss

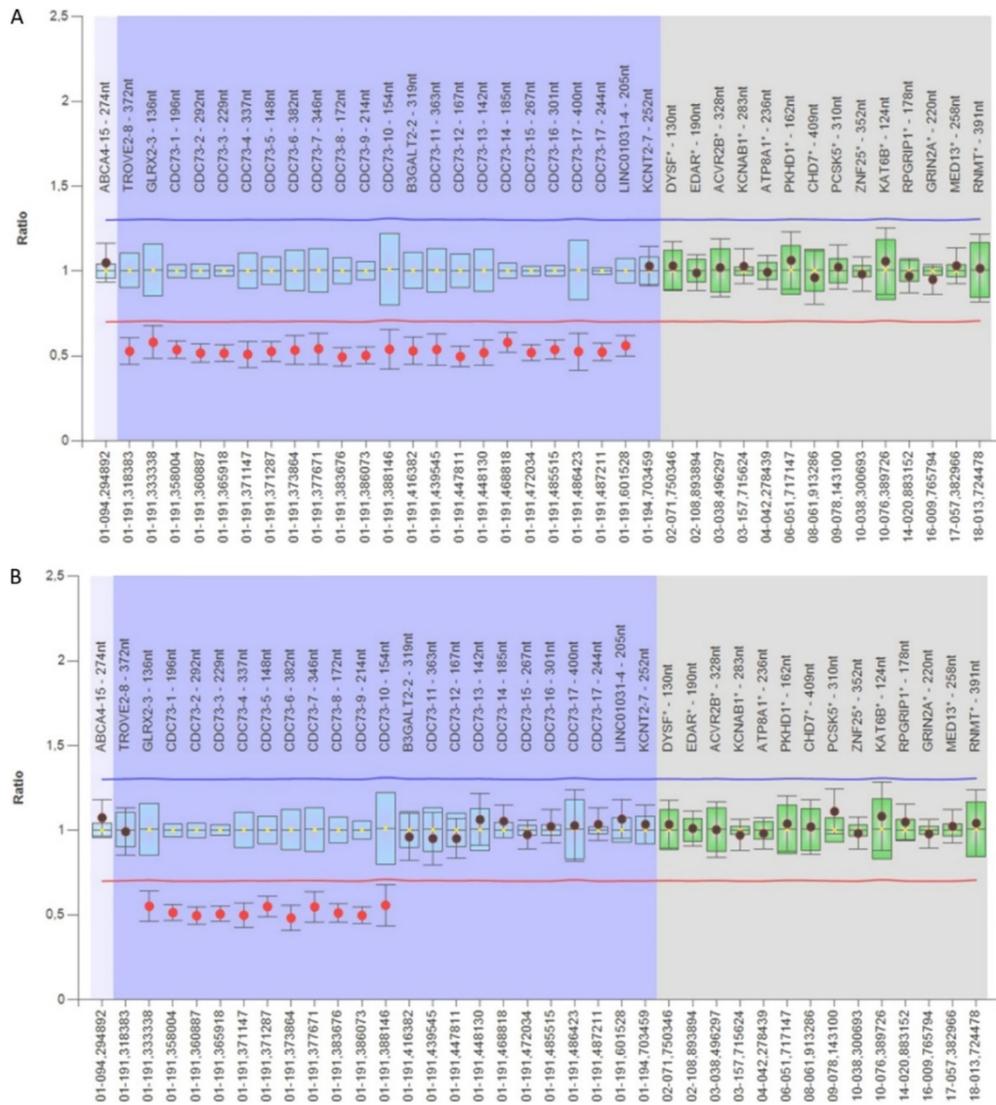


Figure 9: Example of tumour sample analysed by MLPA with graphical output by Cofalyzer software. Red dots display probe ratios and error bars the 95% confidence intervals. The displayed sample contains several aberrations (270)

Table 12: MLPA probe mix used in the Myeloma XI trial

Probemix P425-B1			Probe mix X073-A1		
Gene	Genbank exon	Chrom. Position	Gene	Genbank exon	Chrom. Position
FAM46C	2	1p12	KIAA0319	22	6p22.3
COL11A1	45	1p21.1	TNF	4	6p21.33
DPYD	21	1p21.3	CDKN1A	3	6p21.2
RPE65	14	1p31.2	PKHD1	56	6p12.3
LEPR	5	1p31.3	EYS	42	6q12
PPAP2B	2	1p32.2	TNFAIP3	6	6q23.3
DAB1	2	1p32.2	WTAP	8	6q25.3
DAB1	14	1p32.2	PARK2	3	6q26
FAF1	4	1p32.3	PARK2	12	6q26
CDKN2C	3a	1p32.3	GATA4	5	8p23.1
CKS1B	1	1q21.3	TNFRSF10B	5	8p21.3
CKS1B	2	1q21.3	TNFRSF10A	1	8p21.3
PBX1	9a	1q23.3	NEFL	1	8p21.2
NUF2	14	1q23.3	ZNF703	2	8p11.23
RP11-541J2	-	1q23.3	CHD7	2	8q12.2
RP11-541J2	-	1q23.3	RRM2B	3	8q22.3
RP11-480N10	-	1q23.3	MYC	1	8q24.21
PCDHA1	1b	5q31.3	MYC	3	8q24.21
PCDHGA11	1b	5q31.3	ANO5	22	11p14.3
PCDHAC1	1a	5q31.3	CCND1	3	11q13.3
PCDHB2	1	5q31.3	CCND1	5	11q13.3
PCDHB10	1	5q31.3	BIRC3	2	11q22.2
SLC25A2	1	5q31.3	BIRC2	2	11q22.2
JAK2	6	9p24.1	JAM3	7	11q25
COL5A1	40	9q34.3	NCAPD3	2	11q25
VAMP1	4b	12p13.31	TP53	1	17p13.1
CHD4	40	12p13.31	TP53	1	17p13.1
NCAPD2	32	12p13.31	TP53	2a	17p13.1
CD27	3	12p13.31	TP53	3	17p13.1
CHD4	2	12p13.31	TP53	4b	17p13.1
RB1	8	13q14.2	TP53	5	17p13.1
RB1	26	13q14.2	TP53	6	17p13.1
DLEU1	1	13q14.2	TP53	7	17p13.1
DIS3	6	13q22.1	TP53	8	17p13.1
DIS3	18	13q22.1	TP53	9a	17p13.1
TRAF3	3	14q32.32	TP53	10	17p13.1
TRAF3	11	14q32.32	TP53	11	17p13.1
GABRB3	11	15q12	TP53	11	17p13.1
IGF1R	18	15q26.3	HIRA	3	22q11.21
CYLD	2	16q12.1	SMARCB1	9	22q11.23
CYLD	19	16q12.1	NF2	4	22q12.2
WWOX	1a	16q23.1	LARGE	5	22q12.3
WWOX	7	16q23.1	EP300	19	22q13.2
TP53	4b	17p13.1	BRAF	15; V600E	7q34
TP53	7	17p13.1			
TP53	10	17p13.1			

2.2.7 Multiplexed real-time quantitative reverse transcriptase PCR (qRT-PCR)

Multiplexed real time quantitative reverse transcriptase PCR (qRT-PCR) was used to determine *IGH* translocation status using a translocation and cyclin D (TC) classification-based algorithm, as previously described (271). Although I have conducted this analysis on a number of patient samples, the majority of the laboratory work to process the qRT-PCR was carried out by the Myeloma Group biobanking team. Briefly, complementary DNA was synthesised from 500ng RNA using SuperScript III reverse transcriptase (Life Technologies) and random hexamer primers as per manufacturers' instructions. Multiplexed qRT-PCR assays that used fluorophore-labelled hydrolysis probes (Life Technologies) had been designed in-house or were adapted from the publications that are shown in Table 13 and Table 14. The genes named fibroblast growth factor receptor 3 (*FGFR3*), Wolf-Hirschhorn-Syndrome candidate 1 (*MMSET/WHSC1*), cyclin D1 (*CCND1*), cyclin D2 (*CCND2*), cyclin D3 (*CCND3*), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (*MAF*), v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (*MAFB*), integrin subunit beta 7 (*ITGB7*) and chemokine (C-X3-C motif) receptor 1 (*CX3CR1*) were assayed in multiplex with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Reactions were set up in 20ul volumes using the fast-advanced master mix (Life Technologies) and run in 96-well plates on a real-time PCR cycler (7500 Fast, Life Technologies) in the standard two hour run protocol. Samples were run in triplicate. Inter-assay standard and no-template controls were run in each plate. Δ CT values of target genes relative to concentrations of GAPDH of each sample were calculated to generate relative quantitation (RQ) values. Translocations were allocated by applying the algorithm that had previously been published. RQ values and the algorithm that was used are summarised in Table 13.

Table 13: RT-qPCR algorithm for translocation calling.

Translocation	Algorithm for translocation calling
t(4;14)	FGFR3>1500
	MMSET >5000 (as long as CCND1 not >15000)
t(11;14)	CCND1 >15000
t(6;14)	CCND3>250000
	CCND2
t(14;16)	MAF >2000 & (ITGB7 or CX3CR1 >50000)
t(14;20)	MAFB >20000 & (ITGB7 or CX3CR1 >50000)

Table 14: Primer and probe sequences for RT-qPCR translocation calling

	Primer and probe sequences
MMSET	F: acggcacaccctacgttacc R: ctcaaaggtgacgttgtaa Probe: VIC-caccaccgacaaggagctagaggttctct-MGB
FGFR3	F: gagaaggacagttgaaaaattatgc R: ctcaaaggtgacgttgtaa Probe: VIC-caccaccgacaaggagctagaggttctct-MGB
CCND1	F: ccgtccatgcggaagatc R: gaagacctcctcctcgcact Probe: VIC-tctgttctcgcagacctccagca-MGB
CCND2	F: caccaacacagacgtggattgt R: cggtagctgctcaggctattg Probe: FAM-caaagcttgccaggagcagattgagg-MGB
CCND3	F: ccatcgaaaaactgtgcatctaca R: cctcccagtccecgaaact Probe: VIC-cgaccacgctgtctctccccg-MGB
MAF	F: gcttcgagaaaaacggctc R: tgcgagtgggctcagttatg Probe: FAM-cgacaaccctcctctccccgagttt-MGB
MAFB	F: gcccgaccgaacagaagac R: ctcgggcgtcaggttgag Probe: FAM-agcagatgaacccc-MGB
ITGB7	F: ctacgcttaccctcctct R: gactccagcaacgtgtgaca Probe: VIC-caagggtcacgggtggaagacaggct-MGB
CX3CR1	F: ataggtacctggccatcgtc R: ggtagtcaccaaggcattca Probe: FAM-accgtgcagcatggcgctcac-MGB
GAPDH	F: gaaggtgaaggtcggagtc R: gaagatggtgatgggatttc Probe: NED-caagcttcccgttctcagcc-MGB

2.2.8 Gene expression arrays

Global expression analysis was carried out on patient samples using the Affymetrix U133 Plus 2.0 arrays marked MMProfiler (SkylineDx, The Netherlands), which could analyse the expression of over 50,000 transcripts. The laboratory work that was required to process the arrays was carried out by the Myeloma Group biobanking team. Briefly, 100ng of tumour RNA was amplified and hybridised to arrays of human genome U133 Plus 2.0 by following the manufacturer's instructions. Biotin-labelled cRNA was obtained by the use of the one-cycle eukaryotic-labelling assay (Affymetrix). Arrays were washed on an Affymetrix fluidics station 450 and scanned using Affymetrix GeneChip scanner 3000 7G.

Raw data from expression arrays (CEL files) were processed using the software program R version 3.3.0. Quality control included scale factor and percentage of genes that were present. Expression data were normalised by use of GC-robust multi-array average (GCRMA) (with adjustment for optical noise, non-specific binding, quantile normalisation and median polish) and MAS5 normalisation (antilog of robust average (tukey biweight)). Final quality control of arrays included relative log expression and normalised unscaled standard errors (NUSE) from the "AffyPML" package (www.bioconductor.org). Arrays that showed a NUSE value greater than 1.05 and aberrant relative log expression (RLE) plots were excluded from analysis. Probes that expressed log-fold change of more than 1 and an adjusted *P*-value of <0.05 were considered significant.

Anonymised GEP data were transferred and processed centrally by SkylineDx as per standard protocol and binary SKY92 risk status (high risk/standard risk) were reported back as part of a standard diagnostic report. CEL files were also normalised by SkylineDx, which generated a proprietary 'SKY92' risk score for prognostic validation of this signature.

For exploratory analyses, data were normalised as described previously for the Erasmus University Medical Center (EMC)92 score and batch-corrected to HOVON gene expression arrays that were deposited at the US National Center for Biotechnology Information's gene expression omnibus (NCBI-GEO) repository (GSE19784) (272) through use of the "ComBat" software package. Continuous gene-

expression scores for other risk signatures were calculated as previously described. Continuous gene expression scores for other risk signatures were calculated as previously described (272-277). Differential gene expression between groups was assessed using the `lmfit` function in the “Limma” package. Myeloma XI CEL files that were used in this analysis have been deposited at the European Genome-phenome Archive (EGA) repository (accession EGAS00001004183). Myeloma IX CEL files have been previously deposited at the NCBI-GEO repository GSE15695.

2.2.9 Exome sequencing and mutation calling

DNA samples from both tumours and peripheral blood were used in the exome capture protocol as previously described. They were analysed by the myeloma group team in work that has been previously published by our group (53, 278). A total of 463 trial samples underwent whole exome sequencing (WES) and targeted capture of IGH/K/L and MYC loci. Samples of 200ng of DNA were processed through use of the NEBNext DNA library preparation kit and hybridised to SureSelect Human All Exon V5 Plus. Four samples were pooled and run on one lane of a HiSeq 2000 using 76-bp paired end reads. FASTQC version 0.10.0 was used for basic quality control of illumine paired-end sequencing data. Single-nucleotide variants were called using MuTect (version 1.14; www.broadinstitute.org/cancer/cga/mutect). Copy numbers across the exome were determined using the control-FREE copy number and allelic content caller (FREEC) and the cancer clonal fractions were calculated (279). Data were deposited in the EGA under accession number #EGAS00001001147.

2.3 Data collection, collation and quality control

2.3.1 Myeloma IX data sets

The number of patient samples that contained numerical iFISH calls, iFISH derived clonality information, gene expression arrays and SNP mapping arrays was dependent on the quality and quantity of samples. The iFISH samples required the least amount of material and there is relatively comprehensive data on this. The iFISH data from Myeloma IX have been used for validation of Myeloma XI trial findings with regard to genetics and to perform meta-analyses. Data that were previously unused, such as clonality information from previously recorded iFISH data, have also been re-analysed. Gene expression arrays and copy number arrays have been analysed for validation of the results that were gathered from the Myeloma XI trial. Clinical data

for this trial were provided by the Leeds Institute of Clinical Trials Research at the University of Leeds. Survival data for all analyses that were performed for this thesis used the last 2012 cut-off prior to the closure of the trial to follow up.

2.3.2 Myeloma XI data sets

As Myeloma XI is an ongoing trial that is still in the follow-up stage, several trial endpoints have not been met. As a result, data were released sequentially with different cut-offs for data that were used for analyses that are described in different chapters of this thesis. Additionally, more clinical variables were released over the period in which this PhD was conducted in exchange for the processing of genetic information that was sent to the clinical trials unit. The quality and quantity of samples also determined which analyses were possible in terms of exome sequencing, MLPA analyses, translocation calling by RT-qPCR and gene-expression arrays that were used in this thesis. This has resulted in the analysis of several overlapping data sets over time as these data were processed.

2.3.2.1 Quality control and data cleaning

Clinical data were reviewed and any values that were biologically implausible were set as missing at the time of analysis. Errors were fed back to the clinical trials unit and subsequent data cuts were corrected after manual checks of source data, and these corrections resulted in reductions in the number of missing values in subsequent analyses.

Clinical data in terms of dates of randomisation, date of the start of induction treatment, date of sample collection and receipt were matched to find all presentation samples. Data entry errors were thus corrected in order to obtain a comprehensive list of patients with diagnostic genetics. DNA and RNA extraction followed by the tests detailed above was then carried out on the comprehensive list of presentation samples.

The iFISH data for Myeloma IX were re-analysed with additional data that were provided by Dr Chiecchio from the Wessex genetic laboratory. Clonal deletions and gains were re-analysed and manually checked for errors after initial automated data extraction using pattern recognition in R.

MLPA data quality was checked as per the manufacturer's instructions. Further quality checks for discrepancies between P425 and XO73 were also conducted during joint analyses. *TP53* probes within the XO73 MLPA probe sets were checked for variance and one probe was removed from analyses due to oversensitivity for calling deletion of this probe (detailed in results sections).

Translocation calling that employed RT-qPCR was analysed with removal of any sample with a GAPDH standard deviation of greater than 0.1 within repeat samples. Positive and negative controls were checked for quality. Analysis of samples was repeated if they failed any of the above quality-control measures. Quality control of arrays for gene expression was undertaken as described in the gene expression array section of this chapter.

2.4 Statistical analysis

All statistical analyses were performed through use of the R software. Data were released in stages during the course of this project; therefore different versions of R were used during each analysis procedure. The dates of data cut-off for follow up also varied as a result. Details that are explained in each chapter with data cut-off dates and R versions are listed in Table 15.

Table 15: Summary of R version that was used for analysis and date of data cut off for different results chapters

Results chapter	R version	Date of cut-off for Myeloma XI data	Date of cut-off for Myeloma IX data
Meta-analysis of Myeloma IX and XI	3.3	July 2015	2012
<i>TP53</i>	3.4.1	July 2016	2012
Gene expression	3.5.1	July 2017	2012
Gain (1q)	3.6.2	July 2018	2012

The R packages “Survival” and “Survminer” were utilised for the analyses of overall survival (OS) and progression-free survival (PFS) rates to calculate median survival with 95% confidence intervals. PFS was defined as the time from the date of randomisation to progression, according to IMWG criteria, or to death from any cause. OS was defined as the time from the date of randomisation to death from any cause. Kaplan–Meier survival curves were generated and the homogeneity between groups was evaluated through application of the log-rank test. The R package “rms” was used to perform multivariable and univariate model testing. Variables that were associated with survival in univariate analyses at $P < 0.05$ were entered into multivariable testing. Multivariable analysis of variables that were associated with survival employed a backward elimination Cox proportional hazards model; factors were retained if they were significant at $P < 0.05$. Formal tests for interaction used a Cox regression model, with chi-square values that were derived from the difference of the log likelihood values.

The R package “metafor” (280) was used to perform fixed-effect meta-analyses on individual patient data within the Myeloma IX and Myeloma XI trials. The weighted least squares method that took into account variance within each study was used to assess the overall effect of copy number changes and translocation status in NDMM. This was performed through use of the restricted maximum likelihood estimator “REML” model.

The R package “survC1” was used to test the risk prediction ability of models with censored survival data. This is a measure of goodness-of-fit for binary outcomes in a logistic regression model. It shows the probability that a randomly selected patient who experiences an event has been allocated a higher risk score than a patient who has not experienced the event. It is equal to the area under the receiver operating characteristic (ROC) curve and ranges from 0.5 to 1. A value of 0.5 indicates a poor model. Values of 0.7 indicate a good model and those that are >0.8 indicate a strong model. The package ‘SurvivalROC’ was used for the calculation of time-dependent ROC statistics to assess survival modelling over time. Optimal cut-point estimation was calculated using the AUC_i, log rank statistics, Cox-based Wald P-value and the “maxstat” package using standardised log-rank statistics.

The R packages “JAGS” and “BayesMed” were used to carry out correlation analyses between clinical variables, mutations, copy number changes and translocation status using Bayesian inference. A Bayes factor (BF₀₁) of BF₀₁ <0.01 was considered significant. Correlation between continuous variables was also calculated using Pearson product-moment correlations through use of the base R package. Rank-based measures of association were calculated by application of the Spearman correlation using the base R package.

The association between categorical variables was examined using Fishers exact test. The association between myeloma subtype and gene expression was assessed using the Mann-Whitney test. A two-sided *P*-value <0.05 was considered significant.

3 Meta-analysis of Myeloma IX and Myeloma XI trials

3.1 Introduction

Survival rates for multiple myeloma (MM) have improved over the last decade due to the introduction of novel therapies, earlier detection, and improvements in supportive care. The median five-year survival rates are typically now around 50% and rising (281, 282). Despite this improved response to treatment, survival of newly diagnosed multiple myeloma (NDMM) is heterogeneous; survival ranges from two to >10 years (1). Underlying genetic aberrations are an important cause of this observed heterogeneity and are explored further within this chapter. The focus here is on cytogenetic abnormalities that have a known association with poor prognosis. The introduction chapter of this thesis (Chapter 1) details both primary (t(4;14) (55, 67, 145, 283), t(14;16) and t(14;20) and secondary (deletion 17p, gain 1q and deletion 1p) genetic abnormalities and provides evidence of their association with shortened survival periods.

There are currently areas of debate with regard to these lesions. Studies show that the heterogeneous impact of t(4;14) is related to concomitant chromosomal aberrations (60, 61). Differences in trial results have led to lack of clarity regarding any association between t(14;16) and poor prognosis (68). Translocation t(14;20) is also associated with poor prognosis but this has not been established robustly since it occurs rarely (67, 284, 285) and as a result, it is frequently not included in definitions of cytogenetic high risk in most prognostic scoring systems (69, 225, 226).

Hyperdiploidy, in which multiple copies of odd-numbered chromosomes are produced, is observed in approximately 50% of MM cases and is thought to be a primary cytogenetic abnormality. It is understood to be associated with longer PFS and OS compared with the abovementioned IgH translocations (145). However, studies have demonstrated that coexistence of hyperdiploidy with MM does not abrogate poor prognosis with adverse cytogenetics (46), whereas other studies have found that hyperdiploidy abrogates the risk of co-existent high-risk lesions (45).

Chromosome 1 abnormalities have long been known to be highly prevalent in MM. Gain(1q) (>2 copies 1q) has been found to be associated with short survival times in some studies (125, 286), but others have failed to confirm the negative prognostic association of gain(1q) (84, 134). There is also debate as to whether gain(1q) or only amp(1q) causes the negative association with prognosis in myeloma (100, 287). Additionally, del(1p) is under investigation for its association with survival but is not included in most definitions of high-risk genetics in MM due to heterogeneity in results (67).

As a result of conflicting as well as limited evidence in some cases, there is wide variability regarding what is classified as high risk in NDMM (67, 69, 257). Multiple trials now define high risk in different ways, so comparison of treatments is a challenge. Several studies do not report risk of individual high-risk lesions; instead researchers opt to report only del(17p) and t(4;14) as high risk together (226). As a result of discrepancies in reporting practices, individualisation of therapy according to underlying genetic changes is not routine in myeloma, unlike in several other haematological cancers. Since many of the molecular abnormalities in MM are present only at relatively low frequency, robust establishment of the impact of their presence on prognosis is contingent on the analysis of large patient series that have been uniformly treated.

Although there are several prognostic models that are used to improve risk stratification of patients with NDMM, none of these markers currently explain completely the heterogeneity that is seen in the disease. We and others have recently reported that the co-occurrence of multiple genetic lesions may have greater significance for the prediction of patient outcome than the presence of any single abnormality (67, 137, 288). The work that is described in this thesis has established this association further in the meta-analysis.

To establish robustly the effect of cytogenetic abnormalities and to assess the prognoses that are associated with rare lesions, we report a meta-analysis of the relationship between genetic profile and prognosis in NDMM through the use of data from two UK multi-centre phase III clinical trials that involved 1,905 patients. Validation of the UK-based prognostic tool that combines tumour burden (ISS) and

genetics has also been carried out. This dataset used include previously generated data on the MRC Myeloma IX trial. Additionally, we have analysed molecular copy number profiling in 1,036 Myeloma XI patients to identify sub-groups with specific molecular abnormalities that could be targeted therapeutically (53, 65).

The two trials represent treatment of myeloma over a decade of advances and represent one of the largest, uniformly treated populations of myeloma patients in the world. The Myeloma IX trial treated patients with conventional chemotherapy or thalidomide-based regimens in a randomised setting. The Myeloma XI trial treated patients with a large IMiD-based component and with access to proteasome inhibitors (PIs). Overall survival (OS) in these patients, however, encompassed use of additional novel agents to a growing extent as therapies such as PIs became routinely available off trial during this time.

3.2 Aims

- Confirm association shortened survival in myeloma with high-risk genetic lesions in the context of novel therapy
- Investigate risk associated with low frequency lesions through meta-analysis of two large trials
- Validate Myeloma IX MRC risk signature combining ISS and high risk genetic lesions
- Investigate high risk lesions in the context of hyperdiploid myeloma

3.3 Results: Comparison and meta-analysis of Myeloma IX and Myeloma XI data and analysis of hyperdiploid subsets

3.3.1 Descriptive patient characteristics and structural aberrations in the two trials

The data cut-off for this meta-analysis of Myeloma XI data was July 2016. This is an early timepoint compared with other analyses and the amount of clinical data that was available was limited. The clinical characteristics of the 1,036 newly profiled Myeloma XI trial patients and the 869 Myeloma IX trial patients are detailed in

Overall, there were no significant differences between the groups of trial patients in terms of gender, age range and the proportion of patients who had been in receipt of intensive/non-intensive therapy. These baseline demographics demonstrate the high level of inclusivity of the trials; the median age at trial entry was 65 and 67 in the Myeloma IX and XI trials respectively; poorer performance scores were included, as was markedly impaired renal function (see Chapter 2: Methods for inclusions). The Myeloma XI trial, for example, almost matched the median age of diagnosis for the overall population in the UK, which indicated the broad inclusivity of this trial. More than 40% of patients who were treated in these trials were on the non-intensive, transplant ineligible arm of the trials, which again reflected trial inclusivity. These trials were more representative of real-world data than most commercial and international trials, which were largely restricted to fitter patients. It must be recognised, however, that the Myeloma IX and XI trials of NDMM patients were not fully representative of real-world data in regard to the study of clinical and genetic associations in myeloma.

In terms of baseline genetic differences, the frequencies at which the primary IGH translocations, del(17p), del(1p32), del(13q) and del(16q), occurred in tumours were similar in Myeloma IX and XI trial patients. A higher proportion of Myeloma IX patients had hyperdiploidy (HRD), gain(1q) and del(22q) (Table 17). Among Myeloma XI trial patients, homozygous deletion of *CDKN2C* (1p32), *BIRC2/BIRC3* (11q22) and amplification of *CKS1B* (1q21) and *MYC* (8q24) were the most common focal homozygous copy number changes. These changes were seen at frequencies that were similar to those previously reported. Homozygous focal change information was largely not available for the Myeloma IX trial due to the collection of the genetic information through performance of iFISH for this trial (76).

Table 16. Clinical characteristics and frequency of occurrence of genetic aberrations in Myeloma IX and Myeloma XI trial patients

	Myeloma IX trial		Myeloma XI trial		
	(Total n =869)	Missing Information	(Total n=1,036)	Missing Information	p-value
Clinical characteristics					
Female	339 (39.0%)		398 (38.4%)		0.81
Male	530 (61.0%)		638 (61.6%)		0.81
Intensive treatment pathway	511 (58.8%)		598 (57.5%)		0.64
Non-intensive treatment pathway	358 (41.2%)		438 (42.3%)		0.64
International Staging System (ISS) I	130 (20.7%)	240	225 (23.1%)	61	0.27
ISS II	253 (40.2%)	240	429 (44.0%)	61	0.15
ISS III	246 (39.1%)	240	321 (32.9%)	61	0.01
Median age (years)	65 (range 34-89)		67 (range 34-88)		1.0

Table 17. Frequency of genetic aberrations in Myeloma IX and Myeloma XI trial patients

	Myeloma IX trial		Myeloma XI trial		<i>p</i> -value
	(Total n=869)	Missing Information	(Total n=1,036)	Missing Information	
<i>Primary lesions (translocations, hyperdiploidy)</i>					
t(4;14)	104 (11.9%)		137 (13.2%)		0.45
t(4;14) FGFR3-negative	--		26 (2.5%)		
t(6;14)	8 (0.9%)	1	7 (0.7%)		0.61
t(11;14)	129 (14.8%)		175 (16.9%)		0.23
t(14;16)	27 (3.1%)		38 (3.7%)		0.53
t(14;20)	13 (1.5%)		13 (1.3%)		0.69
Hyperdiploidy	499 (58.9%)	22	488 (47.1%)		3x10 ⁻⁷
<i>Copy number abnormalities</i>					
Del(1p32)	87 (10.7%)	60	107 (10.3%)		0.82
Gain(1q) or Amp(1q)	340 (39.1%)		357 (34.5%)		0.04
Gain(1q)	--		277(26.7%)		
Amp(1q)	--		80 (7.7%)		
Gain(6p) or Amp(6p)	--		122 (12.1%)	29	
Gain(6q) or Amp(6q)	--		69 (6.9%)	29	
Del(6q)	--		157 (15.6%)	29	
Del(8p)	--		164 (16.3%)	29	
Gain(8q)	--		43 (4.3%)	29	
Gain(11q25)	--		418 (41.5%)	29	
Del(12p)	--		78 (7.5%)		
Del(13q)	389 (45.1%)	6	425 (41.0%)		0.07
Del(14q)	--		144 (13.9%)		
Del(16q)	153 (17.6%)	46	175 (16.9%)		0.36
Del(17p)	78 (8.9%)		96 (9.3%)		0.87
Del(22q)	100 (13.1%)	103	103 (10.2%)	29	0.04
<i>Focal copy number abnormalities/mutations</i>					
<i>CDKN2C</i> homozygous del	--		19 (1.8%)		
<i>BIRC2/BIRC3</i> homozygous del	--		22 (2.2%)	29	
<i>MYC</i> amplification	--		28 (2.8%)	29	
<i>CCND1</i> focal gain	--		46 (4.6%)	29	
<i>BRAF</i> V600E mutation	--		36 (3.6%)	29	

3.3.2 Relationship between cytogenetic aberrations and survival

Cytogenetic and ISS associated risk was examined within the two trials. In both trial series, the archetypical high-risk lesions del(17p), gain(1q) and t(4;14) were each found to be significantly associated with shorter PFS and OS by Cox proportional hazards regression. These findings were validated by performance of Kaplan-Meier-based log rank testing of these lesions. The results are displayed graphically (Table 18; Table 19, Figure 10, Figure 11, Figure 12)

In both trials, ISS was found to retain its prognostic association although ISS III appeared to have a relatively higher hazard ratio (HR) in Myeloma XI for both PFS and OS. Levels of LDH were not available to be used as clinical markers in the analysis of R-ISS at this timepoint. Genetic factors that were known to be associated with survival retained this association in both studies except for del(1p32) in the Myeloma IX trial, where there was a trend towards significance. This was also the case for t(14;20) in Myeloma IX, but few cases were included.

Next, we performed fixed-effects meta-analysis to evaluate the two trials together using individual patient data. In this combined analysis, respective HRs for OS were 2.1 for del(17p) ($P=8.86 \times 10^{-14}$), 1.68 for gain(1q) ($P=2.18 \times 10^{-14}$) and 1.60 for t(4;14) ($P=4.77 \times 10^{-7}$) (Table 19). In addition, the t(14;16) and t(14;20) translocations that involved *MAF* and *MAFB* were associated with shorter OS, with respective HRs of 1.74 ($P=0.0005$) and HR 1.90 ($P=0.0089$). Respective inference C-statistic estimates for adequacy of risk prediction are shown in Table 20 and Table 21.

Interestingly, the presence of t(4;14) was associated with a lower hazard ratio and *P*-value when PFS was compared with OS in both Myeloma IX and XI. This finding suggests that the association of this translocation with prognosis may weaken over time compared with other high-risk cytogenetic abnormalities. It may also be a feature of the type of treatment that was administered in these trials. All other known high-risk cytogenetic abnormalities such as the *MAF* translocations, del(17p), gain(1q) and del(1p), showed a worse association with poor survival in terms of OS compared with PFS. This finding suggests that the prognostic role of these cytogenetic abnormalities increases over time or this may be due to selective pressure from therapy. This result

is supported by c-stats comparison of PFS and OS for the above cytogenetic lesions (Table 20; Table 21). This finding could be due to many reasons, and these should be explored once more clinical data are available from the Myeloma XI trial.

Through the meta-analysis of these two trials, the data that were collected for this project confirm the prognostic role of the above cytogenetic abnormalities in both trials, which encompassed 1,905 patients. This is particularly important regarding the MAF translocations, which occur rarely in myeloma and prognosis has not been previously comprehensively established.

Table 18: Cox-based regression showing relationship between presence of genetic abnormalities and patient progression-free survival

	Myeloma IX n=869		Myeloma XI n=1036		Combined n=1905		Heterogeneity
	HR (95% CI)	<i>p</i> -value	HR (95% CI)	<i>p</i> -value	HR (95% CI)	<i>p</i> -value	<i>p</i> -value
t(4;14)	1.88 (1.52-2.23)	5.31x10 ⁻⁹	1.51 (1.22-1.88)	0.0001	1.69 (1.45-1.96)	9.30x10 ⁻¹²	0.16
t(14;16)	1.50 (1.01-2.22)	0.0425	1.51 (1.05-2.17)	0.0256	1.50 (1.15-1.96)	0.0026	0.98
t(14;20)	1.13 (0.64-1.99)	0.6852	1.54 (0.80-2.97)	0.1987	1.29 (0.84-1.98)	0.2509	0.48
Adverse Translocations	1.77 (1.47-2.13)	1.88x10 ⁻⁹	1.58 (1.31-1.91)	2.05x10 ⁻⁶	1.67 (1.46-1.91)	2.69x10 ⁻¹⁴	0.41
Del(17p)	1.54 (1.21-1.95)	0.0003	1.61 (1.26-2.06)	0.0002	1.57 (1.33-1.87)	2.07x10 ⁻⁷	0.79
Gain(1q)	1.53 (1.33-1.77)	6.70x10 ⁻⁹	1.53 (1.31-1.80)	1.34x10 ⁻⁷	1.53 (1.38-1.71)	4.61x10 ⁻¹⁵	1.00
Del(1p32)	0.99 (0.78-1.25)	0.9202	1.30 (1.02-1.66)	0.0331	1.13 (0.95-1.34)	0.1571	0.11
ISS II	1.40 (1.12-1.76)	0.0036	1.54 (1.23-1.92)	0.0002	1.47 (1.25-1.72)	2.50x10 ⁻⁶	0.58
ISS III	1.64 (1.30-2.06)	2.34x10 ⁻⁵	2.46 (1.96-3.09)	6.88x10 ⁻¹⁶	2.02 (1.71-2.37)	1.73x10 ⁻¹⁷	0.01

Table 19: Cox-based regression showing relationship between presence of genetic abnormalities and patient overall survival

	Myeloma IX n=869		Myeloma XI n=1036		Combined n=1905		Heterogeneity
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	p-value
t(4;14)	1.72 (1.36-2.17)	5.12x10 ⁻⁶	1.42 (1.06-1.91)	0.0188	1.60 (1.33-1.92)	4.77x10 ⁻⁷	0.33
t(14;16)	1.52 (0.98–2.35)	0.0607	2.00 (1.28-3.11)	0.0021	1.74 (1.27-2.37)	0.0005	0.39
t(14;20)	1.64 (0.88-3.07)	0.1213	2.35 (1.11-4.97)	0.0259	1.90 (1.17-3.07)	0.0089	0.47
Adverse Translocations	1.74 (1.42-2.14)	1.15x10 ⁻⁷	1.71 (1.33-2.20)	3.23x10 ⁻⁵	1.73 (1.47-2.03)	1.63x10 ⁻¹¹	0.90
Del(17p)	1.92 (1.49-2.48)	6.07x10 ⁻⁷	2.40 (1.77-3.24)	1.61x10 ⁻⁸	2.10 (1.73-2.56)	8.86x10 ⁻¹⁴	0.27
Gain(1q)	1.61 (1.37-1.91)	1.81x10 ⁻⁸	1.80 (1.44-2.24)	1.76x10 ⁻⁷	1.68 (1.47-1.92)	2.18x10 ⁻¹⁴	0.44
Del(1p32)	1.23 (0.94-1.61)	0.1170	1.83 (1.35-2.48)	0.0001	1.46 (1.20-1.78)	0.0002	0.06
ISS II	1.98 (1.47-2.68)	8.11x10 ⁻⁶	1.90 (1.30-2.77)	0.0009	1.95 (1.54-2.47)	2.76x10 ⁻⁸	0.86
ISS III	2.62 (1.94-3.53)	2.69x10 ⁻¹⁰	3.85 (2.66-5.56)	7.41x10 ⁻¹⁴	3.05 (2.42-3.85)	4.38x10 ⁻²¹	0.11

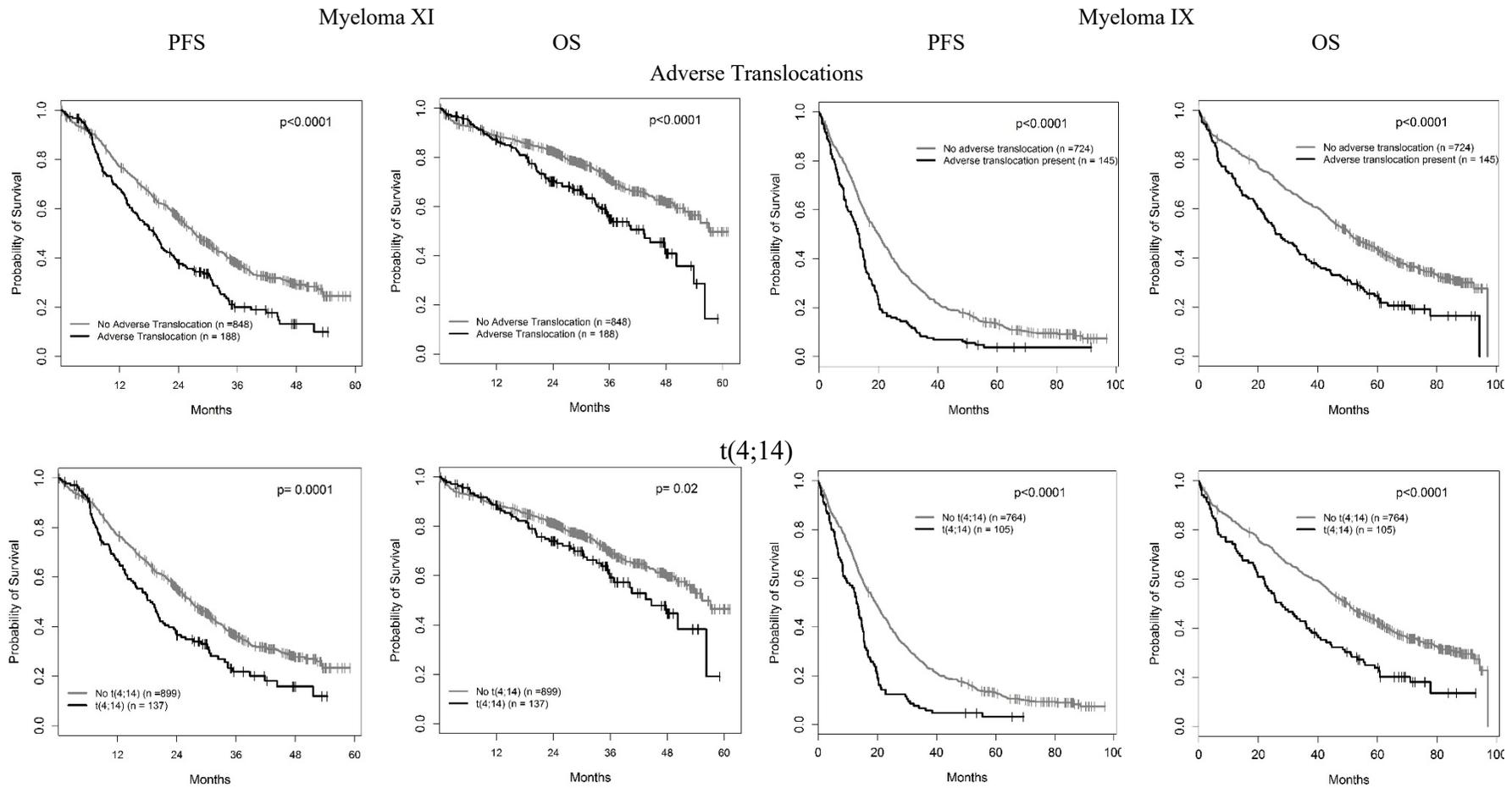


Figure 10: Kaplan-Meier curves and log-rank p-values for 1,036 NCRI Myeloma XI patients and 869 MRC Myeloma IX patients in the context of presence or absence of recurrent genetic aberrations

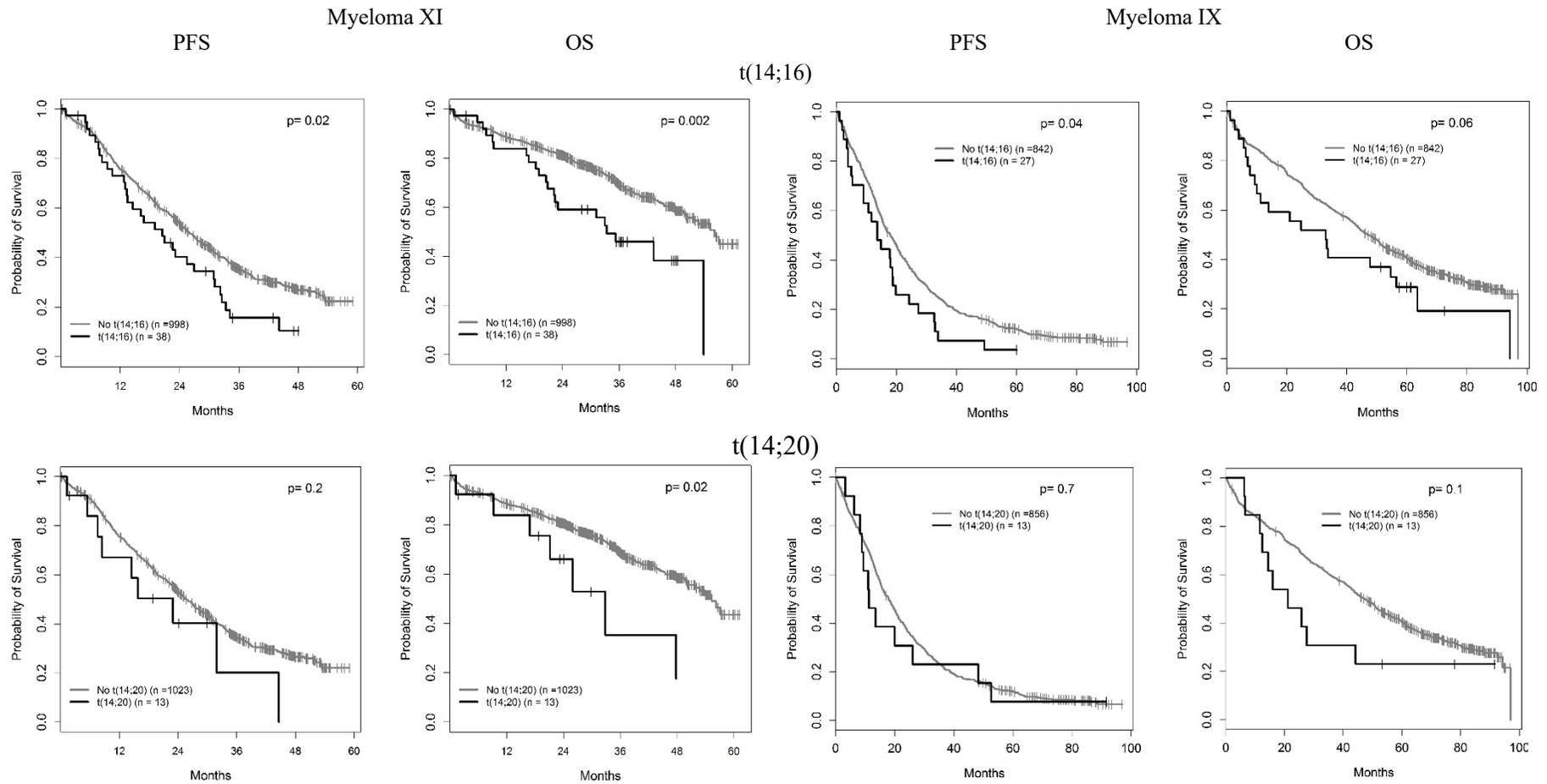


Figure 11: Kaplan-Meier curves and log-rank p-values for 1,036 NCRI Myeloma XI patients and 869 MRC Myeloma IX patients in the context of presence or absence of recurrent genetic aberrations

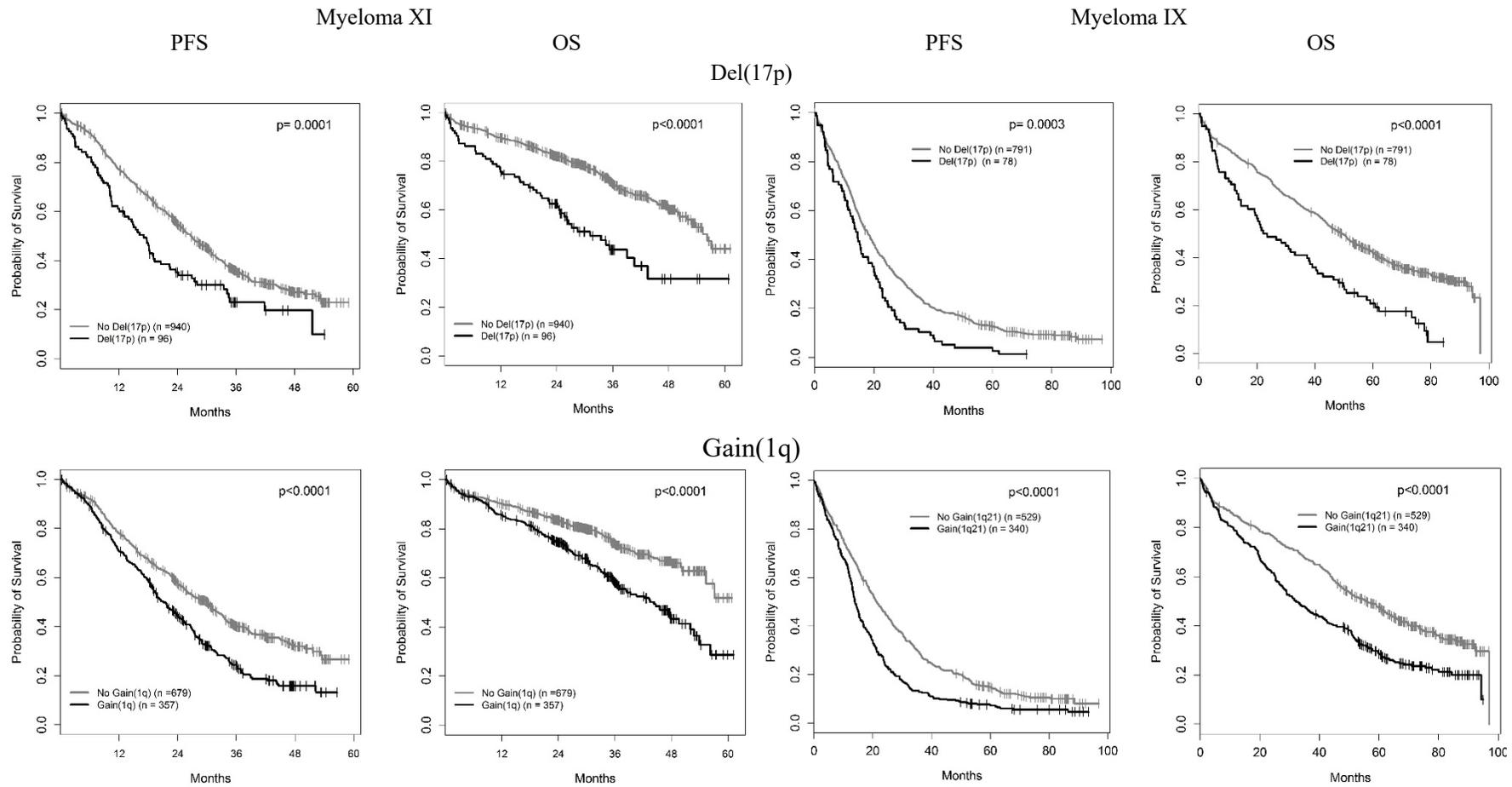


Figure 12: Kaplan-Meier curves and log-rank p-values for 1,036 NCRI Myeloma XI patients and 869 MRC Myeloma IX patients in the context of presence or absence of recurrent genetic aberrations

Table 20: Inference C-values using tau=24 months for PFS in the Myeloma IX and Myeloma XI trials

PFS

	Myeloma IX n=869				Myeloma XI n=1,036			
	Estimate	Standard error	Lower 95% CI	Upper 95% CI	Estimate	Standard error	Lower 95% CI	Upper 95% CI
Adverse translocations	0.5456	0.0080	0.5299	0.5613	0.5387	0.0093	0.5206	0.5568
t(4;14)	0.5379	0.0069	0.5245	0.5513	0.5300	0.0086	0.5131	0.5468
t(14;16)	0.5058	0.0043	0.4974	0.5142	0.5062	0.0047	0.4971	0.5154
t(14;20)	0.5019	0.0035	0.4950	0.5088	0.5025	0.0033	0.4961	0.5089
Del(17p)	0.5149	0.0063	0.5026	0.5272	0.5287	0.0076	0.5139	0.5436
Gain(1q)	0.5576	0.0108	0.5364	0.5788	0.5415	0.0111	0.5198	0.5633
Del(1p)	0.5000	0.0072	0.4859	0.5141	0.5170	0.0082	0.5010	0.5330
ISS	0.5719	0.0140	0.5445	0.5992	0.6036	0.0125	0.5791	0.6282

Table 21: Inference C-values using tau=36 months for OS in the Myeloma IX and Myeloma XI trials

OS

	Myeloma IX n=869				Myeloma XI n=1,036			
	Estimate	Standard Error	Lower 95% CI	Upper 95% CI	Estimate	Standard Error	Lower 95% CI	Upper 95% CI
Adverse Translocations	0.5501	0.0110	0.5285	0.5716	0.5418	0.0120	0.5184	0.5653
t(4;14)	0.5343	0.0094	0.5158	0.5528	0.5194	0.0116	0.4968	0.5421
t(14;16)	0.5096	0.0061	0.4977	0.5215	0.5163	0.0068	0.5029	0.5297
t(14;20)	0.5062	0.0046	0.4972	0.5152	0.5061	0.0038	0.4986	0.5136
Del(17p)	0.5289	0.0084	0.5125	0.5453	0.5462	0.0104	0.5259	0.5665
Gain(1q)	0.5711	0.0131	0.5455	0.5967	0.5658	0.0147	0.5369	0.5946
Del(1p)	0.5124	0.0108	0.4913	0.5335	0.5324	0.0101	0.5127	0.5521
ISS	0.5938	0.0160	0.5625	0.6251	0.6373	0.0155	0.6069	0.6678

3.3.3 Genetic markers and survival in intensively treated patients

Since young, fit patients are most likely to be considered for intensified combination therapy, we focused subsequently on the relationship between molecular profile and survival of this sub-group of Myeloma XI (n=598) and Myeloma IX (n=511) patients.

In these 1,109 intensively treated patients, del(17p), gain(1q) and t(4;14) were consistently associated with shorter PFS and OS; the combined HRs were 2.65 (3.04×10^{-12}), 1.77 (1.65×10^{-8}) and 1.87 (7.62×10^{-7}), respectively (Table 22; Table 23; Figure 13; Figure 14; Figure 15). In this group, t(14;16) was associated with shorter PFS (HR 1.80; $P=0.0021$) and OS (HR 1.82; $P=0.013$). The t(14;20) translocation was not associated with PFS or OS, but the lesion was only present in eight Myeloma IX and five Myeloma XI patients (Table 22, Table 23, Figure 13, Figure 14). The above data are confirmed by inference c-statistics (Table 24; Table 25).

Del(1p32) was significantly associated with shorter OS in both trials in the intensively treated groups and this was found in the combined meta-analysis (HR 1.46; $P=0.0002$). This association was confined to patients in receipt of intensive treatment (Table 23). The association of del(1p32) with OS was independent of gain(1q21) by multivariable analysis ($P<0.05$) in the intensive-treatment groups of both trials.

A study of intensively treated, young, fit patients through the intensive arm of both trials revealed a strong association between poor survival and known poor risk markers. This was also validated by c-stats for the same group. Clinical characteristics such as frailty and co-morbidities are likely to play a more prominent role among non-intensively treated, older and more frail patients than among the young and fit group. However, more clinical information on the trials will be needed for elucidation of the differences between intensively and non-intensively treated patients and regarding important characteristics that are associated with survival in each group.

Table 22: Relationship between presence of genetic abnormalities and progression-free survival of intensively treated patients

PFS

	Myeloma IX n=511		Myeloma XI n=598		Combined n=1109		Heterogeneity
	HR (95% CI)	<i>p</i> -value	HR (95% CI)	<i>p</i> -value	HR (95% CI)	<i>p</i> -value	<i>p</i> -value
t(4;14)	1.96 (1.49-2.59)	1.80x10 ⁻⁶	2.03 (1.56-2.64)	2.18x10 ⁻⁷	2.00 (1.65-2.42)	1.85x10 ⁻¹²	0.88
t(14;16)	1.60 (0.96-2.69)	0.0729	2.03 (1.19-3.47)	0.0099	1.80 (1.23-2.60)	0.0021	0.54
t(14;20)	0.96 (0.46-2.03)	0.9192	0.64 (0.09-4.54)	0.6524	0.91 (0.45-1.84)	0.7987	0.70
Adverse Translocations	1.81 (1.42-2.31)	1.79x10 ⁻⁶	2.09 (1.62-2.68)	8.88x10 ⁻⁹	1.94 (1.63-2.31)	1.07 x 10 ⁻¹³	0.42
Del(17p)	1.81 (1.30-2.51)	0.0004	1.81 (1.29-2.52)	0.0005	1.81 (1.43-2.28)	7.25x10 ⁻⁷	1.00
Gain(1q)	1.48 (1.22-1.80)	7.44x10 ⁻⁵	1.65 (1.31-2.07)	2.03x10 ⁻⁵	1.55 (1.34-1.80)	7.59x10 ⁻⁹	0.49
Del(1p32)	1.05 (0.76-1.47)	0.7556	1.48 (1.04-2.09)	0.0286	1.23 (0.97-1.57)	0.0833	0.17
ISS II	1.34 (1.01-1.77)	0.0409	1.48 (1.11-1.99)	0.0085	1.40 (1.15-1.72)	0.0009	0.61
ISS III	1.43 (1.07-1.91)	0.0168	2.20 (1.61-3.01)	7.88x10 ⁻⁷	1.74 (1.40-2.16)	3.11x10 ⁻⁷	0.04

Table 23: Relationship between presence of genetic abnormalities and overall survival of intensively treated patients

OS

	Myeloma IX n=511		Myeloma XI n=598		Combined n=1109		Heterogeneity
	HR (95% CI)	<i>p</i> -value	HR (95% CI)	<i>p</i> -value	HR (95% CI)	<i>p</i> -value	<i>p</i> -value
t(4;14)	1.74 (1.26-2.41)	0.0008	2.09 (1.41-3.07)	0.0002	1.87 (1.46-2.40)	7.62x10 ⁻⁷	0.49
t(14;16)	1.51 (0.79-2.84)	0.2059	2.31 (1.13-4.73)	0.0218	1.82 (1.13-2.92)	0.01359	0.38
t(14;20)	1.44 (0.59-3.49)	0.4181	1.91 (0.26-13.70)	0.5219	1.51 (0.67-3.39)	0.3169	0.80
Adverse Translocations	1.74 (1.30-2.33)	0.0002	2.30 (1.60-3.31)	6.99x10 ⁻⁶	1.94 (1.55-2.43)	9.91x10 ⁻⁹	0.24
Del(17p)	2.31 (1.61-3.31)	5.87x10 ⁻⁶	3.19 (2.10-4.85)	5.74x10 ⁻⁸	2.65 (2.01-3.48)	3.04x10 ⁻¹²	0.25
Gain(1q)	1.79 (1.40-2.27)	2.33x10 ⁻⁶	1.72 (1.22-2.43)	0.0020	1.77 (1.45-2.15)	1.65x10 ⁻⁸	0.86
Del(1p32)	1.84 (1.28-2.64)	0.0010	1.99 (1.25-3.18)	0.0037	1.89 (1.42-2.52)	1.23x10 ⁻⁵	0.78
ISS II	1.96 (1.32-2.90)	0.0008	1.88 (1.14-3.11)	0.0140	1.92 (1.41-2.63)	3.27x10 ⁻⁵	0.90
ISS III	2.56 (1.72-3.81)	3.59x10 ⁻⁶	3.22 (1.94-5.35)	6.56x10 ⁻⁶	2.79 (2.04-3.81)	1.30x10 ⁻¹⁰	0.49

Table 24: Inference C-values for intensively treated patients using tau=24 months for PFS in the Myeloma IX and Myeloma XI trials

PFS

	Myeloma IX n=511				Myeloma XI n=598			
	Estimate	Standard error	Lower 95% CI	Upper 95% CI	Estimate	Standard error	Lower 95% CI	Upper 95% CI
Adverse translocations	0.5540	0.0122	0.5301	0.5778	0.5742	0.0159	0.5431	0.6052
t(4;14)	0.5458	0.0106	0.5252	0.5665	0.5653	0.0146	0.5367	0.5938
t(14;16)	0.5085	0.0065	0.4957	0.5213	0.5101	0.0067	0.4969	0.5232
t(14;20)	0.5004	0.0037	0.4931	0.5077	0.5012	0.0031	0.4952	0.5072
Del(17p)	0.5190	0.0083	0.5028	0.5352	0.5389	0.0123	0.5149	0.5630
Gain(1q)	0.5567	0.0152	0.5269	0.5865	0.5472	0.0160	0.5158	0.5785
Del(1p)	0.5016	0.0101	0.4819	0.5213	0.5239	0.0137	0.4971	0.5508
ISS	0.5575	0.0229	0.5128	0.6023	0.5969	0.0170	0.5636	0.6302
'Double-hit'	0.5873	0.0166	0.5547	0.6199	0.6003	0.0182	0.5647	0.6359
'Double-hit' - ISS	0.6039	0.0180	0.5686	0.6392	0.6300	0.0176	0.5955	0.6644

Table 25: Inference C-values for intensively treated patients using tau=36 months for OS in the Myeloma IX and Myeloma XI trials

OS

	Myeloma IX n=511					Myeloma XI n=598						
	Estimate	Standard error	Lower CI	95%	Upper CI	95%	Estimate	Standard error	Lower CI	95%	Upper CI	95%
Adverse translocations	0.5546	0.0172	0.5208		0.5884		0.5855	0.0227	0.5410		0.6301	
t(4;14)	0.5352	0.0152	0.5054		0.5649		0.5595	0.0203	0.5198		0.5993	
t(14;16)	0.5139	0.0131	0.4883		0.5394		0.5242	0.0123	0.5001		0.5482	
t(14;20)	0.5056	0.0085	0.4889		0.5223		0.5018	0.0047	0.4927		0.5109	
Del(17p)	0.5303	0.0136	0.5037		0.5570		0.5743	0.0186	0.5378		0.6108	
Gain(1q)	0.5814	0.0204	0.5416		0.6213		0.5636	0.0258	0.5131		0.6141	
Del(1p)	0.5399	0.0154	0.5098		0.5700		0.5420	0.0176	0.5075		0.5766	
ISS	0.6107	0.0248	0.5621		0.6592		0.6280	0.0245	0.5800		0.6759	
‘Double-hit’	0.6163	0.0223	0.5726		0.6601		0.6338	0.0264	0.5821		0.6854	
‘Double-hit’: ISS	0.6600	0.0230	0.6149		0.7051		0.6682	0.0248	0.6196		0.7168	

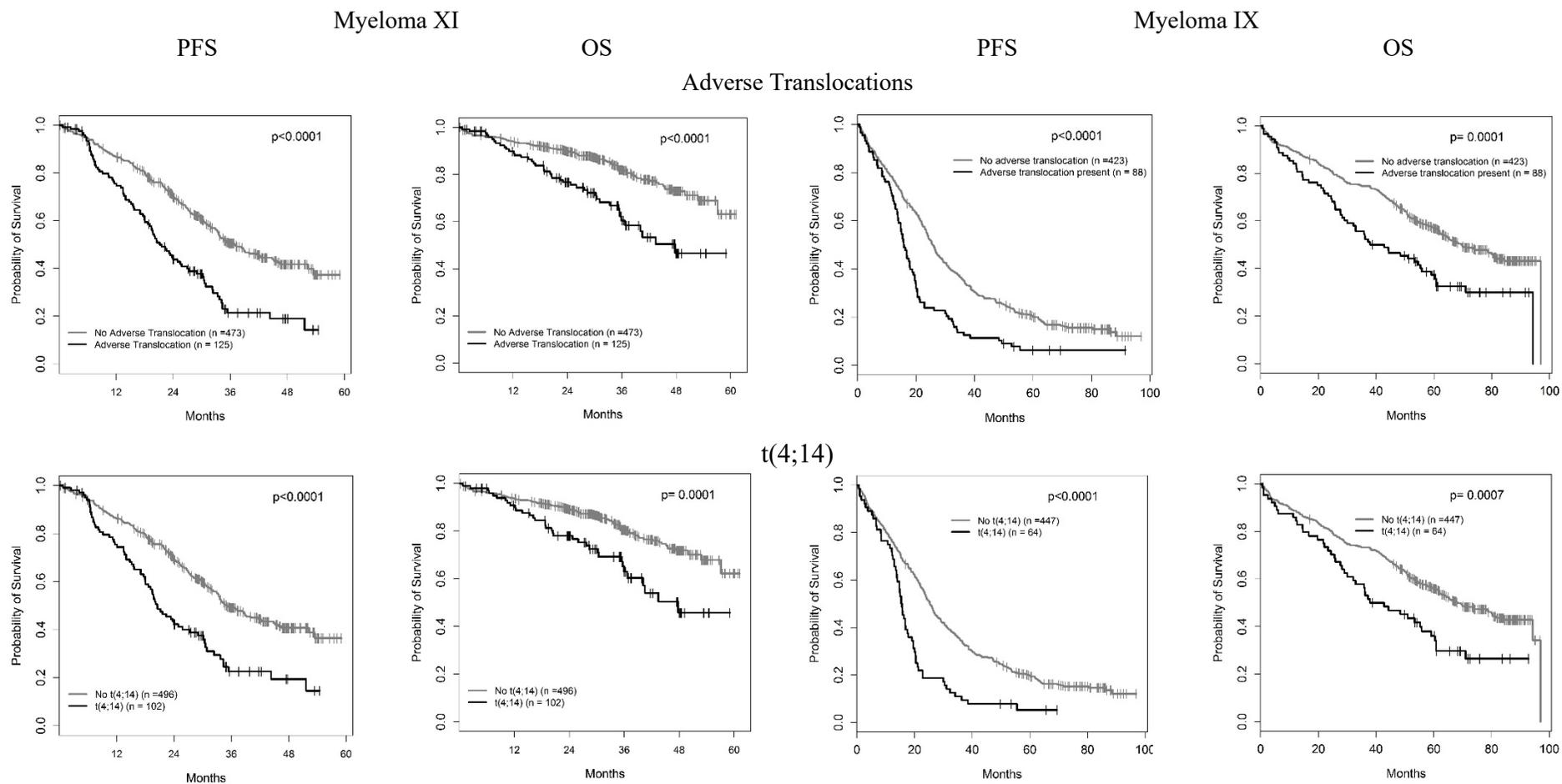


Figure 13: Kaplan-Meier curves and log-rank p-values for 598 intensively treated NCRI Myeloma XI patients and 511 intensively treated MRC Myeloma IX patients in the context of presence or absence of recurrent genetic aberrations

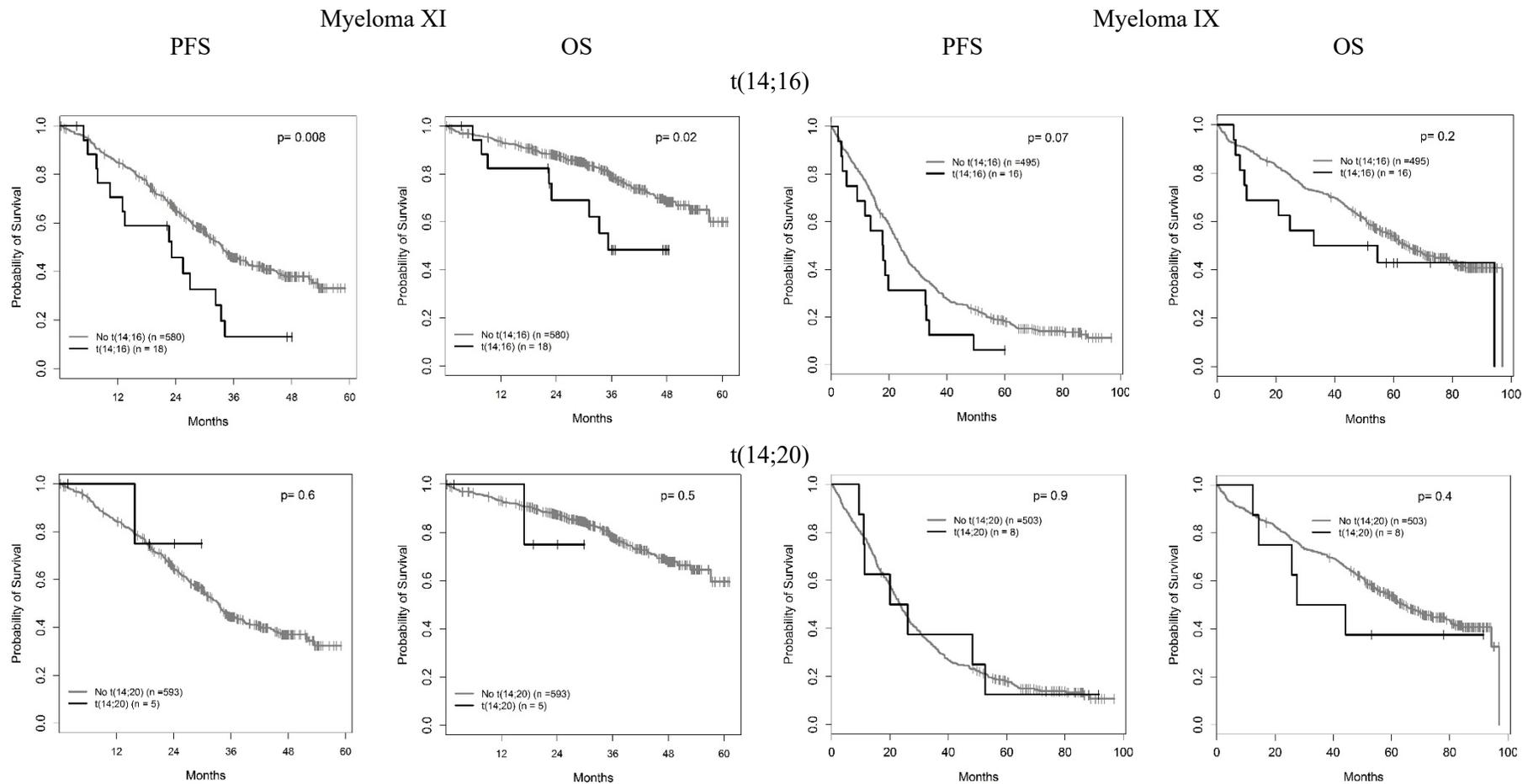


Figure 14: Kaplan-Meier curves and log-rank p-values for 598 intensively treated NCRI Myeloma XI patients and 511 intensively treated MRC Myeloma IX patients in the context of presence or absence of recurrent genetic aberrations

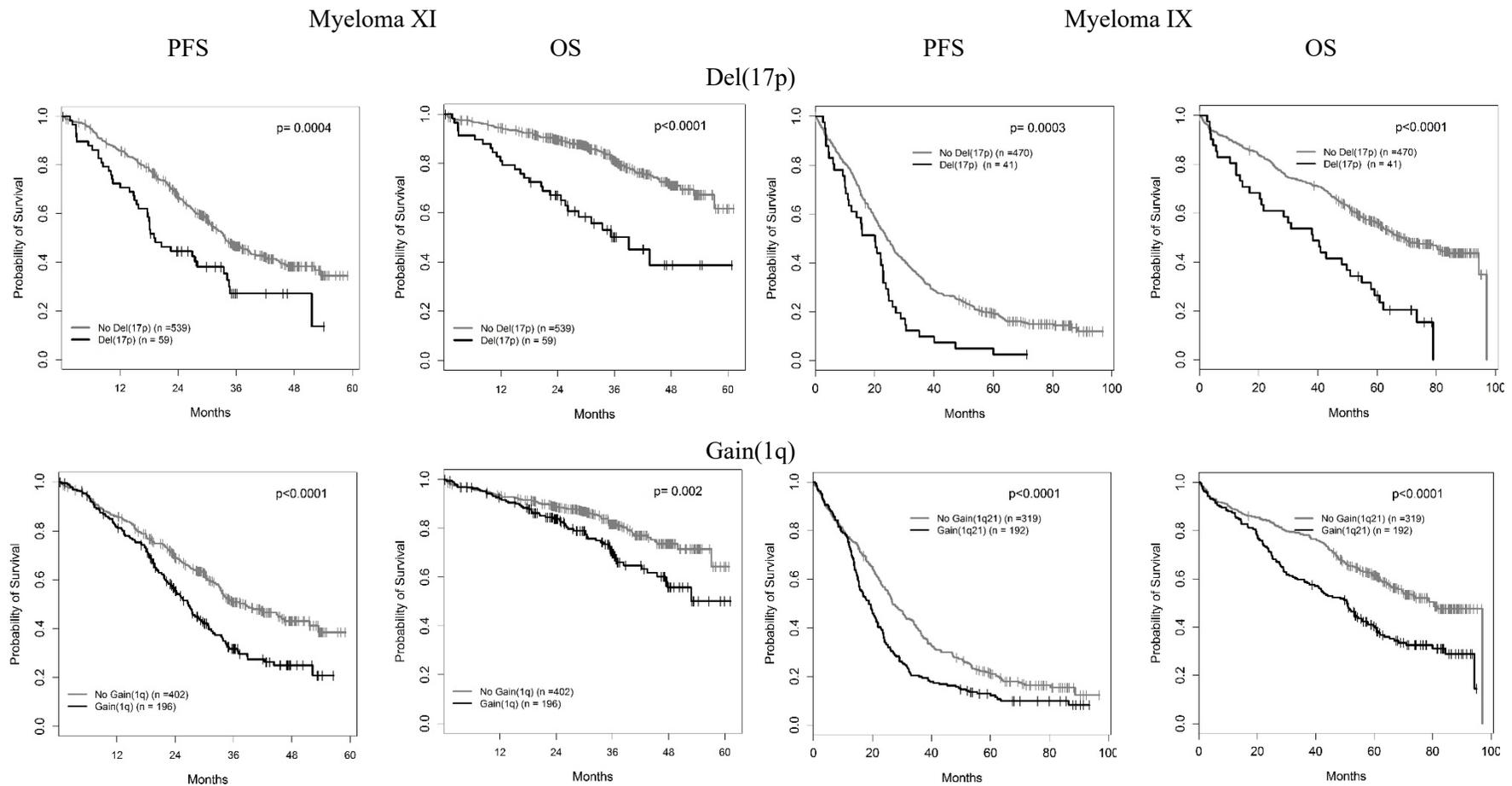


Figure 15: Kaplan-Meier curves and log-rank p-values for 598 intensively treated NCR1 Myeloma XI patients and 511 intensively treated MRC Myeloma IX patients in the context of presence or absence of recurrent genetic aberrations

3.3.4 Gain of chromosome 1q

As discussed in the introductory chapter, the prognostic association of gain(1q21) is debated in the context of survival in NDMM. As there is debate as to whether gain or amp(1q) has prognostic significance, our group sub-classified diploid vs. gain vs. amplification status by application of MLPA to the Myeloma XI trial data that had been previously divided through k-means classification (12). We confirmed the independence of gain(1q21) as a high-risk lesion and its association with significantly shorter OS (HR 1.67; $P=3.30 \times 10^{-5}$) compared with normal 1q copy number. Amp(1q) was also associated with shorter OS (HR 2.28; $P=2.32 \times 10^{-6}$) compared with normal 1q. Amp1q showed no statistically significant difference to gain(1q) in terms of survival (HR 1.36; $P=0.09$ for OS) (Table 26; Figure 16). Results were consistent for PFS. These findings are further explored in depth in the third results chapter of this thesis.

Table 26: Relationship between copy number status of chromosome 1q and progression-free and overall survival in Myeloma XI patients

	PFS n=1036			
	HR	<i>P</i> -value	Lower 95% CI	Upper 95% CI
Gain vs diploid (1q)	1.56	3.53×10^{-7}	1.31	1.85
Amp vs diploid (1q)	1.44	0.01	1.09	1.91
Gain(1q) vs. Amp(1q)	0.91	0.54	0.68	1.23

	OS n=1036			
	HR	<i>P</i> -value	Lower 95% CI	Upper 95% CI
Gain(1q) vs diploid(1q)	1.67	3.30×10^{-5}	1.31	2.12
Amp(1q) vs diploid (1q)	2.28	2.32×10^{-6}	1.62	3.21
Gain(1q) vs. Amp(1q)	1.36	0.09	0.95	1.95

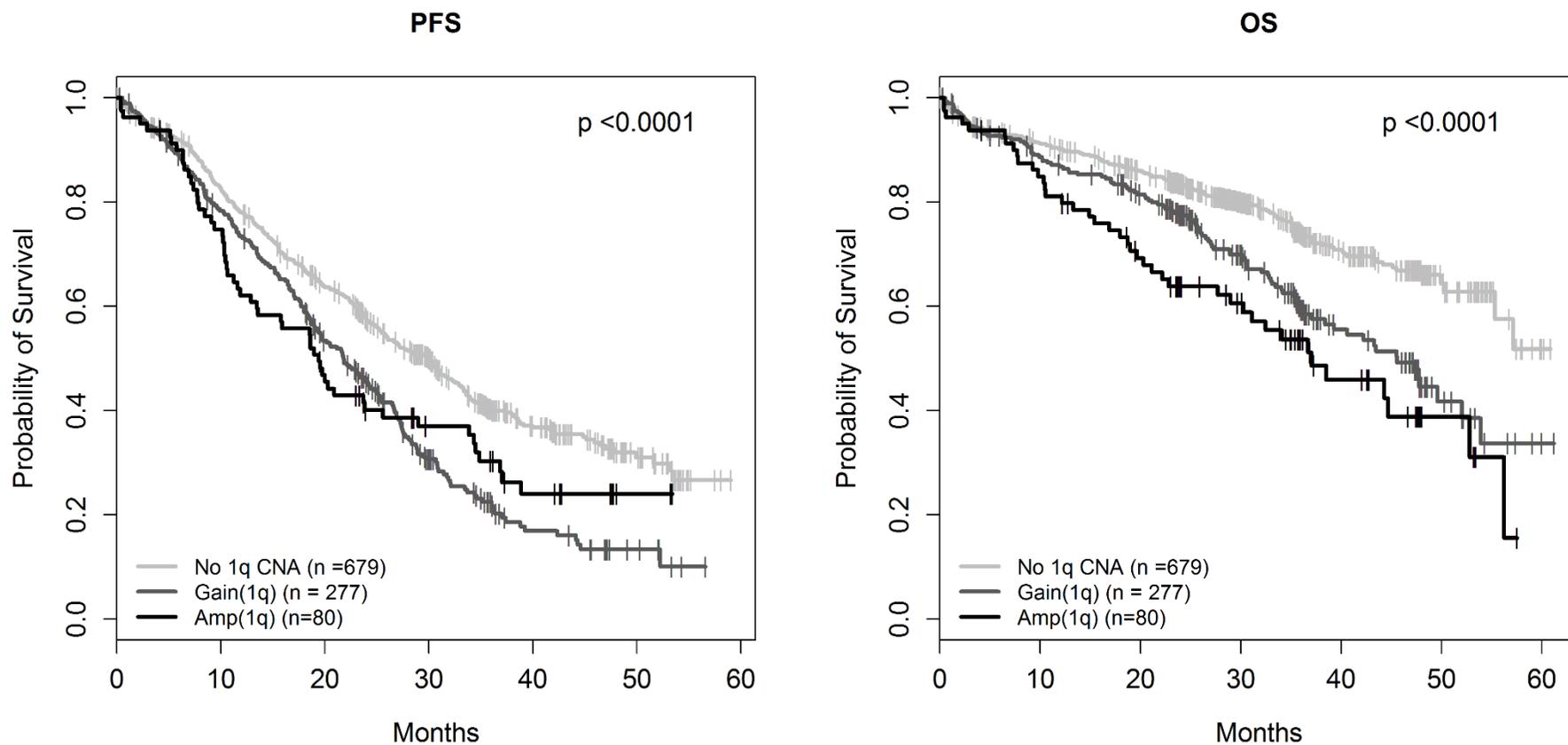


Figure 16: Kaplan-Meier survival analysis of 1036 MRC Myeloma XI patients in the context of gain(1q21) or amp(1q21). Denoted logrank based P value represents difference between most divergent Kaplan-Meier curves. OS: Gain(1q) vs diploid 1q (logrank $P < 0.001$), Amp vs diploid 1q (logrank $P < 0.001$), Amp vs gain 1p (logrank $P = 0.06$). PFS: Gain(1q) vs diploid 1q (logrank $P < 0.001$), Amp vs diploid 1q (logrank $P < 0.02$), Amp vs gain 1p (logrank $P = 0.5$).

3.3.5 ‘Double-hit’ as a high-risk classifier

Next, the we examined the impact of a ‘double-hit’ based on the co-occurrence of at least two of the following: (1) adverse translocations t(4;14), t(14;16), t(14;20); (2) gain(1q); and (3) del(17p) (67). For Myeloma XI data, the three risk groups that were defined as ‘double-hit’, 1 or no adverse lesions were associated with median PFS of 17.0, 24.2 and 31.1 months (log-rank $P=5.7 \times 10^{-13}$), with corresponding median 24-month OS of 66.1%, 76.6% and 86.4% ($P=4.4 \times 10^{-13}$). These findings were consistent with the Myeloma IX data (Table 27; Table 28). In the combined analysis of all 1,905 patients, the HR for ‘double-hit’ was 2.23 for PFS ($P=7.92 \times 10^{-26}$) and 2.67 for OS ($P=8.13 \times 10^{-27}$) (Table 27; Table 28). The increased risk was also validated by c-statistics (Table 29). As has been previously shown in Myeloma IX (67), the triple combination of an adverse translocation, gain(1q) and del(17p) was associated with the worst OS with a HR of 6.23 ($P=1.31 \times 10^{-7}$) (Figure 17 **Table 27: Cox-based regression for presence of genetic abnormalities and patient progression-free survival**).

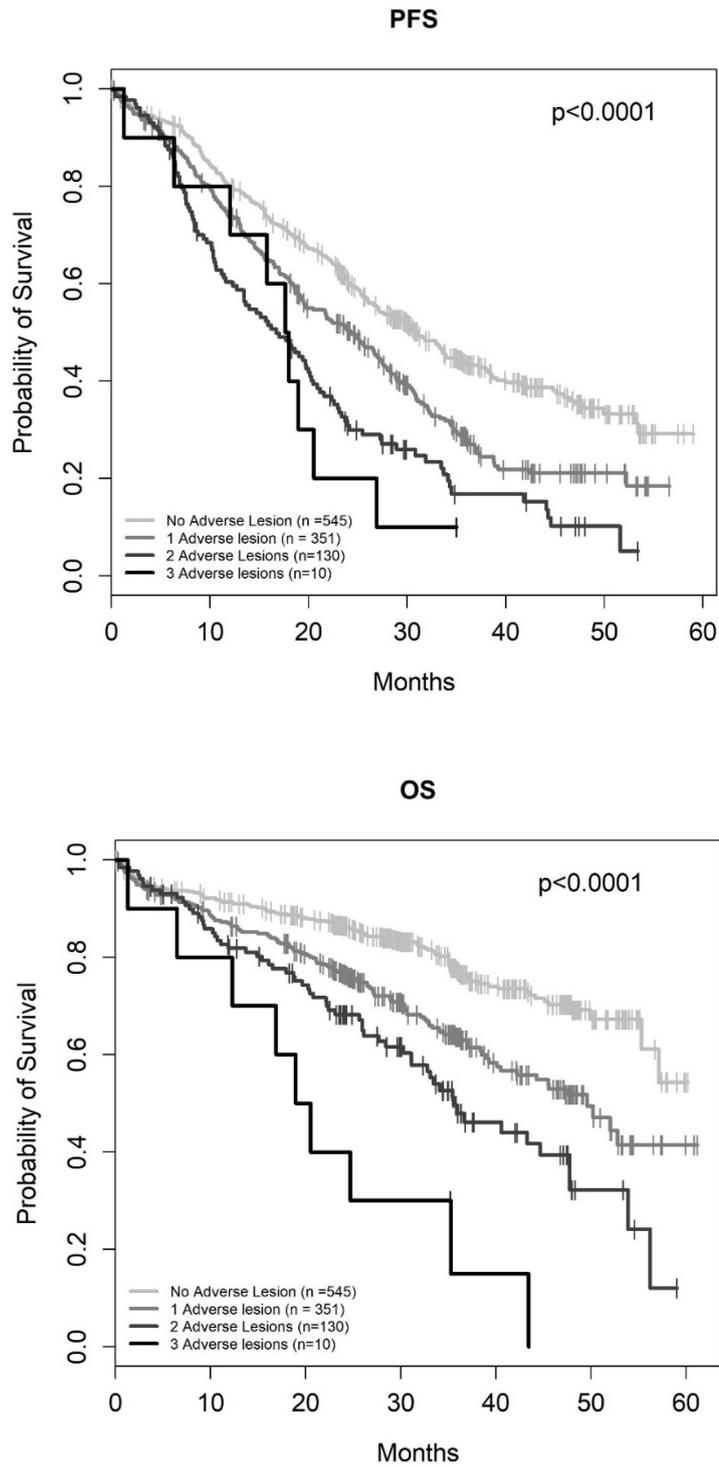


Figure 17: Genetic risk markers and survival: Kaplan-Meier curves and log-rank p-values for 1036 MRC Myeloma XI patients with 0, 1, 2 or 3 adverse genetic lesions. Denoted logrank based P value represents difference between most divergent Kaplan-Meier curves.

Validation of the Myeloma IX signature was sought through the combination of ‘double hit’ lesions and ISS. The integrated ISS and genetic risk signature was defined as ‘double-hit’-ISS ultra high risk (ISS II or III and ‘double-hit’) which comprised 12.0% of the NDMM population in Myeloma XI. The intermediate risk group (ISS I and ‘double-hit’; ISS II and 1 adverse lesion; ISS III and no or 1 adverse lesion) comprised 44.1% of the population. The favourable risk group (ISS I and no or 1 adverse lesion; ISS II and no adverse lesion) made up 43.9% of the population.

Investigation of the independence of ISS revealed that for both Myeloma IX and XI trials, the impact of a ‘double-hit’ on patient outcome was independent of the ISS categorisation (Table 31). In the meta-analysis of Myeloma XI and IX data, the ultra-high risk classification was associated with HR 2.85 ($P=8.32 \times 10^{-31}$) for PFS and HR 4.12 ($P=2.85 \times 10^{-36}$) for OS (Table 27; Table 28). This analysis validates the combined genetics and ISS model that was created for the Myeloma IX trial.

Similarly, our group investigated the effect of combined lesions in intensively treated patients in the Myeloma XI trial. The groups that contained a ‘double-hit’, 1 adverse or no adverse lesion were associated with median PFS of 19.7, 30.9 and 44.8 months (log-rank $P=2.5 \times 10^{-13}$) and 24-months OS of 72.3%, 86.2% and 92.2% ($P=1.6 \times 10^{-10}$). The meta-analysis showed that intensively treated patients with a ‘double-hit’ had a HR for PFS of 2.61 ($P=1.07 \times 10^{-20}$) and HR for OS of 3.19 ($P=1.23 \times 10^{-18}$) (Table 29).

Comparison of the two trials showed that survival time was increased for intensively treated patients in all risk groups who were part of the Myeloma XI trial (figures above) compared with those in the Myeloma IX trial (14.4, 21.9 and 30.8 months; 63.9%, 75.4% and 86.0%, respectively). Median PFS was 5.3 months longer for ‘double-hit’ patients in the Myeloma XI trial vs. those in the IX trial, but 14 months longer for the group without any risk lesion.

The results for the intensively treated group were similar to those for the overall trial. Within this subgroup, we showed independence of ‘double hit’ and ISS (Table 32). On the basis of clinical and genetic information (Table 29), the ‘double-hit’-ISS ultra-high risk group that comprised 12.5% of patients was associated with a HR of 3.11 ($P=1.59 \times 10^{-20}$) for PFS and HR of 4.79 ($P=5.10 \times 10^{-23}$) for OS.

	Myeloma IX n=869		Myeloma XI n=1036		Combined n=1905		Heterogeneity
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	p-value
1 Adverse lesion	1.41 (1.21-1.65)	1.73x10 ⁻⁵	1.46 (1.23-1.74)	1.44x10 ⁻⁵	1.44 (1.28-1.61)	1.07x10 ⁻⁹	0.76
'Double hit' >1 Adverse lesion	2.24 (1.83-2.76)	1.11x10 ⁻¹⁴	2.22 (1.78-2.77)	1.05x10 ⁻¹²	2.23 (1.92-2.59)	7.92x10 ⁻²⁶	0.94
Intermediate risk-ISS	1.50 (1.25-1.79)	1.48x10 ⁻⁵	1.95 (1.63-2.33)	1.56x10 ⁻¹³	1.71 (1.51-1.95)	9.48x10 ⁻¹⁷	0.04
'Double hit'-ISS	2.76 (2.13-3.57)	1.54x10 ⁻¹⁴	2.93 (2.29-3.09)	2x10 ⁻¹⁶	2.85 (2.38-3.40)	8.32x10 ⁻³¹	0.74

Table 27: Cox-based regression for presence of genetic abnormalities and patient progression-free survival

	Myeloma IX n=869		Myeloma XI n=1036		Combined n=1905		Heterogeneity
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	p-value
1 Adverse lesion	1.42 (1.18-1.71)	0.0002	1.81 (1.41-2.32)	3.57x10 ⁻⁶	1.55 (1.33-1.79)	9.97x10 ⁻⁹	0.13
'Double hit' >1 Adverse lesion	2.54 (2.02-3.18)	7.77x10 ⁻¹⁶	2.91 (2.17-3.89)	1.11x10 ⁻¹²	2.67 (2.23-3.19)	8.13x10 ⁻²⁷	0.47
Intermediate risk-ISS	1.96 (1.57-2.45)	4.26x10 ⁻⁹	2.59 (1.96-3.41)	1.62x10 ⁻¹¹	2.19 (1.84-2.61)	1.3x10 ⁻¹⁸	0.13
'Double hit'-ISS	3.93 (2.93-5.27)	2x10 ⁻¹⁶	4.37 (3.13-6.12)	2x10 ⁻¹⁶	4.12 (3.30-5.14)	2.85x10 ⁻³⁶	0.64

Table 28: Cox-based regression for presence of genetic abnormalities and patient overall survival

a)	Myeloma IX n=511		Myeloma XI n=598		Combined n=1109		Heterogeneity
1 Adverse lesion	1.50 (1.21-1.85)	0.0002	1.49 (1.15-1.93)	0.0024	1.50 (1.27-1.76)	1.36x10 ⁻⁶	0.99
‘Double hit’ >1 adverse lesion	2.31 (1.75-3.05)	3.67x10 ⁻¹⁴	3.00 (2.24-4.02)	2.17x10 ⁻¹³	2.61 (2.13-3.20)	1.07x10 ⁻²⁰	0.21
Intermediate risk-ISS	1.47 (1.16-1.86)	0.0015	1.87 (1.45-2.41)	1.45x10 ⁻⁶	1.64 (1.38-1.95)	2.10 x10 ⁻⁸	0.17
‘Double hit’-ISS	2.78 (1.96-3.95)	9.85x10 ⁻⁹	3.42 (2.47-4.75)	1.92x10 ⁻¹³	3.11 (2.45-3.95)	1.59x10 ⁻²⁰	0.40

(b)	Myeloma IX n=511		Myeloma XI n=598		Combined n=1109		Heterogeneity
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	p-value
1 Adverse lesion	1.73 (1.32-2.26)	6.91x10 ⁻⁵	1.62 (1.08-2.44)	0.0207	1.69 (1.35-2.12)	4.30x10 ⁻⁶	0.79
‘Double hit’ >1 adverse lesion	2.84 (2.05-3.94)	3.70x10 ⁻¹⁰	3.88 (2.55-5.92)	2.98x10 ⁻¹⁰	3.19 (2.47-4.14)	1.23x10 ⁻¹⁸	0.25
Intermediate risk-ISS	2.36 (1.71-3.25)	1.44x10 ⁻⁷	2.26 (1.49-3.43)	0.0001	2.32 (1.80-3.00)	7.15x10 ⁻¹¹	0.87
‘Double hit’-ISS	4.51 (2.97-6.83)	1.26x10 ⁻¹²	5.18 (3.24-8.27)	5.78x10 ⁻¹²	4.79 (3.51-6.54)	5.10x10 ⁻²³	0.66

Table 29: Relationship between presence of genetic abnormalities and patient survival for intensively treated patients. (a) Progression-free survival; (b) Overall survival

Table 30: Inference C-values using tau=24 months for PFS and tau=36 months for OS in the Myeloma IX and Myeloma XI trials

PFS

	Myeloma IX n=869				Myeloma XI n=1,036			
	Estimate	Standard error	Lower 95% CI	Upper 95% CI	Estimate	Standard error	Lower 95% CI	Upper 95% CI
'Double-hit'	0.5746	0.0118	0.5515	0.5978	0.5734	0.0117	0.5504	0.5963
'Double-hit' - ISS	0.5988	0.0131	0.5730	0.6245	0.6127	0.0123	0.5886	0.6369

OS

	Myeloma IX n=869				Myeloma XI n=1,036			
	Estimate	Standard error	Lower 95% CI	Upper 95% CI	Estimate	Standard error	Lower 95% CI	Upper 95% CI
'Double-hit'	0.5942	0.0146	0.5655	0.6229	0.6024	0.0156	0.5718	0.6330
'Double-hit' - ISS	0.6326	0.0160	0.6013	0.6639	0.6463	0.0151	0.6167	0.67

Table 31: Multivariate analysis of ‘double-hit’ genetic high risk and ISS in 981 cases with complete information in Myeloma XI trial

a) PFS

Variable	HR	95% CI	P
1 adverse lesion	1.41	1.18-1.68	0.000143
‘Double-hit’ >1 adverse lesion	2.05	1.63-2.56	4.56x10 ⁻¹⁰
ISS 2	1.48	1.18-1.85	0.000719
ISS 3	2.30	1.83-2.89	8.26x10 ⁻¹³

b) OS

Variable	HR	95% CI	P
1 adverse lesion	1.709	1.32-2.22	5.25x10 ⁻⁵
‘Double-hit’ >1 adverse lesion	2.577	1.90-3.49	1.02x10 ⁻⁹
ISS 2	1.763	1.21-2.58	0.00346
ISS 3	3.425	2.37-4.96	7.24x10 ⁻¹¹

Table 32: Multivariate analysis of ‘double-hit’ genetic high risk and ISS in 567 cases treated on the intensive-therapy arm with complete information in Myeloma XI trial

a) PFS

Variable	HR	95% CI	P
1 adverse lesion	1.460	1.123-1.899	0.00468
‘Double-hit’ >1 adverse lesion	2.678	1.978-3.627	1.88x10 ⁻¹⁰
ISS 2	1.414	1.054-1.897	0.02100
ISS 3	1.978	1.443-2.712	2.21x10 ⁻⁵

b) OS

Variable	HR	95% CI	P
1 adverse lesion	1.514	1.000-2.292	0.0497
‘Double-hit’ >1 adverse lesion	3.166	2.045-4.901	2.35x10 ⁻⁷
ISS 2	1.759	1.062-2.912	0.0282
ISS 3	2.783	1.667-4.647	9.13x10 ⁻⁵

3.3.6 Prognostic impact of molecular subgroups in HRD

Next, we focused on genetic sub-groups of hyperdiploid MM. Figure 18 provides an overview of correlations between copy number aberration (CNA) and translocations within the Myeloma XI data set. Only two genetic lesions showed positive correlations with HRD; gain(11q25) and gain(1q21). Although HRD as a whole group was strongly correlated with gain(11q25) ($BF=1.2 \times 10^{-66}$), a subgroup that lacked gain(11q25) was characterised by gain(1q). Of the 488 HRD cases that were involved in the Myeloma XI trial, 68% had gain(11q25) and 29% gain(1q). Bayes correlation analysis on the subset of 488 HRD cases revealed both lesions were negatively correlated and occurred in 15% of HRD cases, which was fewer than expected ($BF=0.0004$).

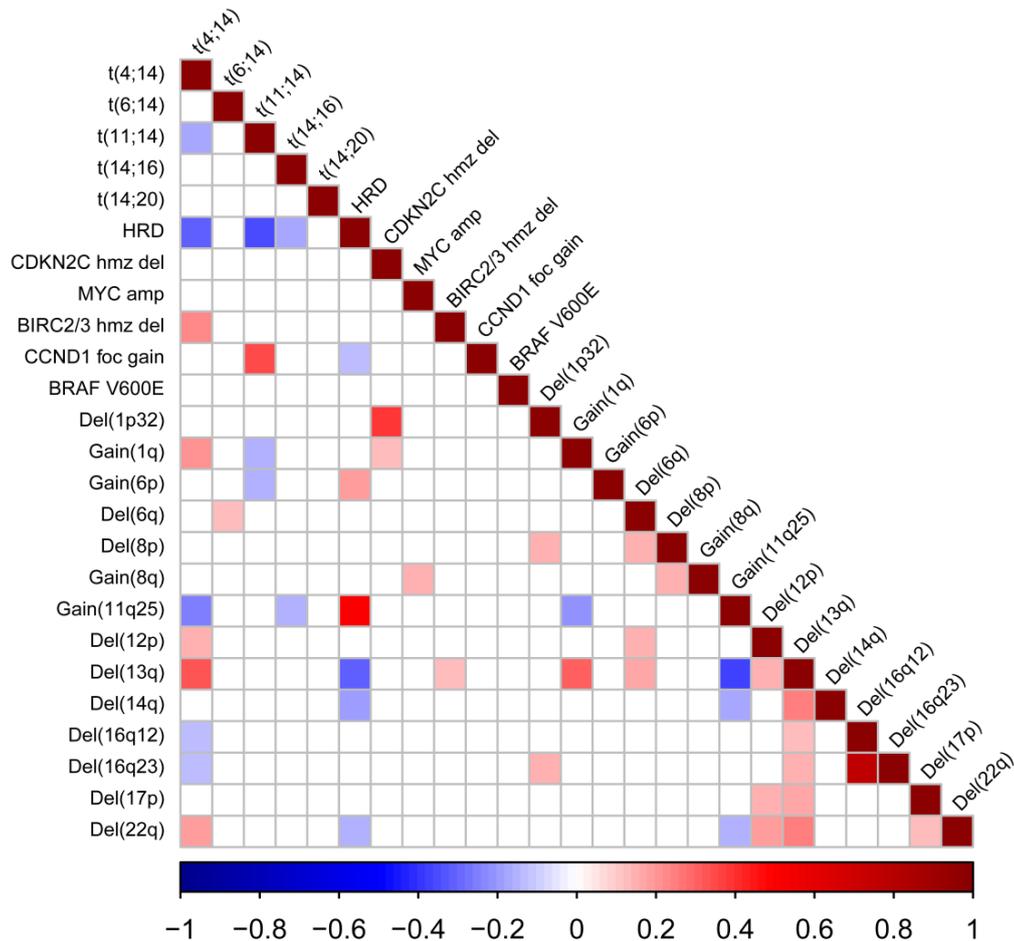


Figure 18: Associations between copy number aberrations and translocations in Myeloma XI trial. A Bayesian approach was used to identify all potential associations between genetic lesions. Significant interactions ($BF < 0.01$) are colour-coded, red representing positive and blue negative associations. amp, amplification; foc gain, focal gain; hmz del, homozygous deletion

I investigated through further analysis whether HRD could be classified according to the above defined copy number abnormalities - gain(1q)-HRD, gain(11q25)-HRD or gain(1q)+gain(11q25)-HRD. We first investigated *CCND1* and *CCND2* expression for these copy number changes within these HRD subgroups. It was found that gain(1q)-HRD was associated with overexpression of *CCND2* and silenced *CCND1* ($P<0.0001$). In contrast, gain(11q25) was associated with *CCND1*-mRNA expression and silenced *CCND2*-mRNA expression ($P<0.0001$). This was demonstrated by performance of qRT-PCR in the 488 Myeloma XI patients with HRD (Figure 19).

Myeloma XI

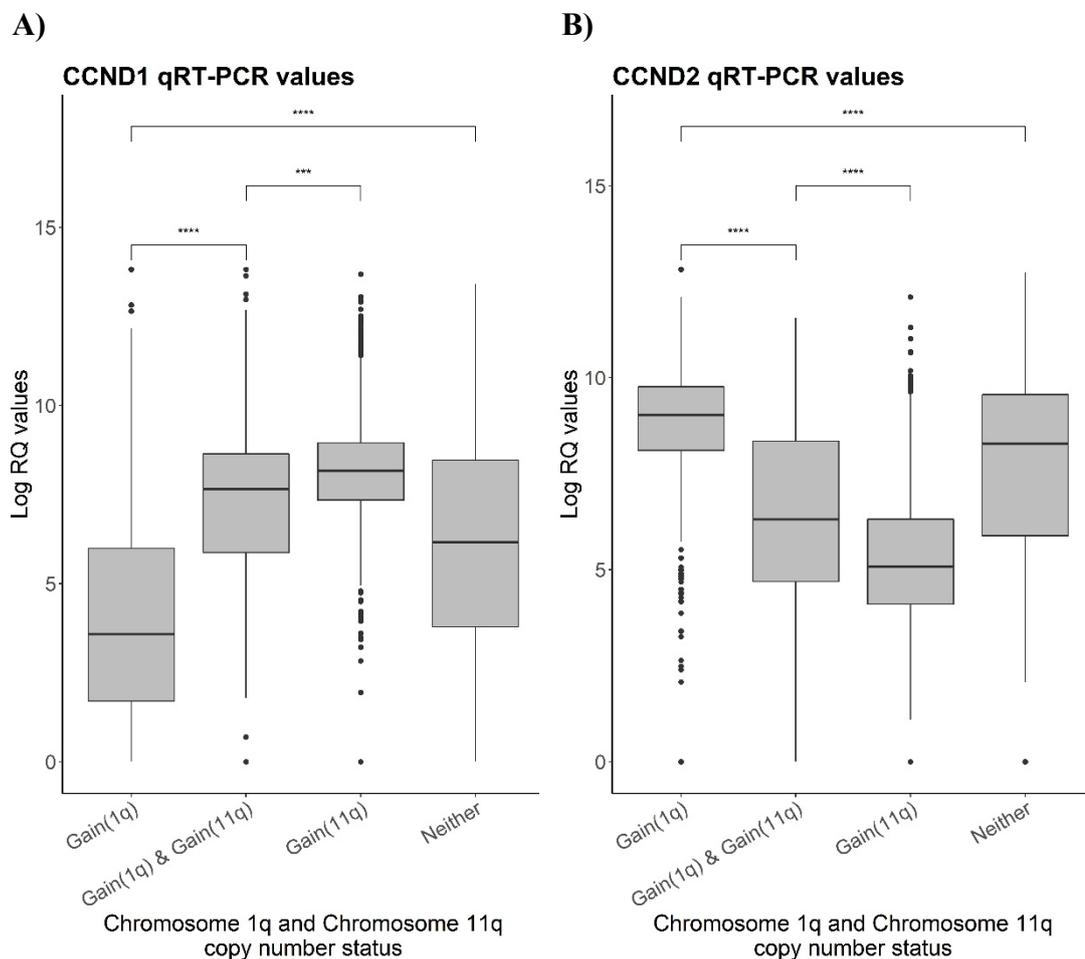


Figure 19: CCND expression in HRD sub-groups. *CCND1* (A) and *CCND2* (B) qRT-PCR expression levels (relative quantitative RQ values, *GAPDH* normalised) for HRD cases with gain(1q), gain(1q) & gain(11q25), gain(11q25) or neither. Gene expression levels were significantly different for all possible group-wise comparisons (two-sided Mann-Whitney-U; ** $P<0.0001$; *** $P<0.001$)**

We validated this correlation between gain(1q)-HRD and *CCND2* expression and between gain(11q25)-HRD and *CCND1* expression in the Myeloma IX dataset by integrating results from copy-number arrays and gene-expression arrays that were available (Figure 20). This shows a similar correlation between *CCND1* and *CCND2* in the gain(11q25) and gain(1q) subgroups of HRD, although overlapping datasets were small (n=46).

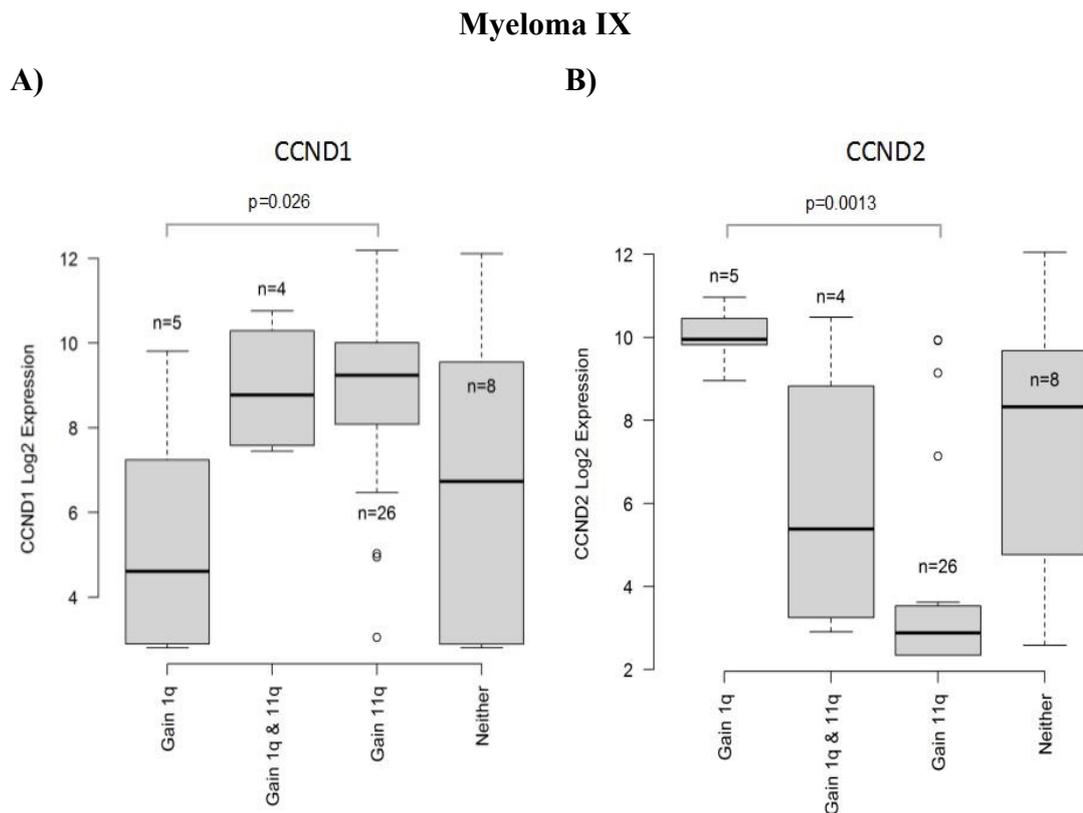


Figure 20: Affymetrix GeneChip Human Genome U133 Plus 2.0, GeneChip Mapping 500K Array data (GSE15695) and iFISH data from the Myeloma IX trial were integrated and analysed for (a) *CCND1* and (b) *CCND2* expression levels in gain(1q)-HRD and gain(11q)-HRD sub-groups

Previous studies of gene-expression arrays and gene expression have classified hyperdiploidy as D1 and D2 subgroups of HRD MM on the basis of *CCND1* and *CCND2* overexpression (48). The findings that are shown by this work suggest similarity between gain(11q25)-HRD and the D1 subgroup, gain(1q)-HRD and the D2 and gain(1q)+gain(11q)-HRD and the D1+D2 TC classification subgroup. These findings demonstrate that subgroups that are defined by gene expression reflect subgroups that are defined by secondary copy number abnormalities.

Further differences between the HRD subtypes were noted: 13q was deleted in 41.1% (58/141) of gain(1q)-HRD (BF=6.0x10⁻⁶; *P*<0.0001) cases, but only in 15.4% (50/325) of gain(11q25)-HRD (BF=5.5x10⁻¹¹; *P*<0.0001). We validated this finding in the Myeloma IX dataset, in which del(13q) was also positively associated with gain(1q)-HRD (*P*=0.024) and negatively associated with gain(11q25) (*P*=0.041).

3.3.7 Molecular subgroups in HRD

The clinical implications of the HRD subgroups that were analysed in the previous section were examined. Gain(1q), del(1p32) and del(17p) were associated with shorter OS (HR 1.81, *P*=0.001; HR 2.44, *P*=0.0004; HR 1.89, *P*=0.022; respectively) in the 488 HRD cases. Gain(1q) and del(1p32) but not del(17p) were also associated with shorter PFS (HR 1.56, *P*=0.0003; HR 1.66, *P*=0.005; HR 1.30, *P*=0.23 respectively). Gain(11q25), del(13q) and del(22q) were not associated with shorter OS or PFS. At least one of the lesions gain(1q), del(1p32) or del(17p) were present in 39.3% (192/488) of HRD cases, and this presence defined a risk population with significantly shorter PFS (*P*=0.0003) and OS (*P*=2.7x10⁻⁶) than HRD MM patients who lacked any of these lesions (Figure 22). These were confirmed on multivariate analysis (Table 33). Interestingly, the 28.5% of all patients (296/1,036) who had HRD MM without any demonstrable adverse lesions showed the longest survival times of all sub-groups, indeed longer than those with t(11;14) MM (Figure 21).

Table 33: Relationship between presence of genetic abnormalities and patient survival for the hyperdiploid sub-group in Myeloma XI.

	PFS n=488			
	HR	P-value	Lower 95% CI	Upper 95% CI
Gain(1q)	1.56	0.0003	1.22	1.99
Del(1p32)	1.66	0.006	1.16	2.37
Del(17p)	1.30	0.23	0.85	1.99

	OS n=488			
	HR	p-value	Lower 95% CI	Upper 95% CI
Gain(1q)	1.81	0.001	1.27	2.59
Del(1p32)	2.33	0.0004	1.46	3.73
Del(17p)	1.89	0.02	1.08	3.30

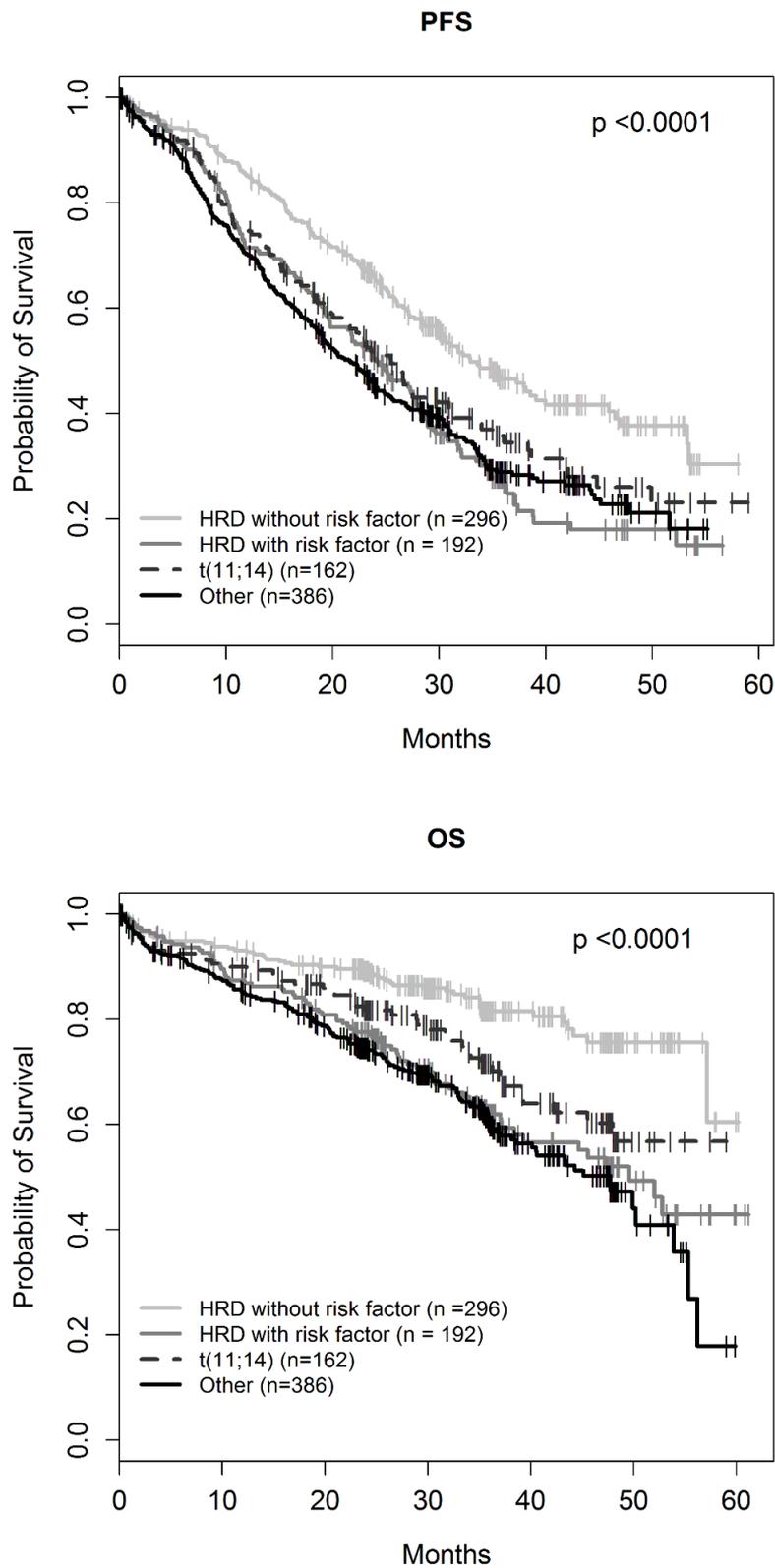


Figure 21: Genetic risk markers and survival. Kaplan-Meier curves and log-rank p-values for 1036 MRC Myeloma XI patients with 0,1,2 or 3 adverse genetic lesions

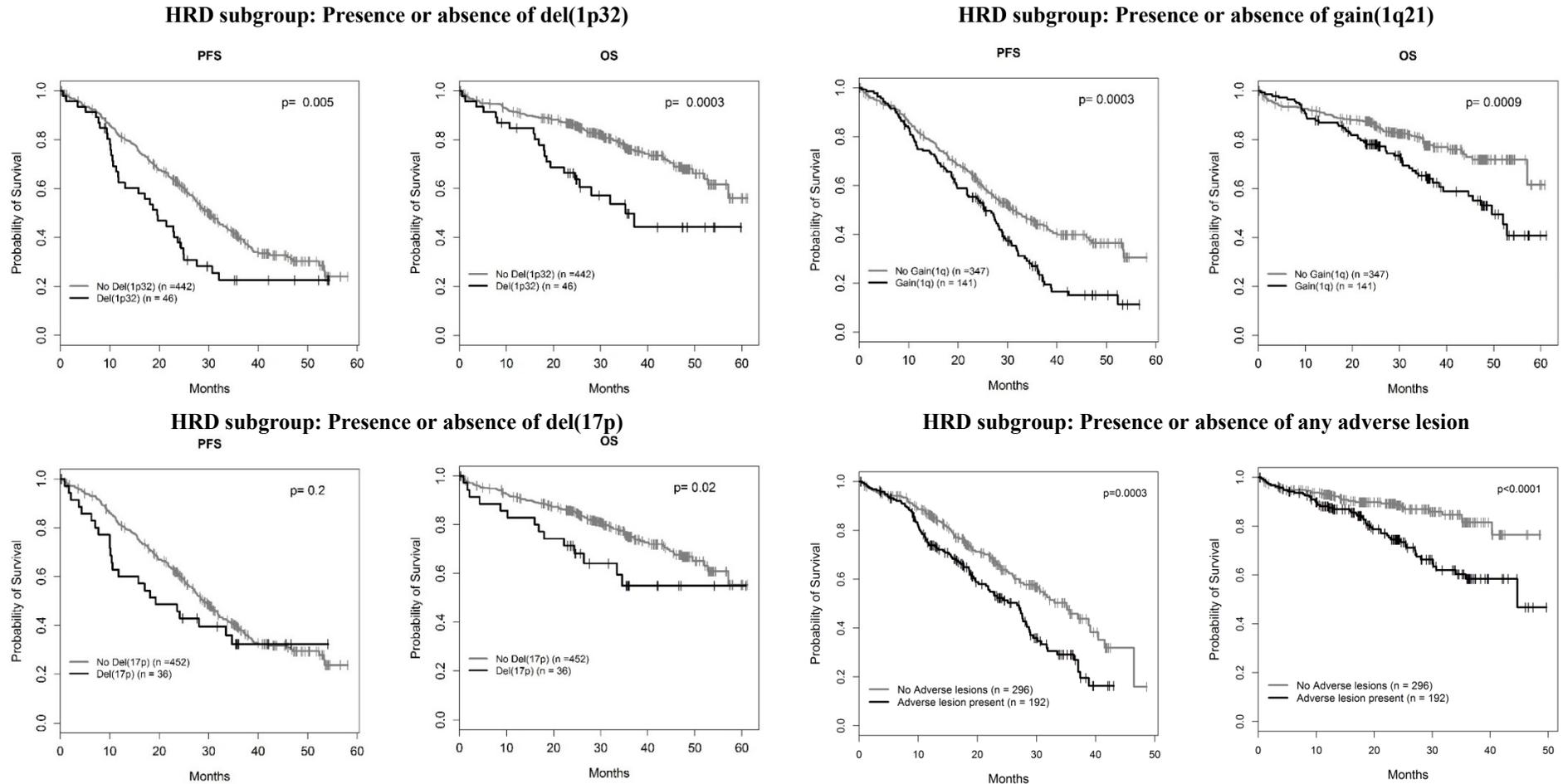


Figure 22: CNA and survival in HRD MM. Kaplan-Meier curves and log-rank p-values for sub-groups of 488 Myeloma XI HRD MM patients characterised by presence or absence of individual risk markers. Dark grey line – high-risk marker present, light grey line – high-risk marker absent

3.4 Discussion

The analysis that was performed as part of the work for this thesis confirms that the association with outcome in MM for the archetypical high-risk lesions del(17p), gain(1q) and t(4;14) is associated with reduced survival rates in our patient group overall. However it must be acknowledged that the lack of proteasome inhibitor therapy at induction may have confounded results for the t(4;14) in our study compared to other studies. Although numbers were low, we were also able to confirm the poor prognosis that was associated with t(14;16) and t(14;20) through meta-analysis of these rare primary genetic abnormalities in myeloma. Gain(1q21) was confirmed as a high-risk lesion that was independent of del(1p32) (84, 289, 290). In particular, the work demonstrated that both gain(1q) and amp(1q) showed prognostic significance within myeloma. The effect of cytogenetics appears to be stronger in younger, fitter, intensively treated patients in all the lesions that are discussed above compared with the older patients who were not intensively treated, apart from t(14;20) as the number of patients within the group was too low to show an effect.

Combinations of cytogenetic abnormalities are common in myeloma and the interpretation of these combinations is important for prognostication (41, 53, 67). The data that are shown here emphasise the importance of ‘double-hit’ as a risk biomarker. Importantly, this work demonstrates that this information can be combined with the ISS score to refine risk prediction further (67, 258, 291-293). The limitations of this model are that it assumes equal weighting of genetic changes. Other modelling that uses combination of lesions such as the R2 ISS (294) and IFM models (295) have added weighting to their models to try and take differences between high risk lesions into account.

This analysis is based on trials that recruited participants between 2003 and 2016. During this timeframe, treatment for MM underwent significant change as detailed within the introductory chapter of this thesis (296). The consistent adverse impact of high-risk genetics on survival among participants in the Myeloma IX and XI trials is striking and highlights the need for intensified efforts to target the biology of high-risk disease. Although survival time had increased for all risk groups in Myeloma XI

versus IX, absolute improvement was smallest for the ‘double-hit’ high-risk group. Median PFS for the ‘double-hit’ group of Myeloma XI patients who received intensive treatment was 19.7 months, meaning that about half of this group of patients relapsed within 12 months after autologous transplant. This analysis also confirms that the impact of recent therapies and supportive care mainly benefited patients who did not have high-risk lesions, and the smallest gains were observed in patients with ‘double hit’ tumours.

The assessment of the inter-relationship of CNAs and translocations in the Myeloma XI trial led to the characterisation of genetic sub-groups within hyperdiploid myeloma that had possible therapeutic relevance. Hyperdiploid MM constitutes the largest genetic sub-group of patients, with substantial heterogeneity (48). We describe two subgroups of HRD with either gain(11q25) and *CCND1* biology or gain(1q21) and *CCND2* overexpression. These groups are similar to the D1 and D2 subgroups of the TC classification, which was the first system that was used to classify HRD MM biologically. Application of the gene-expression-based TC classification in routine diagnostics has unfortunately been restricted due to access limitations to array-based GEP (48). Pragmatic classification of HRD based on gain(11q25) and gain(1q) may facilitate sub-grouping in clinical practice and open opportunities to improve therapy for these patients.

Recently, activity of BCL-2 inhibitors has been reported in *CCND1*-driven t(11;14) MM, and *CCND1*-driven gain(11q25)-HRD may constitute another target group (297). This analysis also discovered a high frequency of del(13q) in gain(1q)-HRD, in contrast with gain(11q25)-HRD. Interestingly, del(13q) and gain(1q) are known to co-occur frequently in t(4;14), which suggests that there are similarities in the genetic sequelae of these pathogenetic groups (298). An interrelationship between del(13q) and gain(1q)-HRD was suggested by Bergsagel et al., who based their suggestion on GEP in the TC classification, but this interrelationship has been demonstrated here for the first time on a DNA level (47, 48). Moreover, HRD MM without the risk lesions gain(1q)-HRD, del(17p) and del(1p32) showed longer remission and survival periods than any other sub-group and may be sufficiently treated with single-novel

agent/IMiD-based approaches. Such treatment could potentially reduce additional side effects and the costs of treatment with novel agent combinations (299-302).

Our study results contrast with those of other studies, which have shown that hyperdiploid myeloma has a protective effect in the presence of high-risk cytogenetic abnormalities (58, 303). However, as the incidence of high-risk lesions in hyperdiploid myeloma is low, this may be a potential confounding factor in several of these studies. The large numbers of patients whose data are included in our analysis enable us to study hyperdiploid myeloma as a subgroup on its own to confirm whether risk markers are abrogated in this sub-group.

The limitation of this study was the lack of whole genome-based analyses, which would be available through use of array-based or sequencing-based methods. These methods are increasingly becoming accessible in the clinic. WES was performed on only 463 patients within the Myeloma XI study and therefore integration of this data into our current study would offer limited findings. However, it is acknowledged that the availability of sequencing data would be helpful for a more comprehensive analysis of prognostic markers. In addition, at the time of this analysis, limited clinical information was available regarding this Myeloma XI subset of patients and there was no information on treatment arms. This limited the ability to analyse these lesions in the context of their treatment randomisation arms. Later in the work that was performed for this project, the data became available from the clinical trials research facility, and analysis of these variables is discussed in subsequent chapters of this thesis.

Another limitation of this study was related to the treatments that were available within these trials. As has been mentioned, the Myeloma IX study investigated induction therapy in patients who were randomised to conventional chemotherapy against thalidomide-based combinations. The Myeloma XI study was also largely IMiD-based. However, during the Myeloma XI study, bortezomib therapy became routinely available under the NHS. As a result, availability of this treatment is likely to be accounted for within the overall survival statistics for this trial. Triple therapy including proteasome inhibitors, IMiD-based therapy and steroids has now come into

routine use, especially for transplant-eligible patients. It must therefore be acknowledged that the associations with cytogenetics that have been found in this study may not be as valid in the modern setting due to changes in treatments that are offered.

The advantages of this study are not only related to the possibility to analyse the data of large numbers of patients with comprehensive chromosomal aberrations. It is also the fact that the two trials themselves were some of the most inclusive compared with most trials that were conducted within this time period, which enabled the study of older-age groups (with adjustment for treatment intensity and type) as well as worse performance status than the average (including performance 3 and 4). These cases are normally excluded from trials. These two trials therefore represent wide inclusivity, which is likely to be more representative of real-world outcomes than are most trials.

The trial also evaluated MLPA as a potential diagnostic tool that could be used in clinical practice for the evaluation of risk in myeloma. Its advantages include ease of use for a large number of samples as well as low inter-operator variability compared with iFISH. Our work has informed diagnostic laboratories and added to evidence in terms of the utility of this method, and has formed part of the basis for the adoption of this tool by the haematology molecular diagnostic services in some centres in the NHS.

In summary, this work has demonstrated the validity of the application of individual chromosomal aberrations to prognosis within the newly diagnosed myeloma setting. It has also validated the concept of increased risk due to the presence of more than one high-risk chromosomal aberration and validated the risk signature that was produced in our centre by the combination of ISS scores and the number of chromosome aberrations.

4 *TP53* in myeloma

4.1 Introduction

Alterations in the *TP53* tumour suppressor gene, which codes for the p53 protein, are the most common genetic lesions that are found in human cancer (128). As detailed in the introductory chapter, chromosome 17p is frequently deleted. Within the chromosome 17p region, *TP53* appears to be the gene of interest in this area; recurrent mutations and focal deletions have been discovered through use of WES and SNP array analysis. Deletion of the *TP53* gene often occurs in the later stages of the disease, rather than at diagnosis, and is thought to be the main driver of disease progression from SMM to MM. Importantly, *TP53* deletion has been found to have one of the strongest genetic associations with poor prognosis in MM (55, 67, 98, 107, 124, 125, 129, 130, 134-141), and reduced survival rates are reported in patients who show this deletion. These findings are discussed in detail later in this chapter.

Mutations rather than deletions in *TP53* have been reported to occur in a small subset of NDMM patients who comprise between 3% and 5% of all patients (37, 53, 125, 126, 304). The majority of *TP53* mutations that occur in MM are missense mutations. Around 10% of *TP53* mutations are nonsense mutations that result in truncation of the p53 protein (305-307). As with deletions, the frequency of occurrence of *TP53* mutations increases with disease progression, which suggests that it is an important driver of MM progression. The presence of these mutations is associated with poor prognosis in MM (114, 131, 132, 304). Sequential studies of the same patients show that mutations often follow deletion or occur simultaneously (304, 308).

TP53 encodes for the p53 tumour suppressor protein. Under normal physiological conditions, the p53 signalling network is kept inactive by low expression levels of p53 and by inactivation through binding by mouse double minute 2 (MDM2) and rapid protein degradation (309). Pathways by which p53 is activated vary according to the type of cellular stress (Figure 23) (310-313).

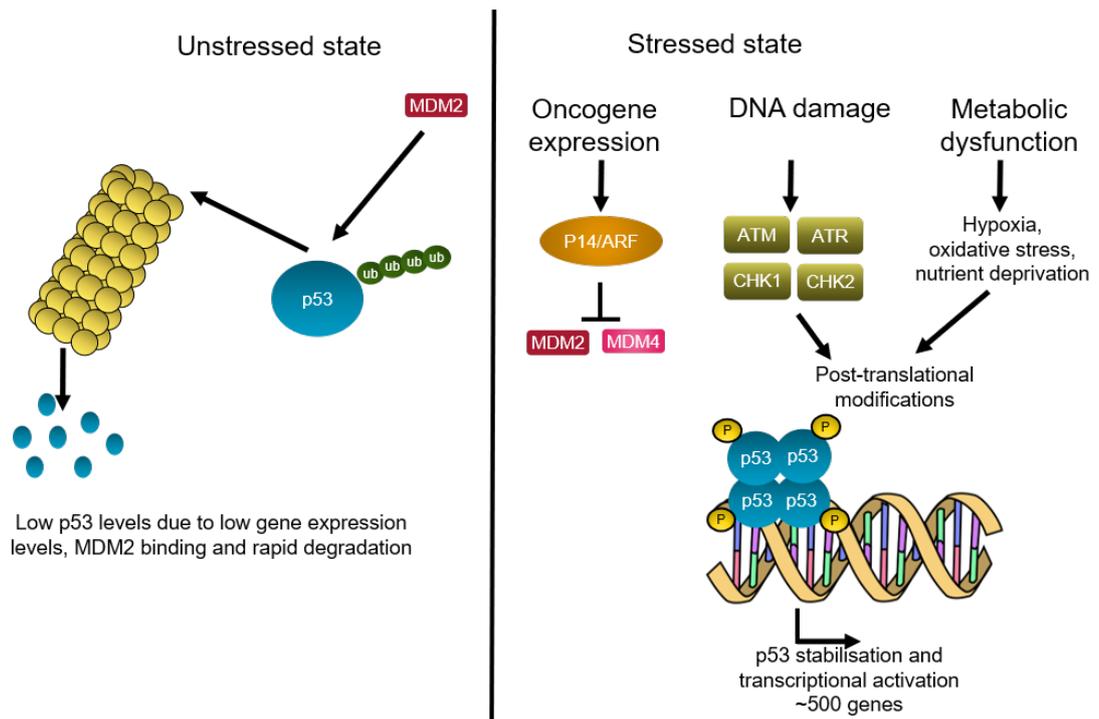


Figure 23: Regulation of p53 control in the unstressed and stressed states. Ub: ubiquitin; P: phosphorylation

This activation of the p53 protein results in its accumulation in the nucleus, which triggers activation of various downstream pathways that cooperate to retain genomic integrity and homeostasis of the cell. The p53 protein induces cell-cycle arrest or apoptosis according to the cellular context by transactivation of *CDKN1A* (which codes for the p21 protein), which leads to cell cycle arrest and senescence, and of *BAX*, *PUMA* and *NOXA*, which triggers apoptosis (314).

The p53 protein plays a crucial role in the maintenance of DNA integrity, through mediation of G1 arrest in response to DNA damage and other acute stresses such as hypoxia, oxidative stress and nutrient deprivation (128). If DNA is not repaired successfully, p53 can initiate apoptosis in the cell (315). Thus, inactivation of the mutations and deletions of *TP53* enables cells to proceed through the cell cycle in a genetically unstable state, which increases the chance of accumulation of other mutations. The suppression of apoptosis is not only important to maintain the tumour and enable its progression, but also facilitates resistance to cytotoxic agents that are commonly used to treat cancer, such as radiotherapy and chemotherapeutic drugs.

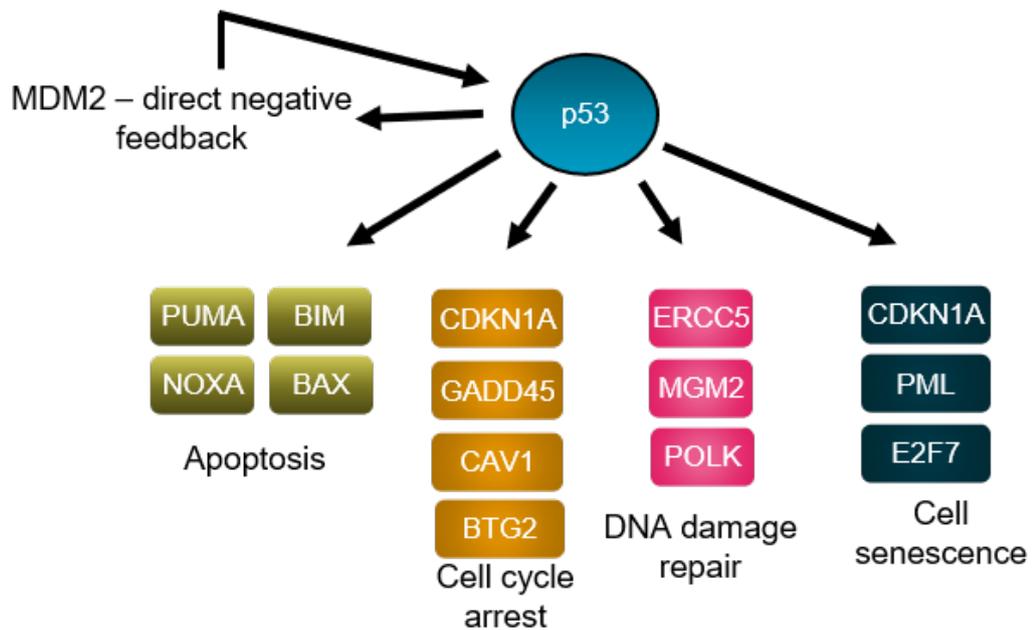


Figure 24: Mechanisms of action of wild-type p53 under conditions of cellular stress

There is no debate regarding the association of this lesion with prognosis in MM, as detailed in the introductory chapter. However, several areas of controversy exist, which limit the way forward in terms of the reporting of this lesion and consequently also limit progress in the discovery of ways to overcome this risk.

One of the main controversies occurs with regard to the reporting of del(17p). It arises due to differences in the association of the subclonal percentage of del(17p) with prognosis. Del(17p) is a secondary subclonal event that shows varying deletional clonal percentage at diagnosis (130). Reports by the IFM group suggest that patients have adverse outcomes only when the deletional percentage of 17p deletion is $\geq 60\%$ (134, 316). Another study by the IFM group shows data that are associated with a lower del(17p) cut-off level; it also shows a poor prognosis with a cut-off level for del(17p) of between 20% and 60% in Kaplan-Meier curves within supplementary data, although provide no statistical analysis for this (62). One study used iFISH data to analyse a cohort of 22 del(17p) cases and defined optimal cut-off at 50%, but this study still demonstrated poorer survival rates in patients with 20-50% del(17p) in Kaplan-Meier survival analysis. However, this study was limited due to its small size (90). The University of Arkansas for Medical Sciences (UAMS) research group has compared the two cut-off levels. They found that prognosis was significant at both

20% and 60% cut-off for del(17p) in the context of high-risk patients that were analysed by GEP (136). This study was limited in its interpretation, as patients were treated differently according to their GEP risk status. In addition, Bolli et al. showed through the sequencing of *TP53* mutations that the mutated tumour variant allele frequency demonstrated poor prognosis irrespective of whether the mutations were sub-clonal or clonal in nature (131). This analysis was limited, however, due to the finding that most *TP53* mutations caused bi-allelic inactivation of the gene. This study may have therefore largely represented bi-allelic inactivation rather than mono-allelic prognostic significance. Previously, both our and other groups have described prognostic significance of del(17p) that is present at levels of 20%, although this data has not previously been compared with higher cut-off levels (82, 125, 140).

Differences in *TP53* deletion-calling has led to difficulties in the ability to compare treatments in the context of this abnormality, as several commercial trials use different cut-off levels (317). The IFM group uses the 60% cut-off for IFM trials (62), while the Arkansas group apply a 10% cut-off for del(17p)-calling and the Heidelberg myeloma group has used a 60-70% cut-off (140). Currently the IMWG has not reached a consensus regarding the optimal clonal deletion fraction that has a prognostic implication (147, 318). The real-world consequence of these different cut-off choices is that comparison between treatments and between trials is not possible and this is an impediment to the assessment of therapy that is based on this lesion.

There is another area of debate regarding mono-allelic and bi-allelic alterations of *TP53*. One study has reported that only bi-allelic rather than mono-allelic alterations in *TP53* have prognostic association (100). However, this study was limited in terms of its follow-up period, which was just 22.9 months (100), and its findings are in contrast with those of other studies, which have shown prognostic association outside the context of bi-allelic *TP53* alterations.

Despite strong evidence of the poor prognostic impact of *TP53* aberrations or del(17p), many clinical trials do not report their effect in isolation; instead, outcomes of patients with high-risk disease are grouped by combining del(17p) with t(4;14), t(14;16) +/- gain(1q) within these analyses. This has led to difficulty in assessment of the effect of this lesion in the context of novel therapy (147). The evidence that exists at present is

summarised within the introduction chapter of this thesis. As a result of this scarcity of evidence, adaptation of therapy for MM according to cytogenetics has not been widely brought into standard clinical practice (148, 149). The aim of this study was to increase knowledge in areas of debate with regard to *TP53* as well as to provide evidence in the context of novel therapies that were used in the UK NCRI Myeloma XI trial.

To assess the prognostic importance of clonal and sub-clonal *TP53* CNA, we profiled the tumours of 1777 NDMM trial patients through use of MLPA. The Myeloma XI trial applied multi-staged randomisation of therapy, which enabled us to study this lesion in the context of novel therapies at different stages of MM therapy in a homogeneously treated group of patients.

Once we had established the optimal cut-off level through application of several different statistical methods, we compared mono-allelic to bi-allelically altered *TP53* to assess the impact on prognosis in a large number of patients with a long follow-up period to see whether the prognostic differences remained within the mono-allelically altered *TP53* group of patients.

Finally, we compared different treatments that were given to patients through induction, consolidation and maintenance stages to see whether there were differences in prognostic impacts at different time-points in the patients' treatment.

4.2 Aims of this study

- To investigate subclonal as well as clonal *TP53* deletions and their prognostic associations in the context of different therapeutic agents that were used in the Myeloma XI trial
- To investigate clinical and genetic associations of *TP53* aberrations
- To characterise the clinical impact of *TP53* deletion in patients in the various treatment contexts of the Myeloma XI trial
- To investigate *TP53* aberrations through deletions and/or mutations by integration of copy number and mutational assays.

4.3 Results

4.3.1 Examination of TP53 probes in MLPA P425 and XO73 probe sets

Historically, the main method that was used for copy-number calling and subclonality measurement in MM was iFISH. There are technical challenges in terms of quantitation of clonal percentages in the use of iFISH due to variability in CD138 cell separation techniques that are used in different laboratories, as well as inter-laboratory and inter-operator variability, which have been shown in previous audits (318).

We chose to analyse Myeloma XI trial data through use of MLPA, which used a similar technique to that employed in SNP arrays and offered technical advantages. These included high throughput and relative independence of the operator from the results. These advantages meant that the performance of uniform profiling across a large trial site such as ours was possible. Later, the findings of this study were validated and confirmed through use of iFISH on a subset of tumour samples to confirm the accuracy of the MLPA findings. This validation process is described later in this chapter.

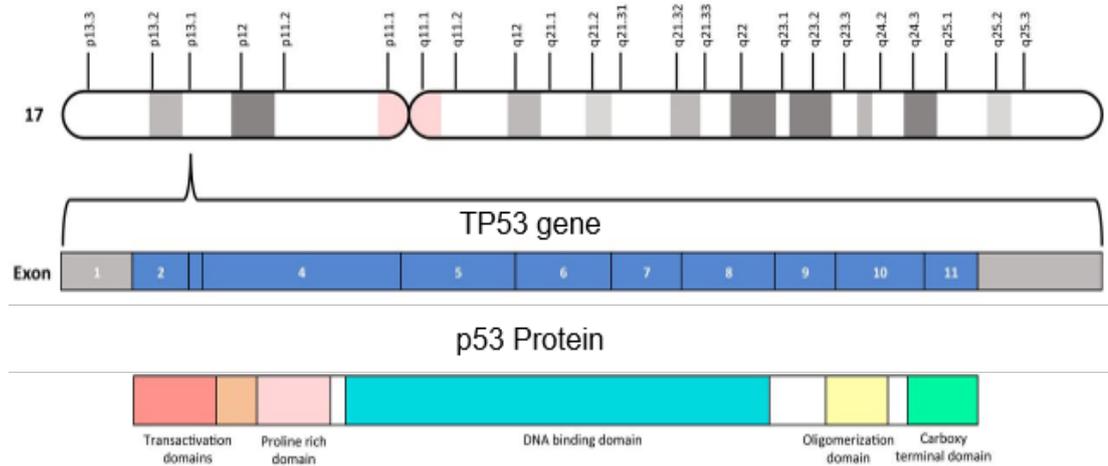


Figure 25: TP53 gene organisation. Mutations in TP53 are concentrated around the DNA-binding domain from exons 4-8, but deletions can be variably distributed (adapted from (53, 76))

We show the diagrammatic representation of the *TP53* gene within chromosome 17 in Figure 25. Two probesets were utilised to discover the MLPA-based copy numbers in this study. These were the P425 and the XO73 probesets, which are described within the methods chapter of this thesis. The P425 probeset contains three probes that function within exons 4, 7 and 10 in the *TP53* gene. At the time of the analysis, DNA from 1777 patients had been tested through use of the P425 probeset on tumour samples and survival results were available for these patients. The XO73 probeset involves 11 probes that cover all 11 exons of the *TP53* gene. This enabled the performance of a more detailed analysis of deletions within the *TP53* gene and enabled us to examine bi-allelic *TP53* deletions, which tend to be focal according to historic SNP-array findings. Samples with a DNA concentration of $<2\text{ng}/\mu\text{l}$ or those that failed MLPA quality metrics were excluded from analysis.

We first examined the distribution of probe values for each of the three *TP53* probes on the P425 MLPA probeset. Relative probe values for each patient were plotted and compared with diploid gene copy numbers for 1777 tumours. Histograms of these values showed a bimodal dataset with two distinct peaks (Figure 26). The major peak, which was observed in the majority of tumour samples, demonstrated the diploid status of *TP53*. These tumour samples are normally distributed around the 0.95-1 normalised MLPA probe value. Values of more than 1.2 represent a small proportion of patients who have a gain of chromosome 17, which is associated with hyperdiploid MM. Lower values represent patients with *TP53* deletion. A minor peak that is centred around 0.55 represents tumour samples with clonal deletion at *TP53*.

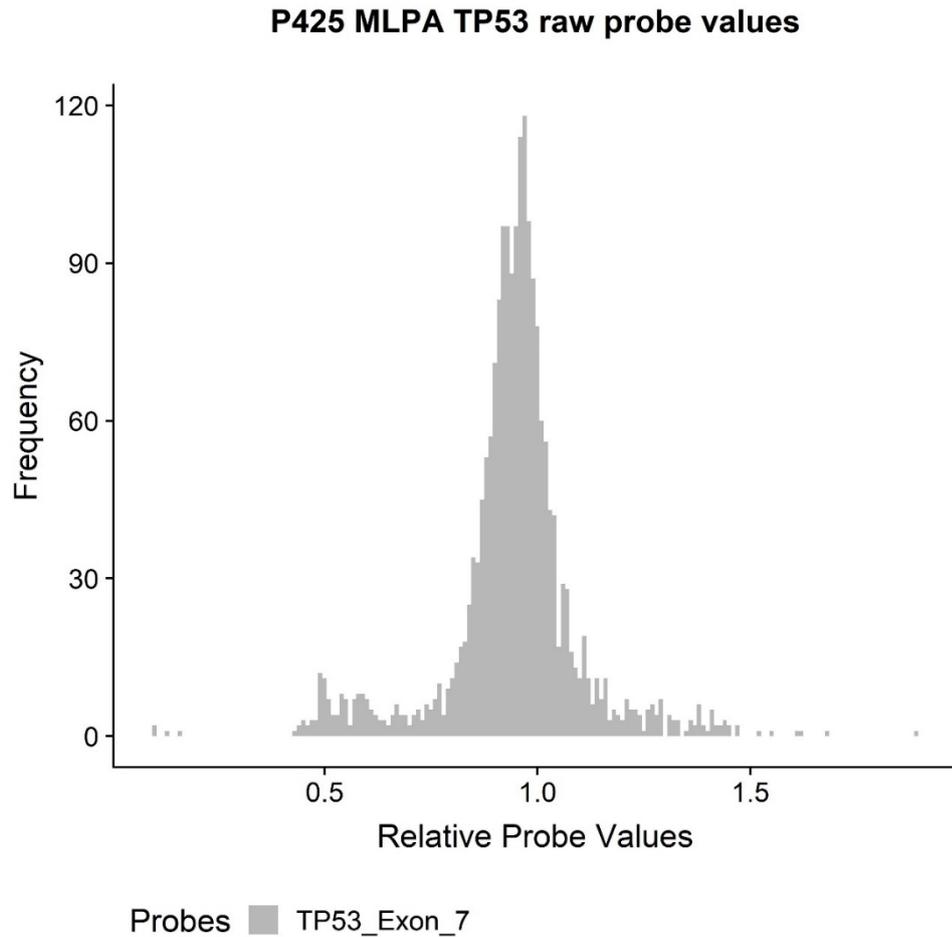


Figure 26: Relative normalised MLPA probe values of exon 7 on P425 probeset

The three probes and their distributions were examined and overlap of the three probes was demonstrated for the calling of deletion in *TP53* (Figure 27). For the MLPA P425 probeset, a two out of three probe approach was chosen for deletion-calling because, although all three probes were associated with survival, studies have shown that it is possible to have a deletion across only part of the *TP53* gene (125, 319). This approach therefore offered the added benefit of identification of more *TP53*-deleted cases due to improved coverage. Additionally, this approach took into consideration the potential for technical failure of a single probe. This is the approach that is recommended by the manufacturer for diagnostic purposes (320).

P425 MLPA TP53 raw probe values

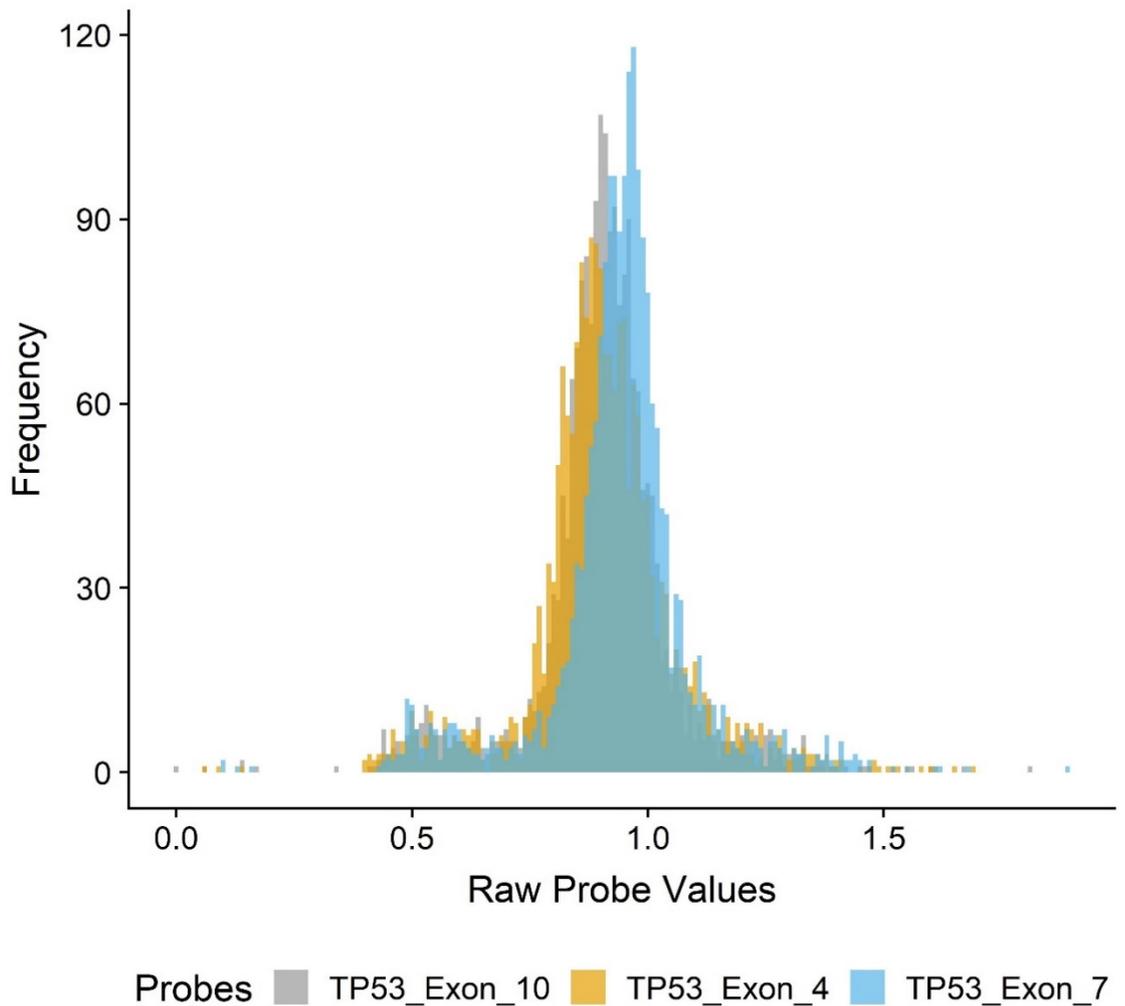


Figure 27: Histogram of MLPA distributions for P425 assay with three probes that covered exons 4, 7 and 10 of *TP53* in 1777 patients

4.3.2 Discovery of the optimal cut-off for survival associated with del(17p)

As shown above, MLPA probe values are continuous. Although the manufacturer defines deletion as a probe value of less than 0.75, which is based on data from germline samples, tumours display subclonal deletions that may cause alterations to these values in terms of calling deletion. Subclonal deletions in *TP53* form an area of debate in the context of MM and survival. We therefore first investigated different ways of defining the optimal cut-off value for calling *TP53* deletion that had an association with overall survival as an important biological end point.

One of the main methods that is used to calculate the optimal cut-off point for survival analysis is the minimal P -value approach. Methods can be based on significance of correlation with survival time through the systematic evaluation of dichotomous data splits with the most significant P -value on long-rank testing. The value chosen is the one that best separates patient outcomes according to a maximum chi-squared statistic and minimum p -value, or maximum relative risk. Similarly, the Cox proportional hazard model can be calculated by cutting continuous variables to binary and evaluating the minimal Wald P -value (321, 322).

Another method that is commonly used for optimal analysis is to find a compromise between the most sensitive and the most specific cut points for measurable data through use of the ROC curve as a global measure of diagnostic accuracy (323). Several researchers use the area under the ROC curve (AUC) to determine the optimal cut-off value. Recent research has incorporated time dependency instead of the standard ROC curve approach. This method has been developed to incorporate modelling of time for variables that may vary with time in terms of their association with survival (324).

In this analysis, we used a variety of approaches to find the optimal cut-off point that showed the highest prognostic association with the clinically significant endpoint of overall survival. We performed this by creation of dichotomous splitting of the continuous data from each of the three *TP53* probes in the P425 probeset by 0.05 step-wise intervals from the cut-off points 0.45 to 0.95, which were taken to cover the spectrum of clonal *TP53* deletions to diploid *TP53* status.

In order to identify a clinically relevant threshold for sub-clonal *TP53* deletions, step-wise increasing fractions of *TP53* deletions were interrogated through use of MLPA and the time-dependent ROC curve analysis method (AUC_i) (325) for OS. The results are shown in Table 34. Through use of this method, a normalised *TP53* MLPA value of <0.8 was identified as the cut-off that provided optimal prognostic power with an AUC_i of 0.557. Use of this method led to identification of 192 of 1777 cases (10.8% of tumours) as *TP53*-deleted. This was confirmed to be the optimal cut-off threshold for both intensively and non-intensively treated groups within the trial, as shown in Table 34. Similarly the area under the ROC curve was plotted for several timepoints

with a polynomial regression fit along different cut-offs for assignment of deletion 17p calling (Figure 28). We showed that the 0.8 cut-off point exhibited a consistently higher area under the curve compared with higher and lower cut-offs at all timepoints, which reflected the results for the AUCi analysis.

Table 34: Integrated AUC (AUCi) for all patients (n=1777), intensively treated patients (n=952) and non-intensively treated patients (n=825) for overall survival over time for step-wise cut-off points of *TP53*-deleted patients for defining optimal cut point.

MLPA cut-off for <i>TP53</i> deletion	AUCi All patients N=1777	AUCi Intensive group N=952	AUCi Non-intensive group N=825
<i>TP53</i> deletion cut-off 0.45	0.506	0.508	0.503
<i>TP53</i> deletion cut-off 0.50	0.509	0.512	0.507
<i>TP53</i> deletion cut-off 0.55	0.519	0.519	0.521
<i>TP53</i> deletion cut-off 0.60	0.527	0.522	0.533
<i>TP53</i> deletion cut-off 0.65	0.539	0.551	0.534
<i>TP53</i> deletion cut-off 0.70	0.541	0.558	0.533
<i>TP53</i> deletion cut-off 0.75	0.546	0.570	0.527
<i>TP53</i> deletion cut-off 0.80	0.557	0.581	0.537
<i>TP53</i> deletion cut-off 0.85	0.550	0.580	0.533
<i>TP53</i> deletion cut-off 0.90	0.543	0.565	0.525
<i>TP53</i> deletion cut-off 0.95	0.505	0.547	0.493

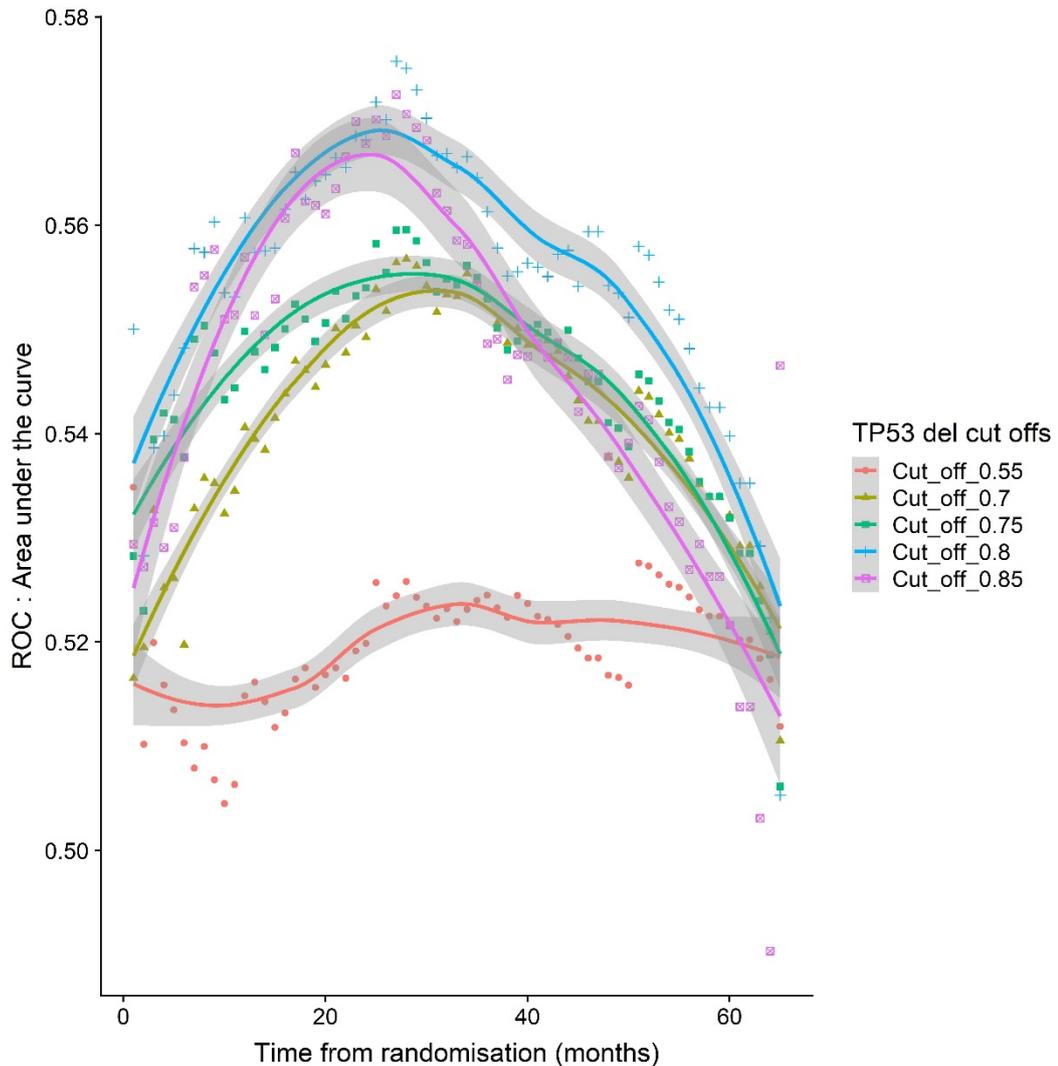


Figure 28: Area under the curve for receiver operating characteristics over time for different *TP53* deletion cut-offs in 1777 newly diagnosed patients

Since methods for calling optimal cut-off points for continuous variables vary according to different researchers, we therefore validated our finding of the optimal cut-off of <0.8 for *TP53* deletion-calling by use of other known methods for optimal cut-off calling that are based on minimum *P*-values.

We have demonstrated through production of the data that are shown in Table 35 that the minimum log-rank-based *P*-value correlated with a cut off of 0.8 with a *P*-value of 6.7×10^{-15} for overall survival. Similarly, as shown in Table 36, the minimum Cox-regression Wald *P*-value of 4.1×10^{-14} was also found at the same cut-off of <0.8 for *TP53* deletion calling. Additionally C statistic values were calculated as specified by Uno et al. (326) and this calculation also demonstrated that the highest C-statistic

coincided with the 0.8 cut-off, as shown in Table 36. The cut-off thresholds that showed the strongest association with PFS were more heterogeneous between tests as shown in Table 35. However, as OS is the more clinically significant endpoint, it was used for cut-off threshold calling. The optimal cut-off of <0.8 in two out of three *TP53* probes was used for all further analyses after this due to its strong and consistent association with poor survival rates.

These results are shown graphically through use of Kaplan-Meier curves (Figure 29), which demonstrate that survival of patients with an MLPA based cut-off of ≥ 0.8 - 0.84 is statistically the same as >0.85 and that lower cut-offs have poorer overall and progression-free survival rates.

In the next section, genetic and clinical characteristics of del(17p) are characterised as defined by the clinically relevant 0.8 cut-off point, which was discovered through use of MLPA as defined in this section.

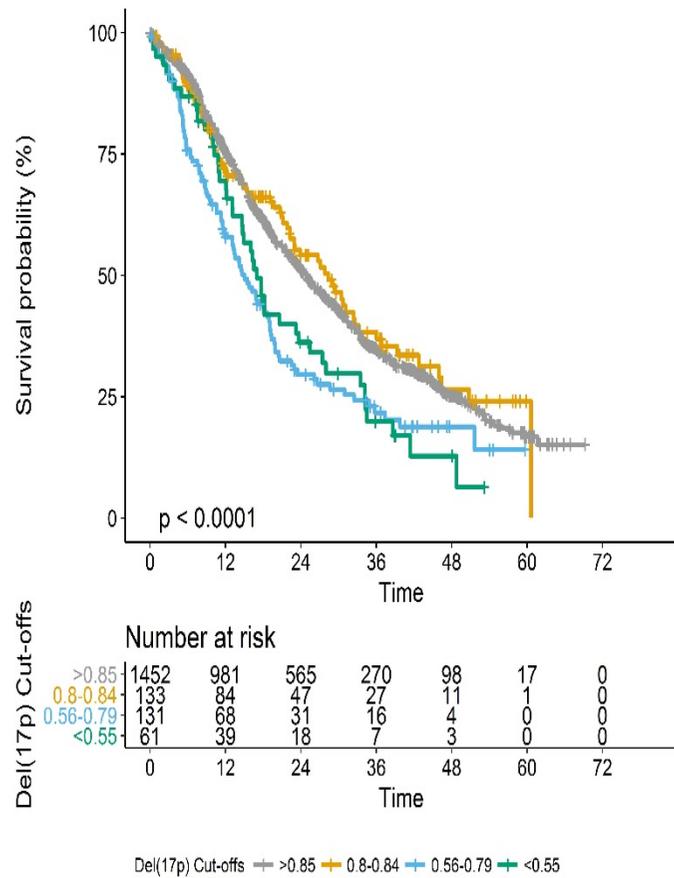
Table 35: Survival statistics for different *TP53* deletional cut-offs by MLPA using univariate Kaplan-Meier-based median progression-free and overall survival rates in months and log-rank *P*-values

	N	Overall survival			Progression-free survival		
		Median survival (months)			Median survival (months)		
		Del(17p)	No Del(17p)	P-Value (logrank)	Del(17p)	No Del(17p)	P-Value (logrank)
Del(17p) cut-off 0.45	7(0.4)	11.89 (2.9-NR)	59.5 (55.4-64)	1.25E-05	7.56 (2.0-NA)	23.8 (22.3-25.5)	4.37E-06
Del(17p) cut-off 0.50	28(1.6)	26.12 (22.4-NR)	59.6 (55.4-64)	4.16E-05	13.14 (10.8-23.2)	23.9 (22.4-25.7)	0.0009
Del(17p) cut-off 0.55	61(3.4)	31.15 (24.2-NR)	59.6 (56-64.3)	1.65E-06	16.99 (14.6-25.4)	24.0 (22.4-25.8)	0.014
Del(17p) cut-off 0.60	87(4.9)	31.15 (22.4-50.5)	60.3 (56-64.3)	4.46E-09	16.49 (13.3-20.5)	24.0 (22.6-26)	0.002
Del(17p) cut-off 0.65	112(6.3)	26.12 (22.4-33.8)	60.3 (56.2-64.3)	5.26E-14	14.69 (12.2-18.1)	24.6 (22.9-26.3)	3.34E-07
Del(17p) cut-off 0.70	125(7.0)	26.61 (23.2-35.4)	60.3 (56.2-64.3)	6.29E-14	15.44 (13.1-18.3)	24.6 (22.9-26.3)	5.15E-06
Del(17p) cut-off 0.75	145(8.2)	28.45 (24.5-39.1)	60.3 (56.2-64.3)	1.89E-13	16.03 (13.3-18.3)	24.7 (23-26.6)	1.95E-05
Del(17p) cut-off 0.80	192(10.8)	31.15 (25.6-42.3)	60.4 (56.2-64.3)	6.66E-15	16.03 (13.5-18.7)	25 (23.4-27.1)	4.11E-07
Del(17p) cut-off 0.85	325(18.3)	45.54 (38.3-NR)	60.3 (56-NA)	1.01E-06	19.15 (16.8-22.5)	24.9 (23.1-26.8)	0.002
Del(17p) cut-off 0.90	676(38.0)	50.50 (45.6-NR)	60.4 (57-NA)	0.001	20.53 (19.1-23.4)	25.2 (23.5-27.7)	0.002
Del(17p) cut-off 0.95	1113(62.6)	59.53 (53.9-NR)	62.8 (53.5-NA)	0.37	23.16 (21.4-25.9)	24 (21.8-26.3)	0.30

Table 36: Survival statistics for different *TP53* deletional cuts by MLPA using Cox-based hazard modelling and Harell's C-statistic by Uno et al. for different *TP53* cut-offs by MLPA in the Myeloma XI trial

	N	Overall survival			Progression-free survival		
		Median survival (months)			Median survival (months)		
		HR	P-value (Wald)	C-stats – 36 months	HR	P-value (Wald)	C-stats – 24 months
Del(17p) cut-off 0.45	7(0.4)	5.7 (2.36-13.79)	0.0001	0.5(0.5-0.51)	4.86 (2.3-10.23)	3.23E-05	0.5(0.5-0.51)
Del(17p) cut-off 0.50	28(1.6)	2.81 (1.68-4.7)	8.80E-05	0.51(0.5-0.52)	1.99 (1.31-3.01)	0.001	0.51(0.5-0.51)
Del(17p) cut-off 0.55	61(3.4)	2.34 (1.64-3.35)	3.30E-06	0.52(0.51-0.53)	1.45 (1.08-1.94)	0.01	0.51(0.5-0.51)
Del(17p) cut-off 0.60	87(4.9)	2.39 (1.77-3.23)	1.30E-08	0.53(0.52-0.54)	1.49 (1.16-1.92)	0.002	0.51(0.5-0.52)
Del(17p) cut-off 0.65	112(6.3)	2.69 (2.06-3.52)	4.88E-13	0.54(0.53-0.56)	1.76 (1.41-2.19)	4.73E-07	0.52(0.51-0.53)
Del(17p) cut-off 0.70	125(7.0)	2.59 (2.00-3.35)	4.87E-13	0.54(0.53-0.56)	1.63 (1.32-2.01)	6.35E-06	0.52(0.51-0.53)
Del(17p) cut-off 0.75	145(8.2)	2.44 (1.91-3.11)	1.08E-12	0.55(0.53-0.56)	1.54 (1.26-1.88)	2.26E-05	0.52(0.51-0.53)
Del(17p) cut-off 0.80	192(10.8)	2.36 (1.89-2.95)	4.12E-14	0.55(0.54-0.57)	1.57 (1.32-1.88)	5.17E-07	0.53(0.52-0.54)
Del(17p) cut-off 0.85	325(18.3)	1.64 (1.34-2)	1.29E-06	0.54(0.52-0.56)	1.27 (1.09-1.48)	0.002	0.53(0.51-0.54)
Del(17p) cut-off 0.90	676(38.0)	1.32 (1.11-1.57)	0.001	0.53(0.51-0.55)	1.21 (1.08-1.37)	0.002	0.53(0.51-0.54)
Del(17p) cut-off 0.95	1113(62.6)	1.09 (0.91-1.3)	0.37	0.51(0.48-0.54)	1.07 (0.94-1.21)	0.3	0.51(0.48-0.53)

a) Progression-free survival



b) Overall survival

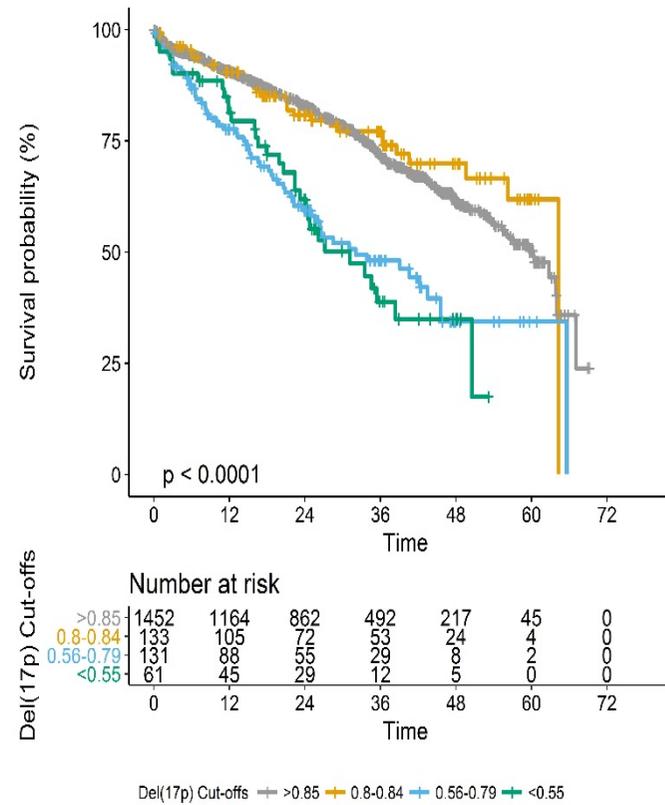


Figure 29: Kaplan-Meier survival curves showing a) PFS and b) OS of different clonal fractions of *TP53* deletion by MLPA in 1777 patients

4.3.3 Characteristics of del(17p) in MM

We first checked for potential confounders between the del(17p) cohort of patients and the cohort that showed no deletion in case these confounders influenced the results. Imposition of a cut-off of <0.8 to call *TP53* deletion led to balance between patients with or without *TP53* aberration between treatment-arm allocations and between intensive and non-intensive treatment-arm allocations (Table 37). We found no statistically different confounders within patient demographics such as age or gender. Furthermore, we also found no statistical differences in treatment randomisation or arms of this cohort within the trial (Table 37).

Table 37: Patient demographics and treatment arms according to *TP53* deletion status

	Missing (N)	No <i>TP53</i> deletion N (%) Total =1585	<i>TP53</i> deletion N (%) Total=192	<i>P</i>
Age >70	0	608(38.4)	72(37.5)	0.88
Male	0	965(60.9))	106(55.2)	0.14
Female		620(39.1)	86(44.8)	
Treatment Characteristics				
Intensive	0	847(53.4)	105(54.7)	0.76
Non-intensive		738(46.6)	87(45.3)	
<u>Induction chemotherapy</u>	0	796(50)	99(52)	0.76
Thalidomide based		789(50)	93(48)	0.76
Lenalidomide based				
<u>Consolidation randomisation</u>	0	188(11.9)	21(10.9)	0.18
Consolidation VCD		119(7.5)	21(10.9)	0.18
No consolidation VCD		1278(81)	150(78.1)	0.44
Not randomised				
<u>Maintenance randomisation</u>	0	359(22.6)	40(20.8)	0.91
Maintenance lenalidomide		292(18.4)	31(16.1)	0.65
Observation only		799(50.4)	102(53.1)	0.49
Not randomised				

4.3.3.1 Genetic association with del(17p) vs. no del(17p)

Associations of *TP53* deletions with other common aberrations in MM were examined (Table 38). The occurrence of *TP53* deletion was positively associated with del(13q) ($P < 0.001$), del(12p) ($P < 0.001$) and del(16q) ($P < 0.001$). It was more weakly associated with del(1p32) ($P = 0.025$) and del(14q) ($P = 0.005$). There was a negative association with hyperdiploidy ($P = 0.02$) that was markedly stronger with increased copy number of certain odd-numbered chromosomes, such as chromosome 9 ($P = 8.8 \times 10^{-5}$). There was no statistically significant association with IgH translocations or gain(1q) although a higher frequency was noted in t(14;16) and t(14;20) and lower frequency in the t(11;14) subsets; however, these did not reach statistical significance.

The most significant association that was observed was the high association of *TP53* deletion with del(13q) and del(17p). This was especially the case within the section 13q14 (which contains *RBI* among other genes). Although this result cannot be conclusive of an association due to the sparsity of probes on chromosome 13 through use of the MLPA P425 assay, it is an interesting finding given the association of *TP53* and *RBI* in the cell cycle as well as other functions. Examples of these include cell plasticity that results in treatment resistance and poor prognosis in several other cancers (327, 328). This association is explored further in this chapter with regard to clonal and subclonal *TP53* deletions.

Table 38: Genetic characteristics compared between patients with *TP53* deletion (n=192) and patients with no *TP53* deletion (n=1585) in the Myeloma XI trial

Genetic associations	Missing (N)	No <i>TP53</i> deletion N (%) Total =1585	<i>TP53</i> deletion N (%) Total=192	<i>P</i>
Del(13q) region	0	534(33.7)	109(56.8)	1.09x10 ⁻¹⁰
Del(13q) <i>RBI</i>	0	574(36.2)	121(63)	1.45x10 ⁻¹²
Del(13q) <i>DIS3</i>	0	521(32.9)	102(53.1)	6.08x10 ⁻⁸
Del(12p)	0	88(5.6)	35(18.2)	1.28x10 ⁻⁸
Del(16q12) <i>CYLD</i>	0	222(14)	54(28.1)	2.35x10 ⁻⁶
Del(16q23) <i>WWOX</i>	0	218(13.8)	54(28.1)	1.22x10 ⁻⁶
Del(14q) region	0	179(11.3)	36(18.8)	0.005
Gain(1q)	0	528(33.3)	68(35.4)	0.57
Del(1p)	0	140(8.8)	27(14.1)	0.025
Hyperdiploidy	0	760(47.9)	75(39.1)	0.022
Gain 5	0	731(46.1)	68(35.4)	0.006
Gain 9	0	765(48.3)	64(35.4)	8.82x10 ⁻⁵
Gain 15	0	768(48.5)	82(42.7)	0.15
t(4;14)	233	156(9.8)	21(10.9)	0.70
t(14;16)	233	37(2.3)	8(4.2)	0.15
t(14;20)	233	12(0.8)	4(2.1)	0.09
t(11;14)	233	239(15.1)	21(10.9)	0.10

4.3.3.2 Clinical association with del(17p)

The association was also examined between *TP53* deletion and biochemical, haematological and clinical markers. Patients with *TP53* deletion showed features of advanced disease and associated morbidity, which were specifically reduced platelet counts $<150 \times 10^9/L$ ($P=0.002$) and poorer performance status (World Health Organization (WHO) performance score ≥ 2) ($P=0.0012$). Although the WHO performance score was found to be independently associated with shortened survival on multivariable analysis, this association suggests an interrelationship with genetic and clinical features that are normally thought of as patient-related rather than disease-related. A weak association only was noted with calcium ≥ 3 mmol/l. The association between clinical features and *TP53* deletion is shown in Table 39.

Table 39: Baseline table: clinical and biochemical characteristics comparing patients with *TP53* deletion (n=192) with those without the deletion (n=1585) in the Myeloma XI trial

Clinical characteristics	Missing (N)	No <i>TP53</i> deletion N (%) Total =1585	<i>TP53</i> deletion N (%) Total=192	<i>P</i>
Hb >100 g/l	1	997(62.9)	113(58.9)	0.34
Plt <150 x10 ⁹ /l	0	170(10.7)	38(19.8)	0.002
Corrected Calcium >3 mmol/l	8	46(2.9)	11(5.7)	0.04
LDH <300 U/l	301	572(36.1)	73(38)	0.55
eGFR <30	25	144(9.1)	20(10.4)	0.5
B2M >5.5 mg/l	112	456(28.8)	64(33.3)	0.85
Albumin ≥ 35 g/l	5	834(52.6)	101(52.6)	1.0
Lytic bone lesions present	18	1038(65.5)	127(66.1)	1.0
ISS III	113	456(28.8)	64(33.3)	0.24
WHO performance status ≥ 2	72	314(19.8)	58(30.2)	0.0012

4.3.3.3 Response to induction treatment

Next, the association between induction response and *TP53* deletion was investigated (Table 40). No significant difference was found in the response, with an overall response rate (ORR) of 80.7% vs. 83.2%. However, complete response (CR) was achieved at lower frequency in the patients with deletion (3.6% vs. 7.3%) and these patients also showed a higher frequency of disease progression (5.7% vs. 2.9%), although neither of these rates reached statistical significance. This is a surprising finding considering the association of the deletion with shorter progression-free survival and overall-survival rates that has been found in this and previous studies. It suggests that the effect of this genetic lesion over time is similar to that suggested by the AUC:ROC curve in Figure 28. These results show that the predictive power of *TP53* is highest at around 25-30 months and low from the first few months of induction.

Table 40: Response to induction treatment based on *TP53* deletion. VGPR = very good partial remission, CR = complete remission, PR = partial remission, MR=minimal remission, NC=no change, PD = progressive disease

Response to Induction	Missing (N)	No <i>TP53</i> deletion N (%) Total =1585	<i>TP53</i> deletion N (%) Total=192	<i>P</i>
≥VGPR	76	857(54.1)	101(52.6)	0.70
CR	76	116(7.3)	7(3.6)	0.07
VGPR	76	741(46.8)	94(49)	0.59
PR	76	461(29.1)	54(28.1)	0.80
MR	76	71(4.5)	7(3.6)	0.71
NC	76	36(2.3)	2(1)	0.42
PD	76	46(2.9)	11(5.7)	0.05
Death within 60 days of induction	76	46(2.9)	9(4.7)	0.18

4.3.4 Survival in the context of anti-myeloma therapy and *TP53* deletion

The Myeloma XI trial enables the study of both intensively and non-intensively treated patients. Additionally, as Myeloma XI is a multi-staged trial with three stages of randomisation, it enables the study of the prognostic association of *TP53* deletion at different stages of the disease and in the context of treatment. The first randomisation is at induction between thalidomide and lenalidomide-based induction (with cyclophosphamide and dexamethasone).

The second randomisation step involved only a subset of patients who had achieved only a partial response (PR) or minimal response (MR) to the above induction treatment. These patients were randomised to either PI-based intensification (bortezomib with cyclophosphamide and dexamethasone (VCD)) or to no intensification. Patients who achieved stable disease or progressive disease were automatically eligible for VCD treatment and did not undergo randomisation, as detailed in the methods chapter.

The third randomisation step in the trial was at the end of induction +/- consolidation. Patients were randomised to no maintenance/lenalidomide maintenance or to lenalidomide and vorinostat maintenance. At the time of analysis, patients who had been randomised to lenalidomide and vorinostat had not been evaluated in terms of the main trial outcomes, so these data have not been included within this analysis due to trial restrictions. We have compared maintenance with lenalidomide only vs. no maintenance in the context of *TP53* deletion. As shown in Table 37, all randomisation arms look balanced in the context of *TP53*-deleted patients.

Table 41: Landmarked univariate analysis: median survival in months and Cox-based hazard ratios for PFS and OS of three randomisation stages of the Myeloma XI trial: thalidomide vs. lenalidomide induction; response-based VCD vs. no VCD consolidation; and lenalidomide vs. no maintenance randomisation. Also shown are non-randomised treatment parameters including intensive and non-intensive and HDM-ASCT in the context of *TP53* deletion

	PFS					
	N	Median/ months (95% CI)		P	Cox HR	P
					(95% CI)	
		Deletion of <i>TP53</i>	No <i>TP53</i> deletion		<i>TP53</i> del	
Intensive	952	19.8 (18.1-28)	33.6 (31.7-36.9)	5.0x10 ⁻⁶	1.8 (1.4-2.3)	6.8 x10 ⁻⁶
Non-intensive	825	11.5 (9.1-14.7)	16.2 (15.2-17.5)	0.0030	1.5 (1.1-1.9)	0.0032
High dose melphalan + ASCT	600	20.4 (14-32.6)	39.0 (33.3-44.5)	1.4x10 ⁻⁵	2.1 (1.5-3.0)	2.2x10 ⁻⁵
Thalidomide-induction	895	16.2 (11.7-19.3)	23.2 (21.2-26.6)	0.00028	1.6 (1.2-2.0)	0.00031
Lenalidomide-induction	882	16.0 (13.4-19.9)	26.0 (24.1-28.5)	0.00049	1.6 (1.2-2.1)	0.00055
VCD-consolidation	142	22.5 (10.1-NA)	35.9 (28.1-54.7)	0.082	1.9 (0.9-4.1)	0.087
No consolidation	130	7.7 (3.5-12.6)	20.0 (11.9-31.3)	0.00024	2.8 (1.6-4.9)	0.00044
Maintenance lenalidomide	387	19.1 (15.9-NA)	36.5 (30.8-42)	0.025	1.7 (1.1-2.6)	0.026
No maintenance	314	7.8 (6.3-25.4)	18.0 (14.6-21.8)	0.0025	1.9 (1.2-2.9)	0.0029
	OS					
	N	Median/ months (95% CI)		P	HR(95% CI)	P
					Deletion of <i>TP53</i>	
Intensive	952	38.34 (31-NA)	67.1 (63.8-NA)	2.3x10 ⁻¹⁴	3.3 (2.4-4.6)	6.0x10 ⁻¹³
Non-intensive	825	23.75 (19.7-40.6)	44.6 (40-50.2)	2.4x10 ⁻⁵	1.9 (1.4-2.6)	3.3 x10 ⁻⁵
High dose melphalan + ASCT	600	35.84 (27.4-NA)	NA (56.1-NA)	3.6x10 ⁻¹²	4.4 (2.8-6.9)	1.6x10 ⁻¹⁰
Thalidomide-induction	895	32.07 (19.9-45.5)	60.3 (53.9-NA)	3.3x10 ⁻⁸	2.3 (1.7-3.1)	7.7 x10 ⁻⁸
Lenalidomide-induction	882	31.05 (26.1-NA)	60.4 (56-NA)	6.1x10 ⁻⁸	2.5 (1.8-3.5)	1.6 x10 ⁻⁷
VCD-consolidation	142	39.13 (29.5-NA)	NA (NA-NA)	0.058	2.5 (0.9-6.7)	0.067
No consolidation	130	19.68 (13.9-NA)	NA (NA-NA)	7.0x10 ⁻¹⁰	8.7 (3.8-19.6)	2.2 x10 ⁻⁷
Maintenance lenalidomide	379	42.18 (25.9-NA)	NA (50.9-NA)	0.00011	3.1 (1.7-5.6)	0.00024
No maintenance	309	32.89 (23.4-NA)	57.3 (NA-NA)	0.067	1.9 (0.95-3.7)	0.072

4.3.4.1 Survival in intensive and non-intensive arms of treatment

Patients who took part in the Myeloma XI trial were selected for treatment under either the transplant-eligible (intensive) arm or the transplant non-eligible (non-intensive) arm, as decided by the patient's physician, who took the patient's age and physical fitness into account. In general, patients are usually thought to be transplant eligible if they are below the age of 70; this age limit is extended at several centres for fit and healthy patients with no or few co-morbidities. *TP53* deletion was found to be associated with a poorer PFS and OS in both groups (Figure 30; Table 41). This was shown to be the case in multivariable analysis with other factors known to influence prognosis in MM. The size of the prognostic impact of *TP53* deletion was found to be more pronounced in intensively treated patients compared with non-intensively treated patients, particularly for OS, which showed a hazard ratio of 3.3 (95% CI 2.4-4.6; $P=6 \times 10^{-13}$) in the intensively treated arm vs. 1.9 (95% CI 1.4-2.6; $P=3.3 \times 10^{-5}$) in the non-intensively treated.

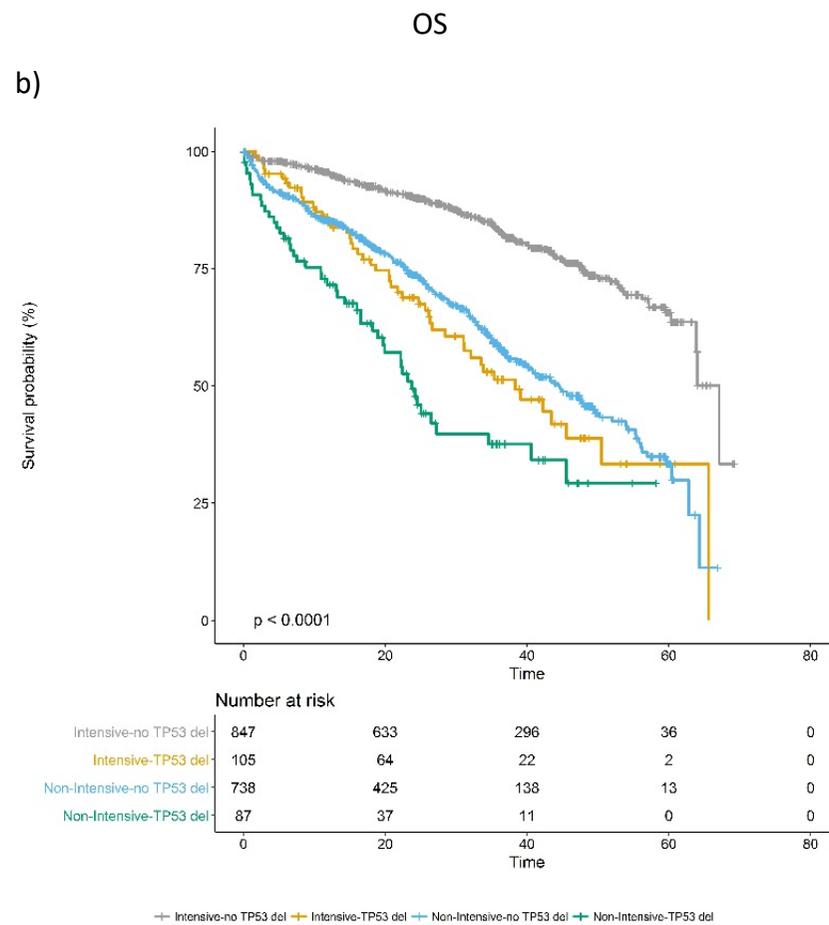
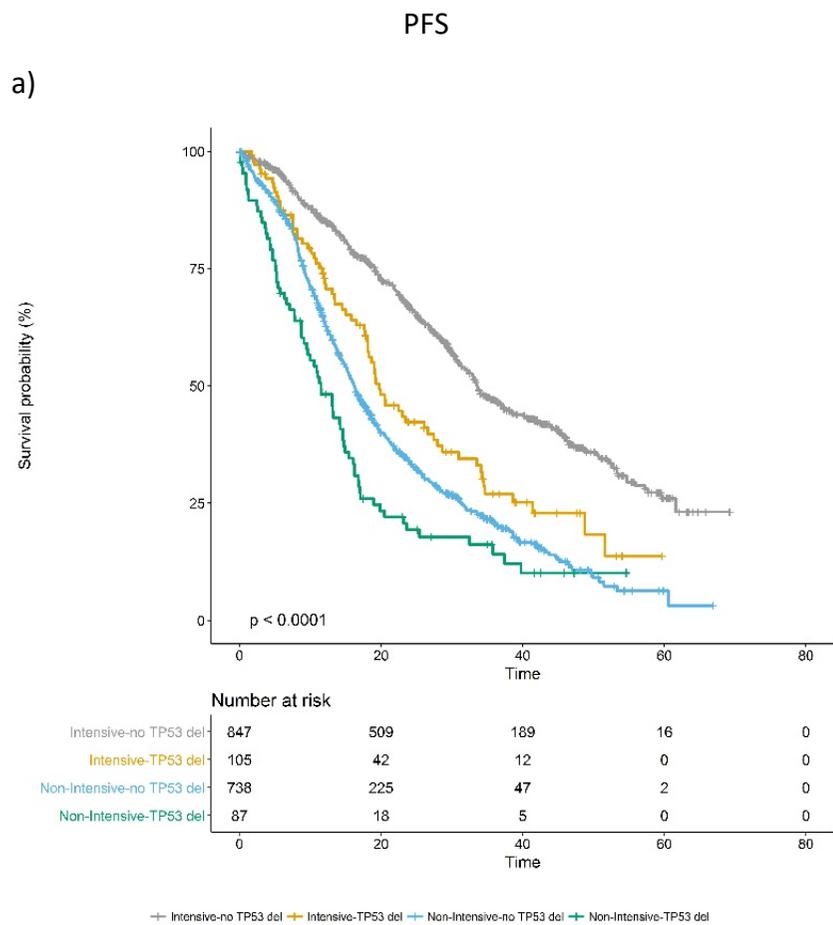


Figure 30: Kaplan-Meier survival curves showing a) PFS and b) OS of intensively and non-intensively treated patients with or without *TP53* deletion by MLPA in 1777 patients

4.3.4.2 Survival in the context of type of IMiD induction

The association of *TP53* deletion with inferior outcome was consistent in landmark analyses for all randomisations within the Myeloma XI trial. Specifically, *del(TP53)* was associated with shortened survival periods for patients who were randomised to either CRD or CTD induction randomisation. Randomisation of thalidomide vs. lenalidomide induction made no statistical difference in terms of PFS or OS (PFS: HR = 1.1(0.9-1.6); $P=0.332$, OS: HR = 1.3(0.9-1.9); $P=0.24$) within the 192 patients with *del(TP53)* (Figure 31, Table 41).

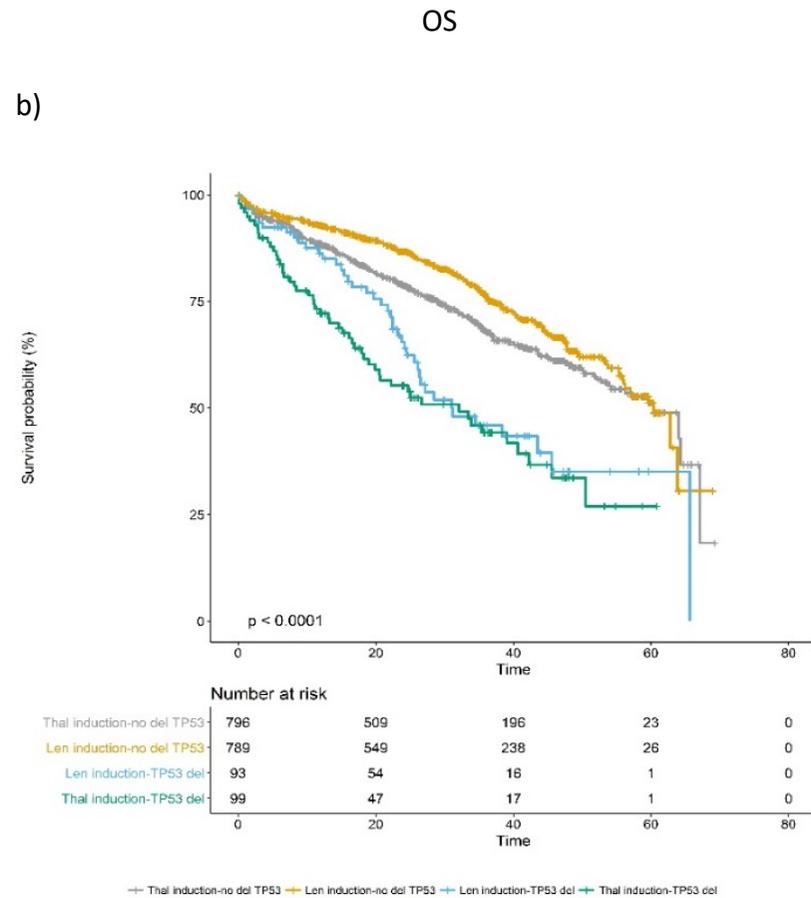
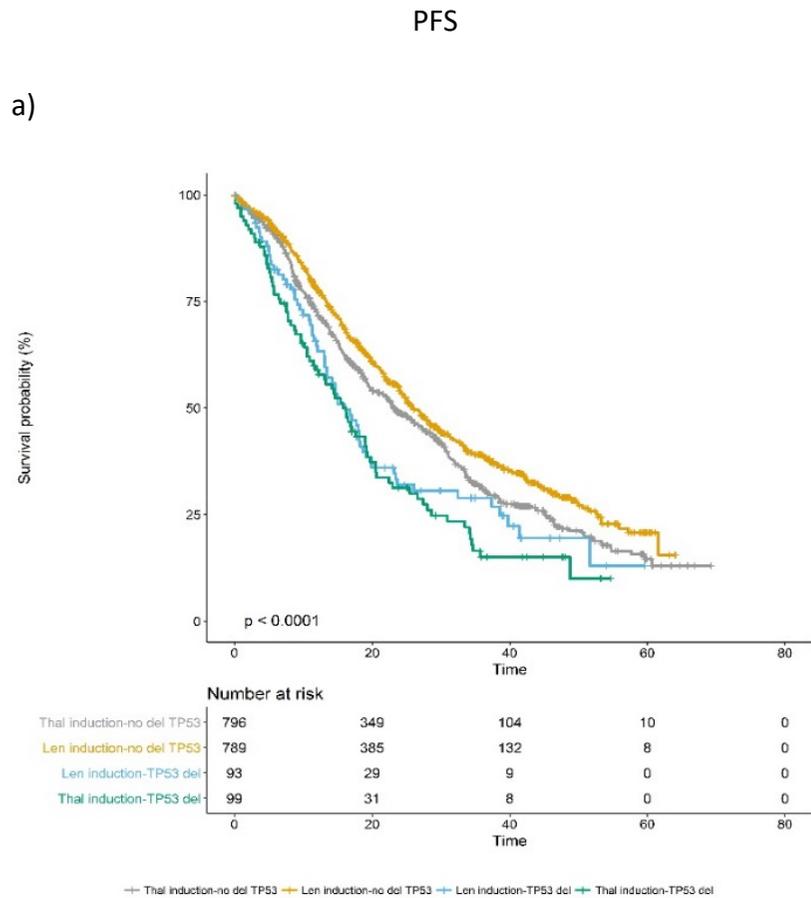


Figure 31: Kaplan-Meier survival curves showing (a) PFS and (b) OS of patients who were randomised to induction with thalidomide or lenalidomide (with cyclophosphamide and dexamethasone)

4.3.4.3 Survival in the context of CVD consolidation

As detailed above, the ORRs after inductions were similar regardless of del(*TP53*) status. Patients with sub-optimal response (MR or PR) to IMiD induction were randomised to receive proteasome inhibitor combination therapy (CVD) consolidation or no CVD consolidation. For 272 patients, landmarked PFS and OS outcomes were available for this randomisation. Overall, *TP53* deletion appeared to be associated with shortened survival periods in these patients, although this did not reach statistical significance. Del(*TP53*) was associated with a PFS hazard ratio of 1.9 (95%CI 0.91-4.05; $P=0.09$) in the CVD treatment arm and 2.8 (95%CI 1.57--4.9; $P=0.0004$) in the arm without consolidation. OS was similarly affected by del(*TP53*) with an HR of 2.5 (95% CI 0.9-6.7; $P=0.07$) in patients who were treated with VCD consolidation vs. 8.7 (95% CI 3.8-19.6; $P=2.2 \times 10^{-7}$). Within the subset of patients with del(*TP53*), CVD treatment was associated with improved survival, with a significantly longer median OS of 39.1 months (95% CI: 29.5-not reached) vs. 19.7 months (95% CI: 13.9-not reached, log-rank $P=0.027$) and an HR of 0.3 (95% CI 0.1-0.9; $P=0.03$) (Table 41, Figure 32). Our data in the context of patients who achieved only a PR or MR to induction IMiD-based treatment suggest that VCD may be beneficial in patients with a *TP53* deletion although it should be acknowledged that the number of patients in this subgroup were low and our results would need further validation.

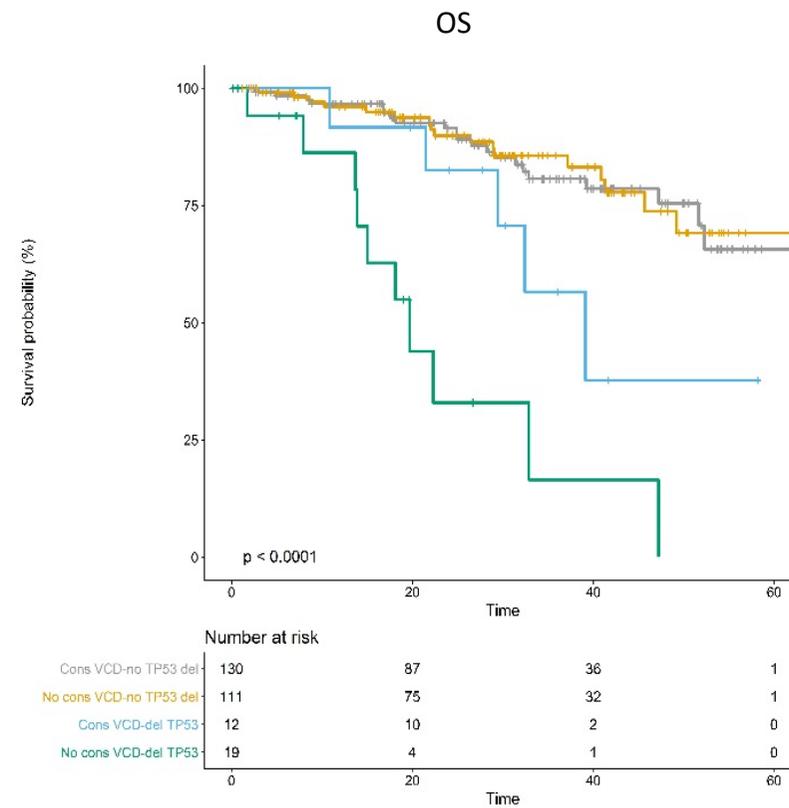
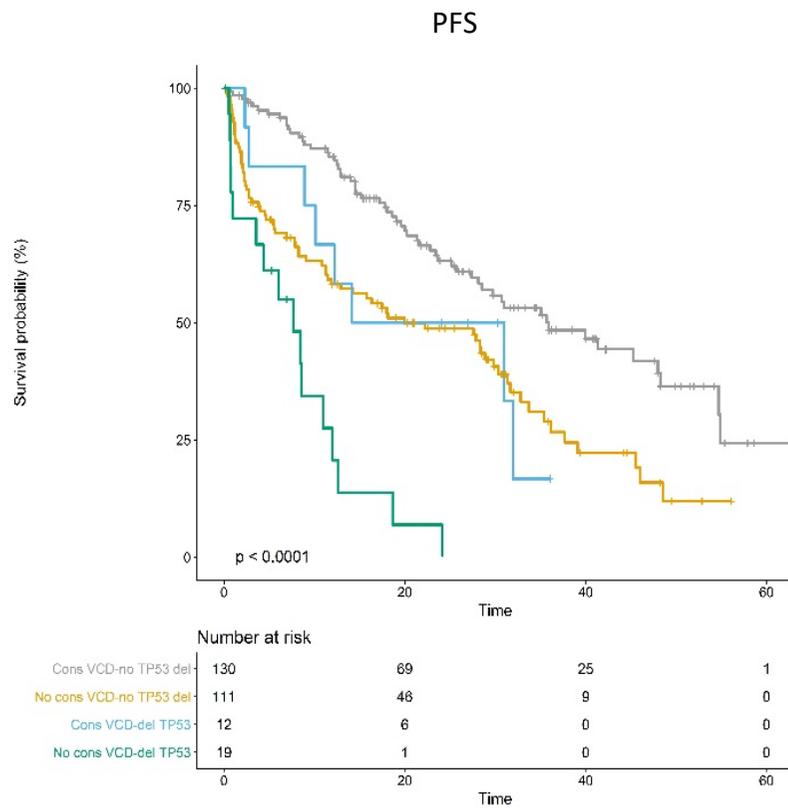


Figure 32: Kaplan-Meier survival curves showing PFS and OS of randomisation stages of the Myeloma XI trial; response-based VCD vs. no VCD consolidation was given to patients who achieved minimal or partial remission only

4.3.4.4 Survival landmarked from time of transplantation

The effect of *TP53* deletion after HDM-ASCT treatment was analysed and landmarked from the time of transplant and the data are therefore limited only to patients who underwent a transplant. *TP53* deletion was associated with a significantly worse PFS (HR 2.1; 95% CI 1.5-3.0; $P=2.23 \times 10^{-5}$) and OS (HR 4.4; 95% CI 2.8-6.9; $P=1.61 \times 10^{-10}$) from the time of transplantation. This finding suggests that transplantation does not overcome the association with poor risk (Figure 33; Table 41). The question of whether transplantation has any benefit in the context of this lesion can only be answered through randomisation and so is not addressed here.

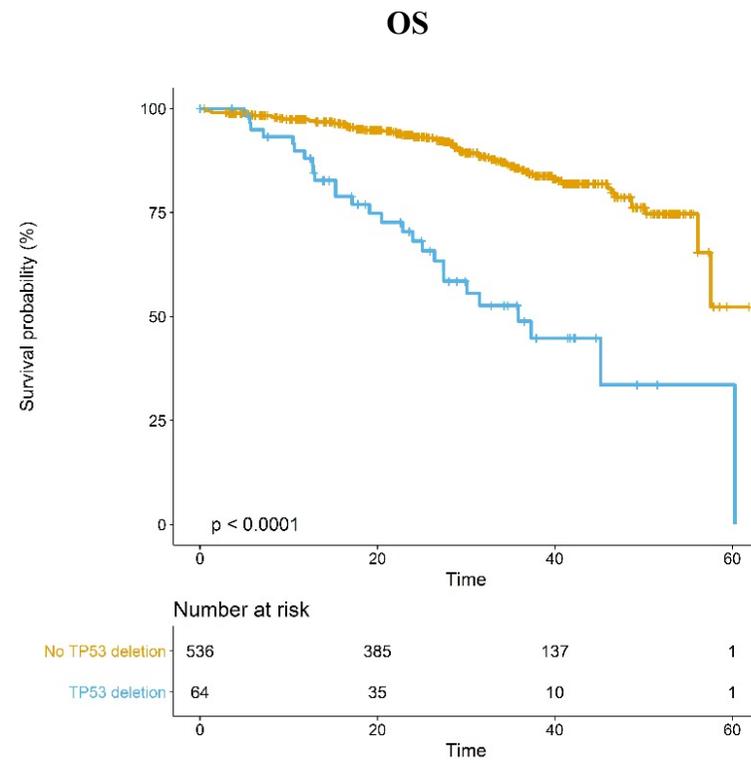
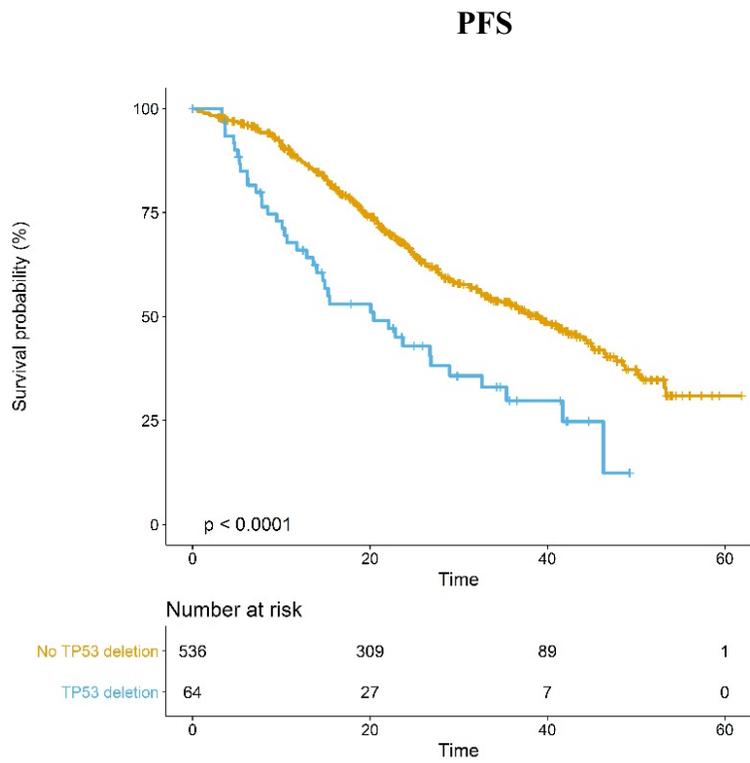


Figure 33: Kaplan-Meier survival curves showing PFS and OS of patients who underwent HDM-ASCT according to whether or not they had a *TP53* deletion

4.3.4.5 Survival in the context of IMiD-based maintenance randomisation

The final randomisation step in the Myeloma XI trial involved randomisation to lenalidomide maintenance or to monitoring only (data for patients who were randomised to lenalidomide and vorinostat were not available at this timepoint). In a landmark analysis from the date of maintenance randomisation, patients with del(*TP53*) were associated with poorer PFS rates with a HR of 1.9 (1.3-3.0; $P=0.001$) compared with those who showed no deletion; this improved to 1.7 (95%CI 1.1-2.6; $P=0.03$) when lenalidomide maintenance was given. In patients with a *TP53* deletion, by univariate analysis, patients who received lenalidomide maintenance had a PFS of 19.1 months vs. 7.8 months ($P=0.02$) with no maintenance. However, lenalidomide maintenance did not abrogate its negative impact of del(*TP53*) completely ($P=0.00021$). This benefit in PFS that was conferred by lenalidomide maintenance was not carried through to OS, as there was no significant difference in OS between patients who received maintenance and those who did not (Table 41, Figure 34).

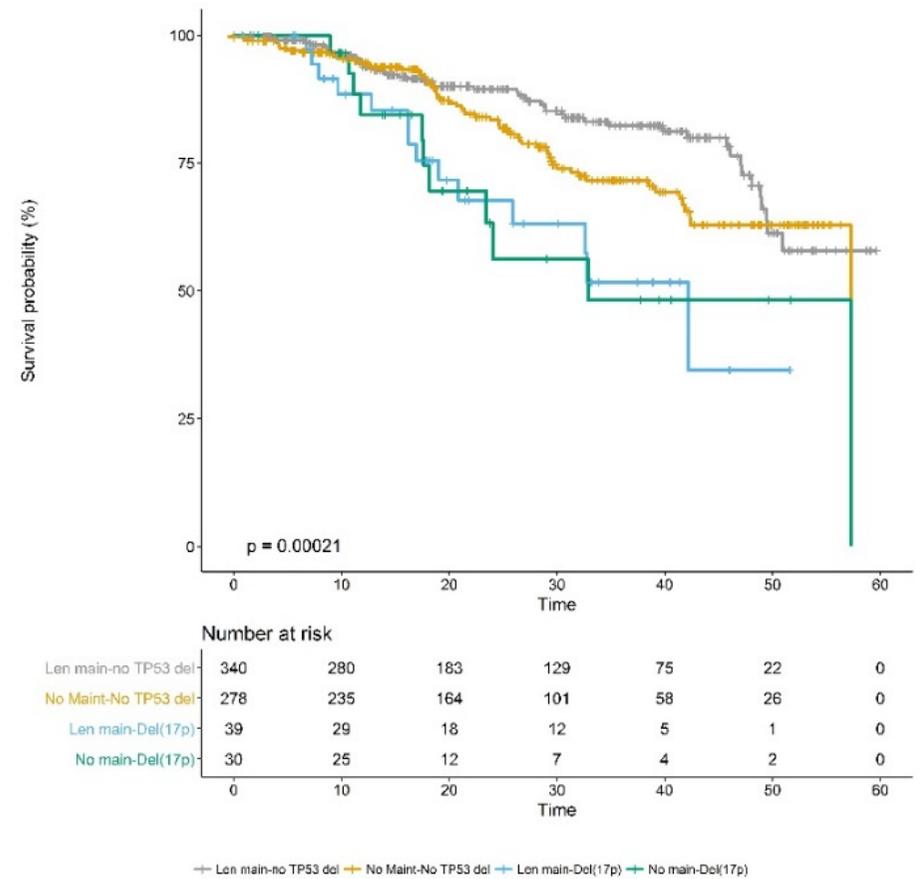
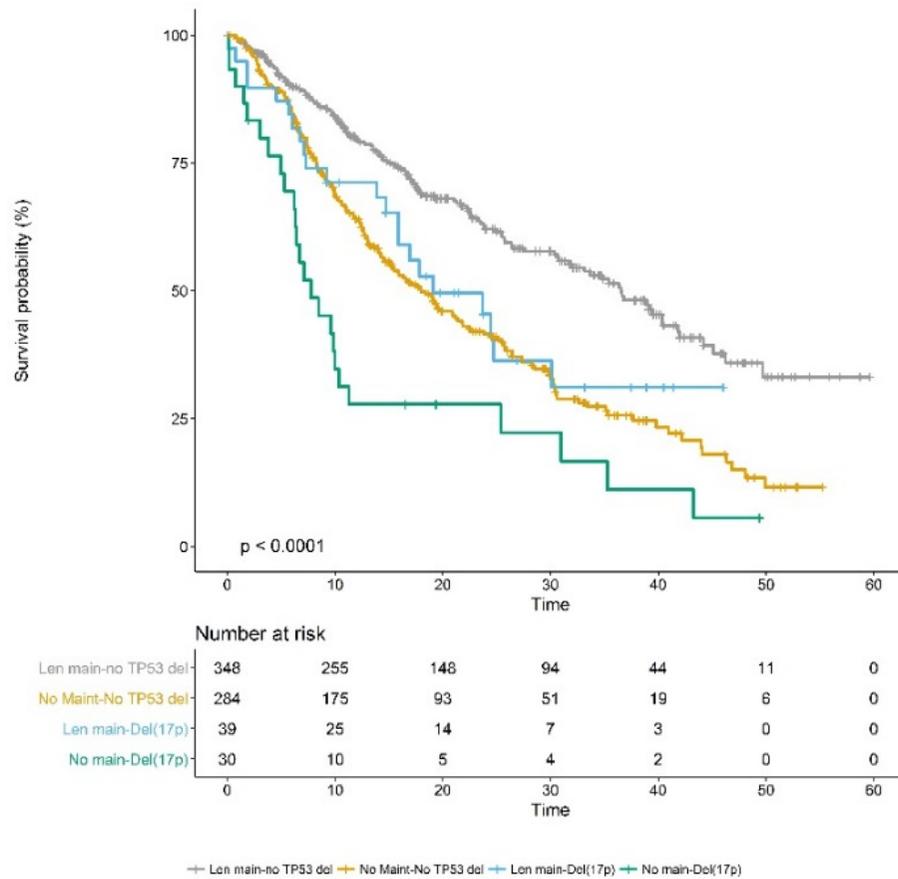


Figure 34: Kaplan-Meier survival curves showing PFS and OS for treatment with lenalidomide vs. no maintenance treatment in the context of TP53 deletion

4.3.5 Definition and validation of sub-clonal vs. clonal del(17p) by MLPA

In order to study the effect of tumour heterogeneity in the context of *TP53* and to identify potential correlation between the clonal fraction of *TP53* deletion and clinical parameters, the group that contained *TP53* deletions was divided into three sub-groups that were defined by increasing cut-off values by MLPA of approximately equal size: a low clonally-deleted fraction (MLPA cut-off ≥ 0.7 : <0.8) with mostly sub-clonal deletions; an intermediate clonally-deleted fraction (MLPA cut-off ≥ 0.55 : <0.7); and a high clonally-deleted fraction (MLPA cut-off <0.55).

As most studies to date on subclonal del(17p) have been based on iFISH data, we demonstrated the equivalence between MLPA-based cut-offs and percentage deletion by iFISH in a matched subset of the same patients for whom iFISH fixed material had been stored. In order to validate the three chosen MLPA-based cut-offs, we performed iFISH deletional percentage estimation on a subset of 64 patients who showed *TP53* deletions by MLPA and for whom iFISH fixed material was available (Figure 35). Individual per patient data points were plotted to demonstrate variability in percentage deletion that was detected despite the efforts of two operators and the counting of a larger than normal number of cells during the process. We showed that the cohort of patients with MLPA cut-off <0.55 were found by iFISH in all but one case to be $>60\%$ *TP53*-deleted. The cohort of patients with an MLPA cut-off of between 0.7 and 0.8 showed variation in *TP53* deletion of between 4% and 59%; only one case had deletion of $>50\%$. These findings confirmed that the upper and lower cut-offs that were used for the study of subclonal vs. near-clonal del(17p) by MLPA were valid. Differences between these patients were then studied in terms of survival, clinical characteristics and genetic characterisation.

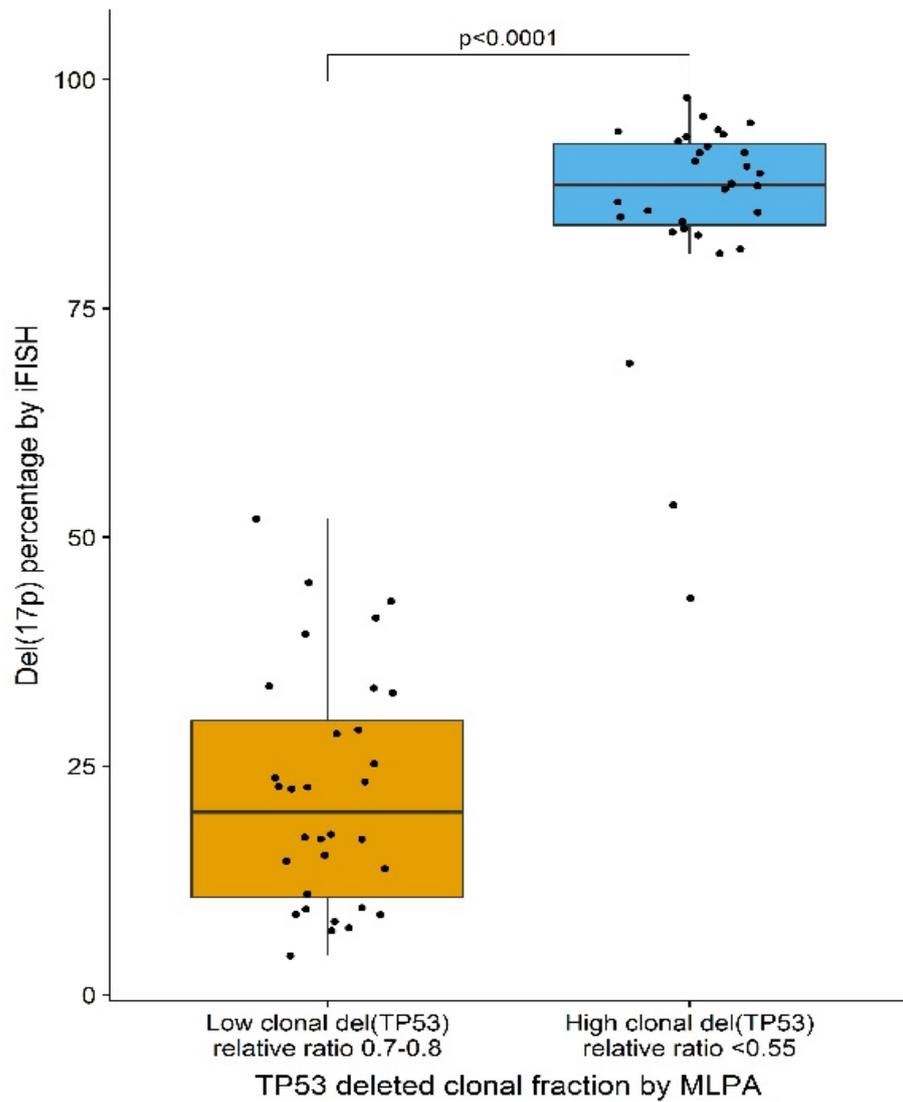


Figure 35: Box plot with individual data points compares low clonal deletion by MLPA with relative ratio between 0.7 -0.8 vs. relative ratio <0.55 with deleted percentage by iFISH in a subset of 64 patients with available material. Percentage deletion calculated as mean percentage deletion from two operators who counted between 200 and 400 cells, depending on the availability of material. *10% is the limit of del(17p) detection by iFISH at our diagnostic laboratory

4.3.5.1 *TP53* deletion clonal fraction and survival

When survival rates were compared among the three del(17p) cut-offs, all three groups were independently associated with sub-clonally deleted *TP53* (HR of 1.8 (95% CI: 1.2-2.8; $P=0.01$)) for OS, intermediate-deleted *TP53* (HR 2.9 (95% CI: 1.9-4.4; $P=5.6 \times 10^{-7}$)) and clonally deleted *TP53* (HR 2.2 (CI: 1.4-3.2; $P=0.0002$)) (Figure 36, Table 42). Additionally, landmarked analyses from autologous stem cell transplant and lenalidomide maintenance randomisation showed consistent results for all three groups (Table 42; Figure 37). We also demonstrate the independence of three del(17p) cut-offs by multivariable analysis in Table 43.

Some studies suggest that bi-allelic deletion rather than mono-allelic alteration drives prognosis in myeloma (100). In order to investigate whether the findings of this study with regard to a similarly poor prognosis in both sub-clonally and clonally deleted *TP53* might have been due to bi-allelic alterations within this group, we investigated the incidence of bi-allelic deletion and mutation in these groups. WES data were used that we had from a subset of 455 patients who were investigated in the above study. We found that mutations of *TP53* were present in 1/21 (4.7%) in the sub-clonally deleted *TP53* group by MLPA vs. 3/18 (16.7%) of the clonally deleted *TP53*. Similarly, bi-allelic deletion of *TP53* was present in 0/47 of sub-clonally *TP53*-deleted cases vs. 5/47 (10.6%) of clonally deleted cases in patients for whom we had available MLPA XO73 assays ($n=1307$). These assays employ probes on all exons of *TP53* (exons 1-11). These results suggest that bi-allelic *TP53* loss in the sub-clonal deleted group does not explain the poor prognosis that is associated with this group. In fact, bi-allelic inactivation was more common in the clonally deleted *TP53* group.

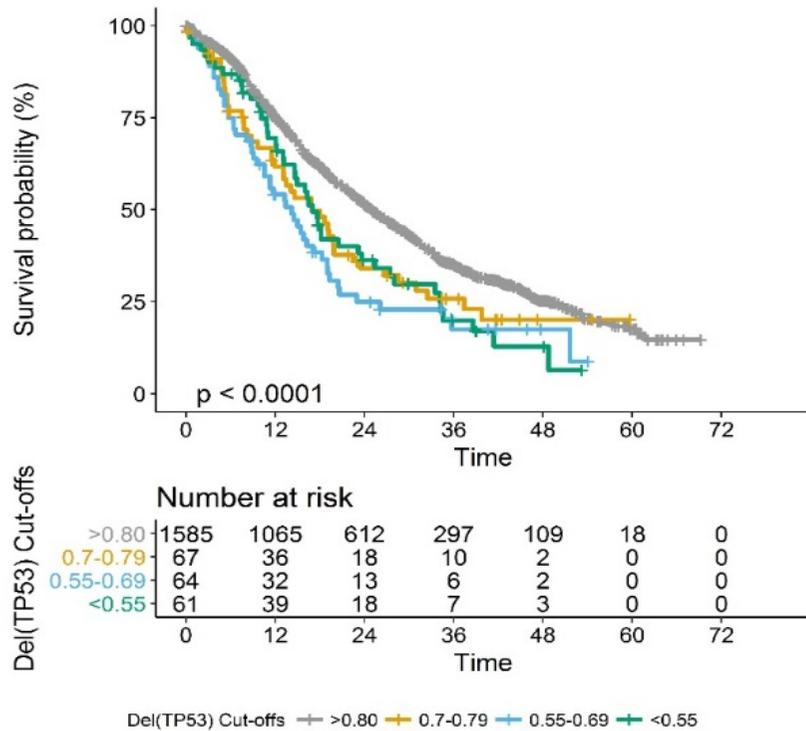
Table 42: Survival statistics for deleted *TP53* grouped into approximate thirds by clone size: Overall survival: univariate median survival and Cox model hazard ratios of different del(*TP53*) cut-offs after induction randomisation/after ASCT and after maintenance randomisation to lenalidomide; b) Cox model-based multivariable analysis of PFS and OS including three different clonal cut-offs of *TP53* deletion and known adverse prognostic markers of myeloma in 1239 patients with complete data in the Myeloma XI trial from time of induction randomisation

From induction randomisation: all patients	Total	N	Median OS (months)		P	OS: Cox-based regression	
			Del(<i>TP53</i>)	No Del(<i>TP53</i>)		HR	Wald P
Del(<i>TP53</i>) cut off 0.8	1777	192	31.2 (25.6-42.3)	60.4 (56.2-64.3)	6.7x10 ⁻¹⁵	2.4 (1.9-3.0)	4.12x10 ⁻¹⁴
Del(<i>TP53</i>) ≥0.7 < 0.80	1652	67	43.5 (26.3-NR)	60.4 (56.2-64.3)	0.0012	1.9 (1.3-2.7)	0.0014
Del(<i>TP53</i>) ≥0.55 < 0.70	1649	64	26.5 (19.7-NR)	60.4 (56.2-64.3)	1.14x10 ⁻⁹	2.8 (2.0-4.0)	5.5x10 ⁻⁹
Del(<i>TP53</i>) < 0.55	1646	61	31.2 (24.2-NR)	60.4 (56.2-64.3)	1.26x10 ⁻⁷	2.6 (1.8-3.7)	3.47x10 ⁻⁷
Landmarked post HDM +ASCT							
Del(<i>TP53</i>) cut off 0.8	600	64	20.4 (14-32.6)	39 (33.3-44.5)	1.45x10 ⁻⁵	2.1 (1.5-3.0)	2.23x10 ⁻⁵
Del(<i>TP53</i>) ≥0.7 < 0.80	556	20	14.0 (11.8-NR)	39 (33.3-44.5)	0.0034	2.3 (1.3-4.2)	0.0045
Del(<i>TP53</i>) ≥0.55 < 0.70	553	17	15.4 (7.1-NR)	39 (33.3-44.5)	0.019	2.1 (1.1-4.0)	0.021
Del(<i>TP53</i>) < 0.55	563	27	26.7 (10.1-NR)	39 (33.3-44.5)	0.0061	2.0 (1.2-3.2)	0.0072
Landmarked post lenalidomide maintenance randomisation							
Del(<i>TP53</i>) cut off 0.8	379	39	42.2 (25.9-NR)	NA (50.9-NR)	0.00011	3.1 (1.7-5.6)	0.00024
Del(<i>TP53</i>) ≥0.7 < 0.80	355	15	32.8 (16.2-NR)	NA (50.9-NR)	0.013	2.8 (1.2-6.5)	0.017
Del(<i>TP53</i>) ≥0.55 < 0.70	349	9	NA (20.8-NR)	NA (50.9-NR)	0.24	2.3 (0.6-9.5)	0.25
Del(<i>TP53</i>) < 0.55	355	15	42.2 (16.9-NR)	NA (50.9-NR)	0.00072	4.0 (1.7-9.3)	0.0017

Table 43: Survival statistics for deleted *TP53* grouped into approximate thirds by clone size: Cox model-based multivariable analysis of PFS and OS including three different clonal cut-offs of *TP53* deletion and known adverse prognostic markers of myeloma in 1239 patients with complete data in the Myeloma XI trial from time of induction randomisation

	OS		PFS	
	HR(95% CI)	Wald <i>P</i>	HR(95% CI)	Wald <i>P</i>
Del(<i>TP53</i>) ≥ 0.7 < 0.80	1.8(1.2-2.8)	0.010	1.5(1.04-2.02)	0.028
Del(<i>TP53</i>) ≥ 0.55 < 0.70	2.9(1.9-4.4)	5.64x10 ⁻⁷	1.9(1.3-2.7)	0.00026
Del(<i>TP53</i>) < 0.55	2.2(1.4-3.2)	0.00025	1.3(0.9-1.8)	0.12
Gain(1q21)	1.4(1.2-1.8)	0.0009	1.3(1.1-1.4)	0.12
Adverse translocation	1.5(1.2-1.9)	0.0015	1.4(1.2-1.7)	0.00032
LDH	1.0003(1.00-1.001)	1x10 ⁻⁵	1.0004(1.000-1.0001)	0.007
ISS	1.8(1.6-2.2)	2.4x10 ⁻¹⁵	1.5(1.4-1.7)	1.03x10 ⁻¹⁴

a) PFS: All patients from time of induction randomisation



b) OS: All patients from time of induction randomisation

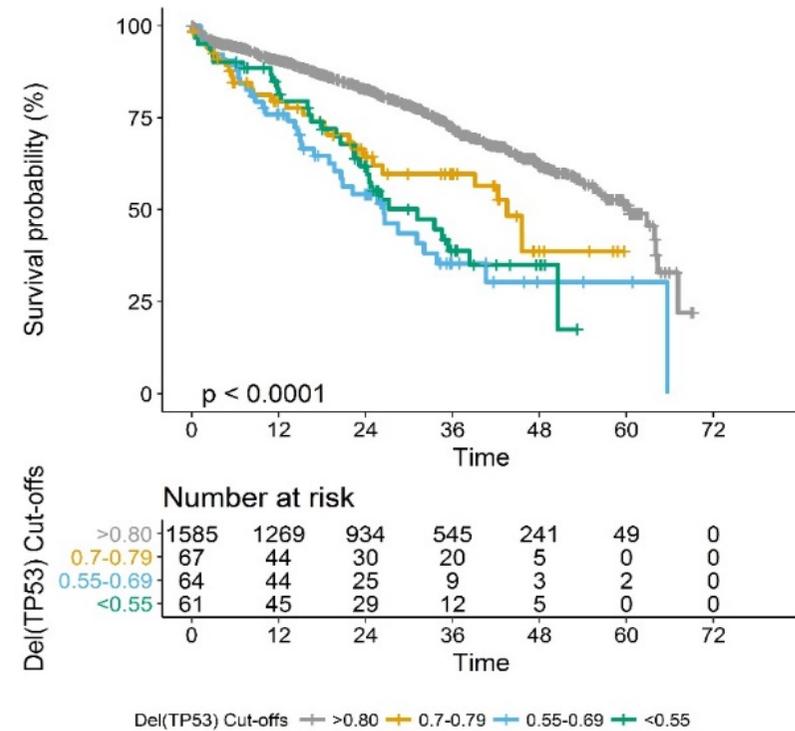
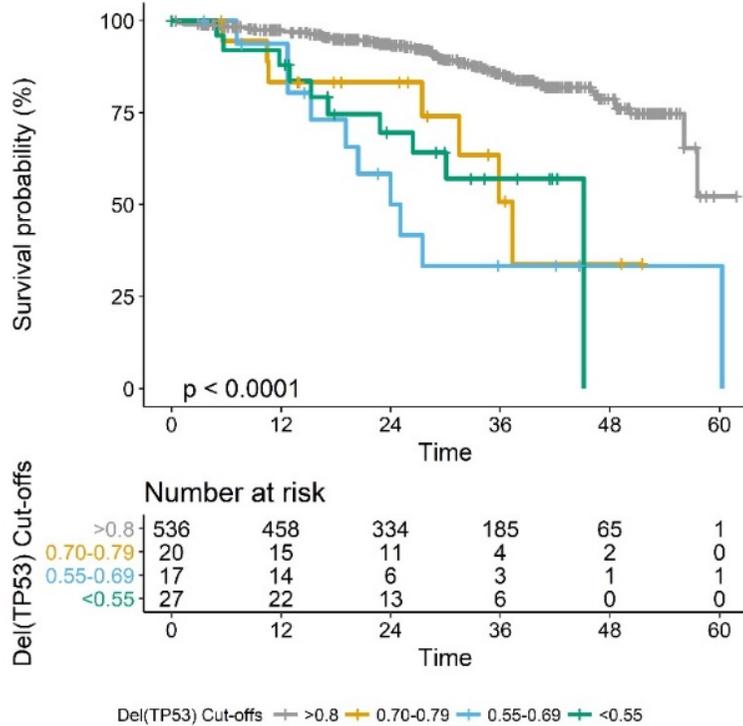


Figure 36: Association between sub-clonal and clonal *TP53* deletion and survival in newly diagnosed myeloma. Kaplan-Meier survival curves showing (a) PFS and (b) OS of three approximately equal sized *TP53*-deleted clonal subgroups versus no *TP53* deletion in 1777 patients in the Myeloma XI trial from the time of induction randomisation

a) OS: Landmarked from time of autologous transplantation



b) OS: Landmarked from time of maintenance randomisation

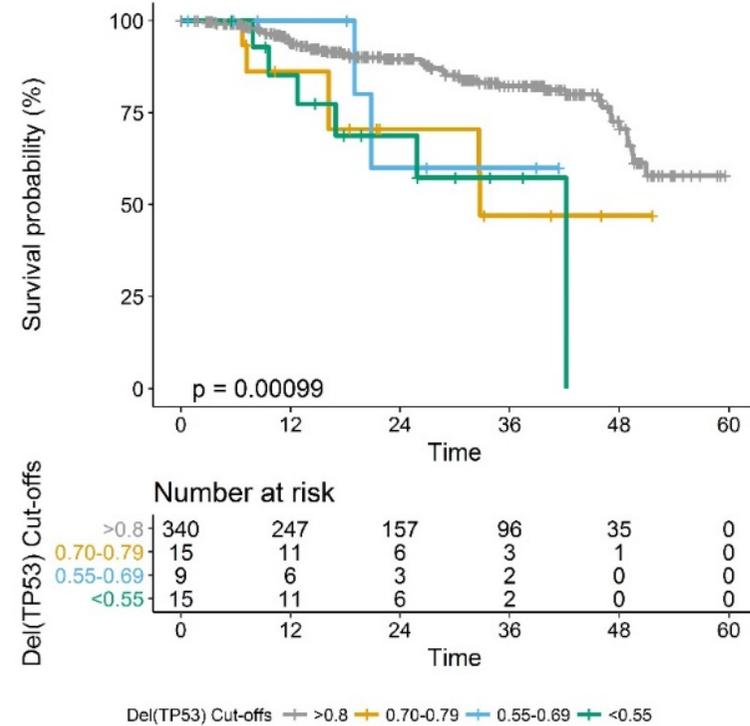


Figure 37: Association between sub-clonal and clonal *TP53* deletion and survival in newly diagnosed myeloma. Kaplan-Meier survival curves showing (a) OS evaluation of above subgroups in landmarked analysis from time of HDM-ASCT; and (b) OS evaluation of above subgroups in landmarked analysis from time of maintenance randomisation

4.3.5.2 *TP53* deletion clonal fraction and its association with other genetic lesions in myeloma

The frequency of occurrence of tumours with no del(17p), low clonal, intermediate clonal and high clonal burden of *TP53* deletions was compared as defined above. Tumours with mostly clonal *TP53* deletions had a significantly higher rate of concurrent del(13p) (68% vs. 40%; $P=0.002$) and a higher rate of del(1p) (4% vs. 21% respectively; $P=0.006$) compared with the group characterised by sub-clonal deletions (Figure 38). The incidence of hyperdiploidy fell significantly (48% vs. 33%) in the highest clonal fraction of *TP53* deletion. IgH translocations and gain(1q) were not statistically significantly different in frequency across the clonal *TP53* deleted fractions (Figure 38). Homozygous deletion at 1p32 at the *CDKN2C* locus was only detected in the high clonally deleted *TP53* group (7% vs. 0%, $P=0.04$).

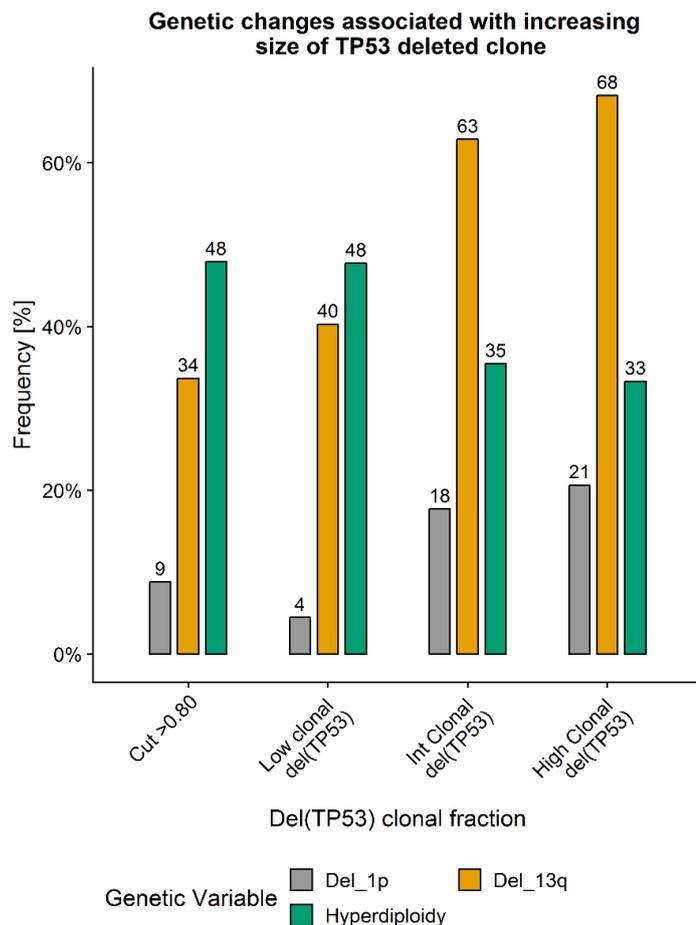


Figure 38: Bar plot of frequency of genetic copy number abnormalities across different del(17p) clonal sizes

As shown above, the strongest association with increasing clonality of *TP53* was observed with a rising frequency of concurrent deletion of 13q. Therefore, the two lesions were analysed by iFISH and by MLPA to investigate this relationship. Figure 39 demonstrates the deletional percentage that was shown by iFISH of individual patients with both deletion 13q and deletion 17p. It shows that del(13q) is a near-clonal lesion with deletion in 80-90% of tumour cells. This is consistent with its early evolution in precursor states of MM (e.g. MGUS). Del(17p), on the other hand, has a wider spread of deletional percentage, consistent with it being a later secondary lesion. We show that 22/24 (91.7%) of patients with del(13q) patients had a higher percentage of del(13q) than del(17p) when individual paired data are compared, as shown in Figure 39 which suggests that the dominant lesion is del(13q) in the majority of tumours.

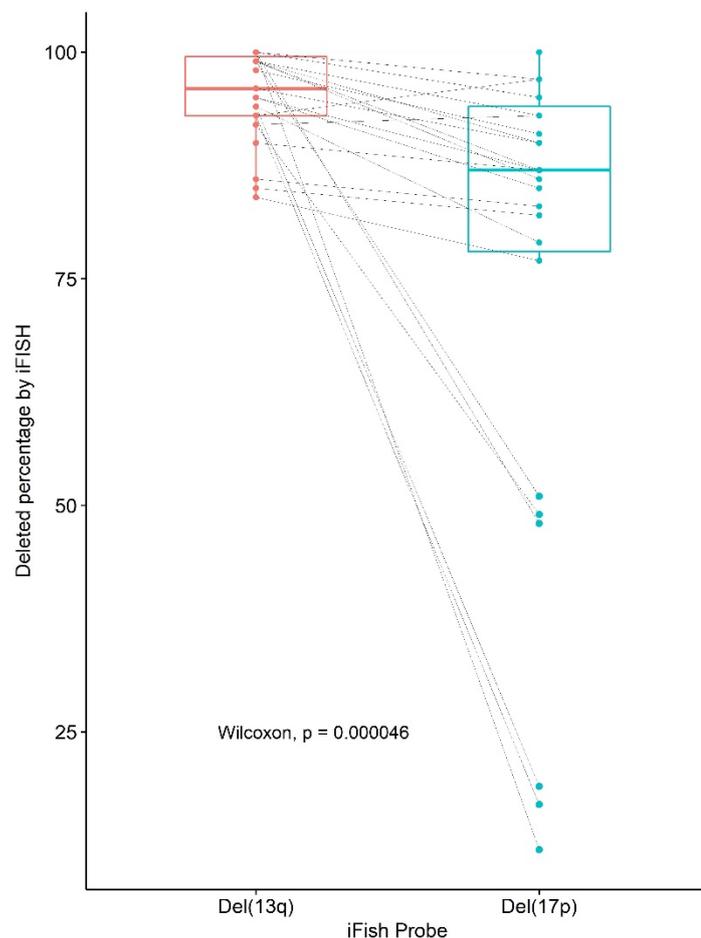


Figure 39: Box plot of iFISH probe percentage deletion in a subset of patients' tumours with simultaneous del(13q) and del(17p) with paired data points of individual patient tumour samples

MLPA data are shown in Figure 40 shows that, in tumours with both 13q and 17p deletion, overall del(13q) clone has a lower MLPA relative ratio. This finding suggests that it is the dominant clone as per the iFISH data in Figure 39. Tumours with 13q deletions are near clonal, with a median MLPA value of 0.53 in sub-clonally *TP53* deleted tumours, and fully clonal in the clonally *TP53*-deleted cases with a median MLPA value of 0.48. These findings also suggest that del(13q) is the dominant lesion. The iFISH and MLPA data that are presented here suggest that the presence of del(13q) may increase the ability of a subclone of del(*TP53*) to grow and that the almost clonal del(13q) also increases in its deletional percentage as the bi-deleted (with both del(13q) and del(*TP53*)) clone takes over.

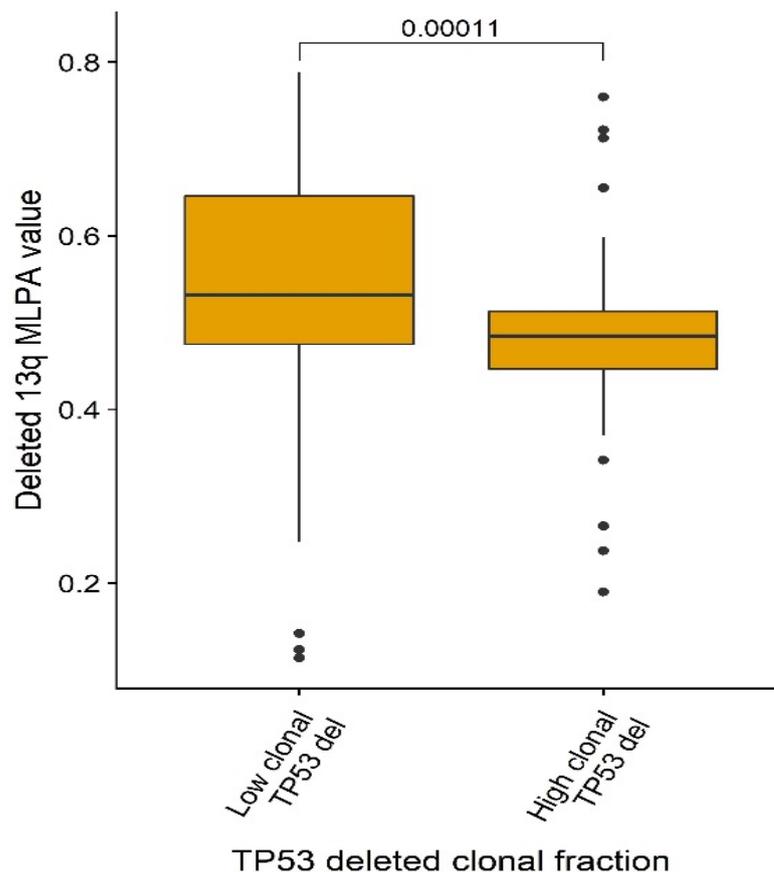


Figure 40: Relationship between sub-clonal and clonal *TP53* deletion with clinical and genetic characteristics of myeloma. Box plot of cumulative MLPA values of del(13q) clone in patients with both del(13q) and del(*TP53*) compared with del(13q) MLPA values of sub-clonally *TP53* deleted vs. clonally deleted tumours. MLPA values <0.5 indicate clonal 13q deletions

Co-evolution of the two lesions is further suggested by evaluation of MLPA-based clonality in Figure 41. Figure 41a demonstrates that the number of tumours with the probe at 13q MLPA values falls with increasingly clonal *TP53* deletion Figure 41b similarly shows that in tumours with *TP53* deletion, the *TP53* clone size falls with increasing clonal size of del(13q).

The co-increase of these two genetic copy number abnormalities in terms of clonal burden and incidence therefore suggests that del(13q) may synergise with del(17p) to provide tumour cells that have both lesions with a survival advantage over other clones.

However, despite the associations with other genetic abnormalities shown above, we show that *TP53* deletion retains its independent prognostic association as with known genetic markers associated with poor prognosis (Table 43).

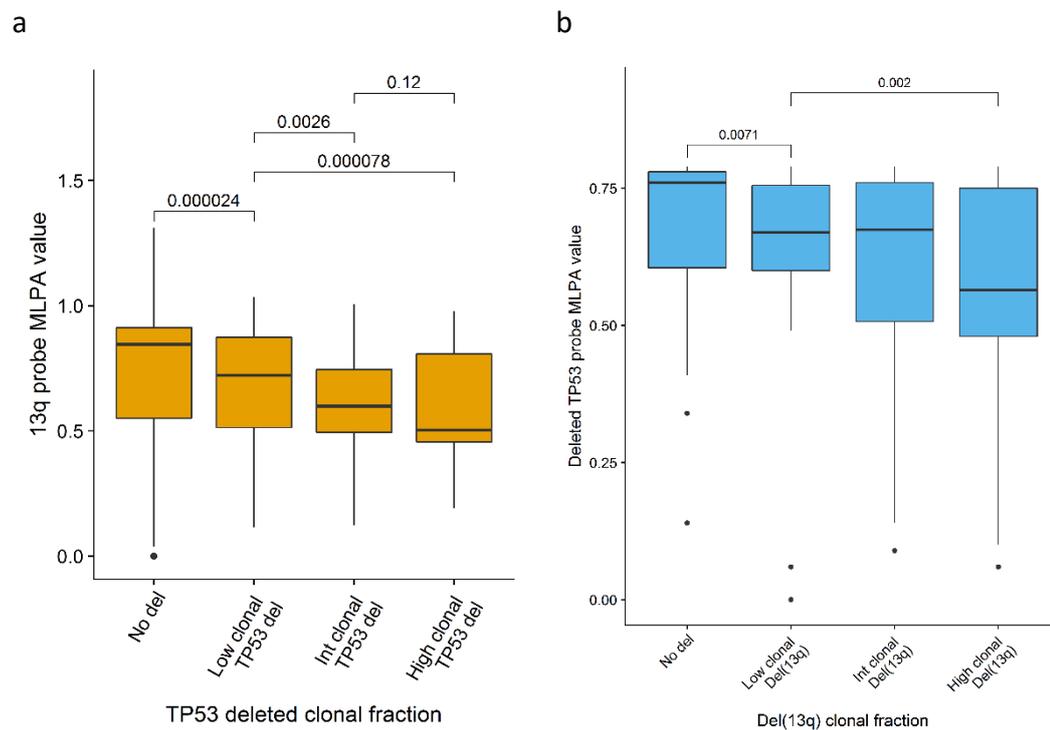


Figure 41: Relationship between sub-clonal and clonal *TP53* deletion with clinical and genetic characteristics of myeloma. (a) MLPA values normalised values for 13q probes in the same patients with low, intermediate and high deletion of *TP53* clone (b) MLPA values across subset of patients with del(*TP53*) in patients with increasing size of del(13q) clone. Lower MLPA values represent increasing size of deleted clone

It was also observed that as incidence of *CDKN2C* increased at the chromosome 1p locus, so did *TP53* clonal size. However, no difference was found in clonal size of the two lesions, nor was there any evidence of clonal dominance by iFISH or MLPA. Both *TP53* and *CDKN2C* showed a spread of clonal and subclonal deletion and there was no trend in clonal size. The association of these two lesions was weak compared with the association with del(13q) and the significance of this clonal lesion with increasing clonal size is uncertain.

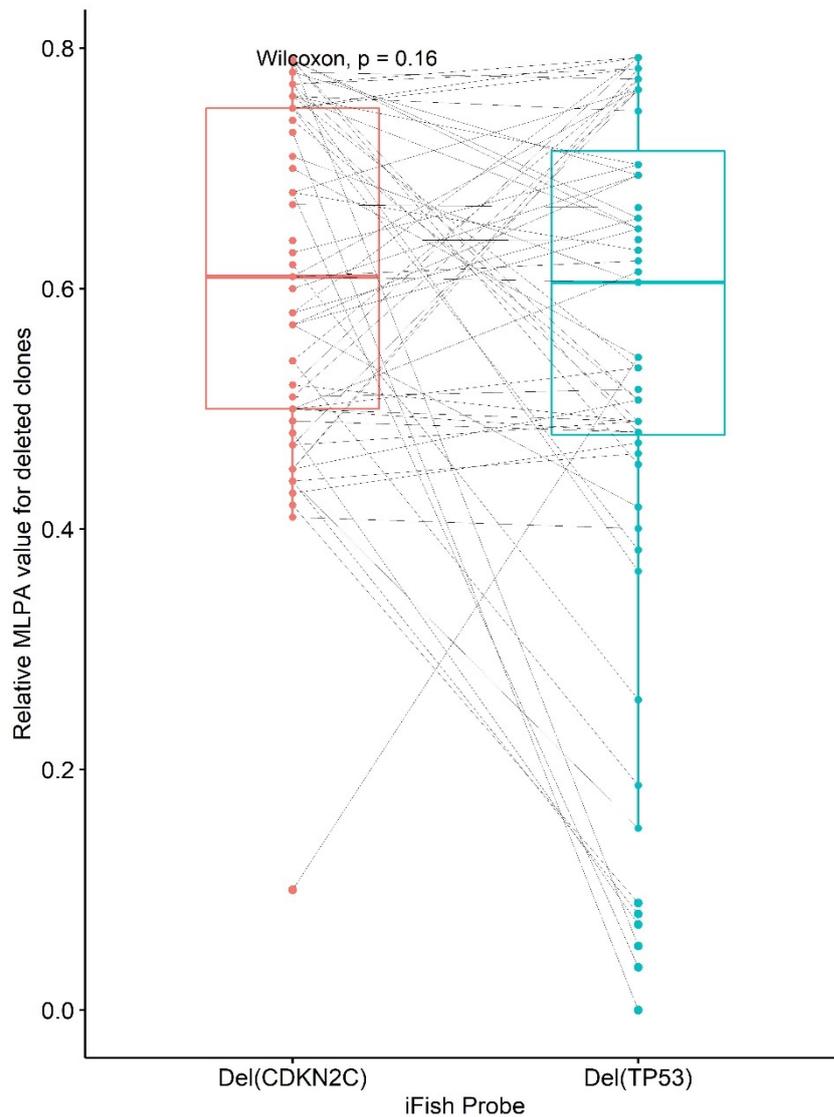


Figure 42: Box plot of relative normalised MLPA values of tumours with both deletion 1p (*CDKN2C*) and deletion 17p (*TP53*)

4.3.5.3 TP53 deletion clonal fraction and its association with clinical characteristics

4.3.5.3.1 Biochemical characteristics

Correlation of biochemical blood results showed that patients with clonal rather than sub-clonal *TP53* deletion were associated with elevated LDH level >300U/l (57% vs. 32%; $P=0.012$) (Figure 43). No statistically significant association was found with albumin <35g/l (44% vs. 48%, $P=0.72$). We observed a trend towards a higher level of β 2-microglobulin (β 2M) >5.5 in the clonal *TP53*-deleted subset but this was not statistically significant (38% vs. 27%, $P=0.2$). There was also no association with a high ISS score, which is traditionally believed to be associated with disease burden. LDH is also known to be associated with a high proliferation rate, so it may be that the association that was observed is due to an increased proliferation rate rather than, or in addition to, a high disease burden, especially as *TP53* is known to play a key role in cell cycle regulation.

4.3.5.3.2 Bone-marrow-related characteristics

Additionally, we observed that high clonal *TP53* deletion showed a higher frequency of patients with thrombocytopenia 35% vs. 9% with subclonal *TP53* deletion ($P=0.00047$) (Figure 43). Previously in this chapter we have shown a strong association between thrombocytopenia and del(*TP53*). A fall in platelet count is frequently observed later in disease progression, in the relapsed/refractory phase of MM (329). The association that was observed with thrombocytopenia was not as significant in terms of haemoglobin; a higher proportion of patients with a haemoglobin concentration of <80g/l was observed in high clonally deleted *TP53* patients, but this was not statistically significant (8% vs. 2%, $P=0.1$). While anaemia is an early marker of MM at diagnosis, thrombocytopenia is relatively uncommon at diagnosis. The observation that deletion *TP53* is associated with thrombocytopenia more than with anaemia suggests a potential interaction with megakaryocytes and MM cells, perhaps outside of plasma cell infiltration alone. This has also been suggested in in-vitro and in-vivo studies (330, 331).

4.3.5.3.3 Response to induction treatment

As with the occurrence or non-occurrence of deletion of *TP53*, we also found no association between induction response and clonal vs. sub-clonal *TP53* deletion. This suggests that, although there is an initial disease response to treatment, this is not long lasting, as evidenced by the poor overall survival rate that is associated with this lesion.

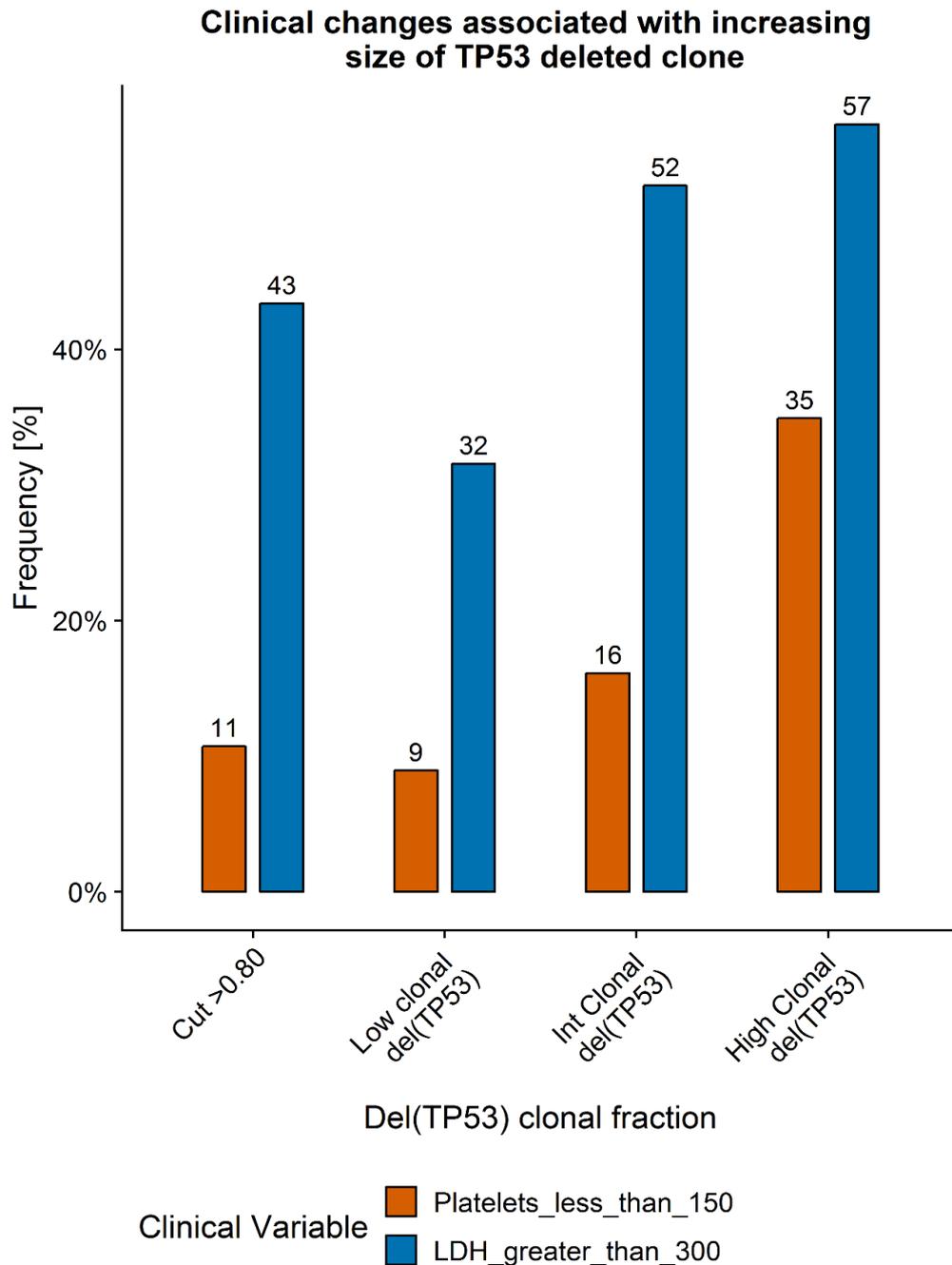


Figure 43: Relationship between sub-clonal and clonal *TP53* deletion and clinical characteristics. Percentage frequency of clinical changes associated with low, intermediate and high deletion of *TP53* clone

4.3.6 Homozygous and heterozygous deletions of *TP53* and their association with survival

As mentioned, studies have suggested that bi-allelic inactivation of *TP53* but not mono-allelic inactivation is important for prognostication in MM. Therefore, for this study, homozygous deletions of *TP53* were investigated in terms of their association with survival. It had already been observed that bi-allelic *TP53* deletions could be focal in nature. Therefore, an alternative assay was used for this study after quality checks that are described below.

Using the manufacturer's recommendations, we considered samples with a normalised MLPA copy number value of <0.25 to be homozygously deleted for *TP53*. Within the 1777 patient samples that had been assayed for *TP53* exons 4, 7 and 10 through use of the P425 MLPA probemix, nine patients (0.50%) had a homozygous deletion of *TP53*. It was found that 8/9 homozygous deletions of *TP53* were focal and involved only one of three probes for *TP53*, while the other exons showed deletion levels that were consistent with clonal heterozygous deletions elsewhere within the gene. Among the eight patients with focal bi-allelic deletions, the remaining probes all showed a cut-off value that was indicative of a heterozygous deletion with cut-off values of <0.55 in six of the nine patients and <0.6 in 2/9 patients. As discussed in the previous section, almost all bi-allelic deletions were found in the context of a mono-allelic deletion within the other probes of *TP53*. The mono-allelic deletion was near clonal (MLPA <0.55) in the majority of cases; only 1/9 cases had a subclonal deletion.

The P425 MLPA probeset has been used by several laboratories including our own with previously published results. To further investigate this focal pattern of homozygous deletion, all 11 *TP53* exons (detailed in Figure 25) were profiled using a specifically designed MLPA probemix (X073) in a sub-group of 1,357 patients. As this was a new probeset design by the manufacturer that had not been validated in the past, we performed quality checks on the probeset in the first instance against the *TP53* probes on the P425 probeset. These quality checks revealed that a probe at exon 4b of the X073 probemix was faulty; it showed discrepant results to all other probes for calling deletion (Figure 44). We therefore used the probe on P425 for exon-4 deletion calling while the X073 probeset was used for all other probes for the investigation of focal bi-allelic deletions of *TP53*. Quality checks were performed against P425 probes.

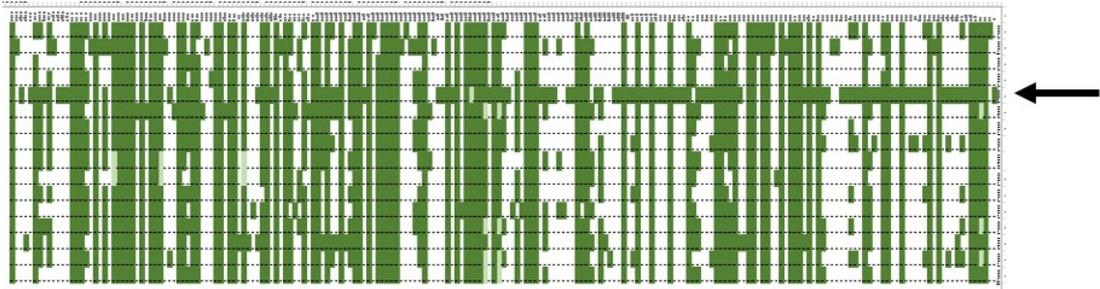


Figure 44: Position of *TP53* deletion across different XO73 and P425 probes. Green coloured boxes denote deletion detected by probes running horizontally. Black arrow points towards a faulty probe in the XO73 probeset with an increased number of deletions called. This probe was subsequently removed from all future analyses

As demonstrated in Figure 45, eight of these patients (0.6%) carried homozygous deletions of at least one exon of *TP53*. Again, homozygous deletions were focal and did not appear to be restricted to exons 4-8, the DNA binding domain, as is the case in most *TP53* mutations. Homozygous deletions involved exons 10 and 11 only in three patients, exon 7 only in three patients and exon 1 only in one patient. One non-focal homozygous deletion spanned between exons 3 and 10. This finding demonstrates that the reason why iFISH does not frequently pick up bi-allelic *TP53* deletions easily is due to their focal nature. It also emphasises that any assay that is used for the diagnosis of *TP53* deletions should include probes across all exons of this gene.

	Exon Probes											
	1	2	3	4	5	6	7	8	9	10	11	
Patient 1												
Patient 2												
Patient 3												
Patient 4												
Patient 5												
Patient 6												
Patient 7												
Patient 8												

Figure 45: Position of focal deletion at *TP53* according to probes positioned at the above exons. Red boxes denote bi-allelic deletion detected by MLPA probes across the 11 *TP53* exons

We investigated the association of homozygous *TP53* deletion with other genetic abnormalities in myeloma and found that bi-allelic *TP53* deletions were strongly associated with del(13q) in 88% of cases and negatively associated with hyperdiploidy, which was present in only 11% of cases. In terms of clinical characteristics, homozygous *TP53* deletions were associated with a particularly high LDH of >300U/l in 67% and platelet count <150x10⁹/l in 67% of bi-allelically deleted tumours. The clinical results suggest the development of aggressive tumour characteristics with the acquisition of a second *TP53* deletion.

We also investigated the association of homozygous *TP53* deletion with survival in the 1,307 patients from the above subset for whom survival data were available. Univariate survival analysis demonstrated that homozygous *TP53* deletion was associated with a very short median OS of 22.4 months and a HR for OS of 3.7 (95% CI: 1.5-8.9; $P=0.004$). Heterozygous deletions were detected in 10.4% of 1,307 patient tumours with extended profiling and were associated with shorter median OS of 33.5 months and a hazard ratio of 2.2 (95% CI: 1.7-2.8; $P=3.5 \times 10^{-10}$) (Figure 46, Table 44). Both homozygous and heterozygous deletions retained their significance in terms of survival in multivariate testing for both OS and PFS in a model that included other established molecular and clinical risk factors (Table 45 **Table 45**). It must be acknowledged however that survival data was only available for 7 patients within the homozygous deletion group so our data should be further validated in future studies.

Table 44: Univariate survival and Cox proportional hazard ratios for heterozygous *TP53* deletion and homozygous *TP53* deletion in 1307 patients in the Myeloma XI trial

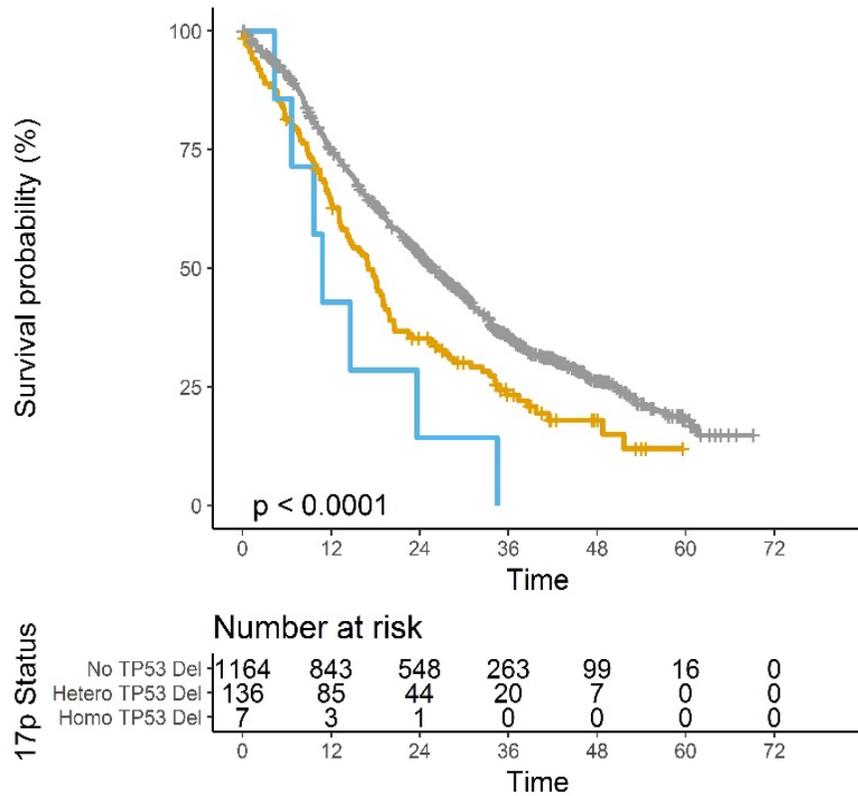
	PFS			
	Median months (95% CI)	<i>P</i>	HR(95% CI)	<i>P</i>
No <i>TP53</i> deletion	25.8 (24.0-27.9)	n/a	1.0	n/a
Heterozygous <i>TP53</i> deletion	17.1 (14.1-19.7)	0.00014	1.5 (1.2-1.8)	0.00015
Homozygous <i>TP53</i> deletion	10.8 (6.7-NR)	0.011	2.6 (1.2-5.4)	0.014

	OS			
	Median months (95% CI)	<i>P</i>	HR(95% CI)	<i>P</i>
No <i>TP53</i> deletion	60.3 (56-64.3)	n/a	1.0	n/a
Heterozygous <i>TP53</i> deletion	33.5 (25.6-45.5)	1.2x10 ⁻¹⁰	2.2 (1.7-2.8)	3.5x10 ⁻¹⁰
Homozygous <i>TP53</i> deletion	22.4 (10.1-NR)	0.0020	3.7 (1.5-8.9)	0.0040

Table 45: Multivariate analysis of overall survival of homozygous and heterozygous deletion of *TP53* vs. no deletion with known clinical and genetic risk features in 960 patients who were involved in the Myeloma XI trial

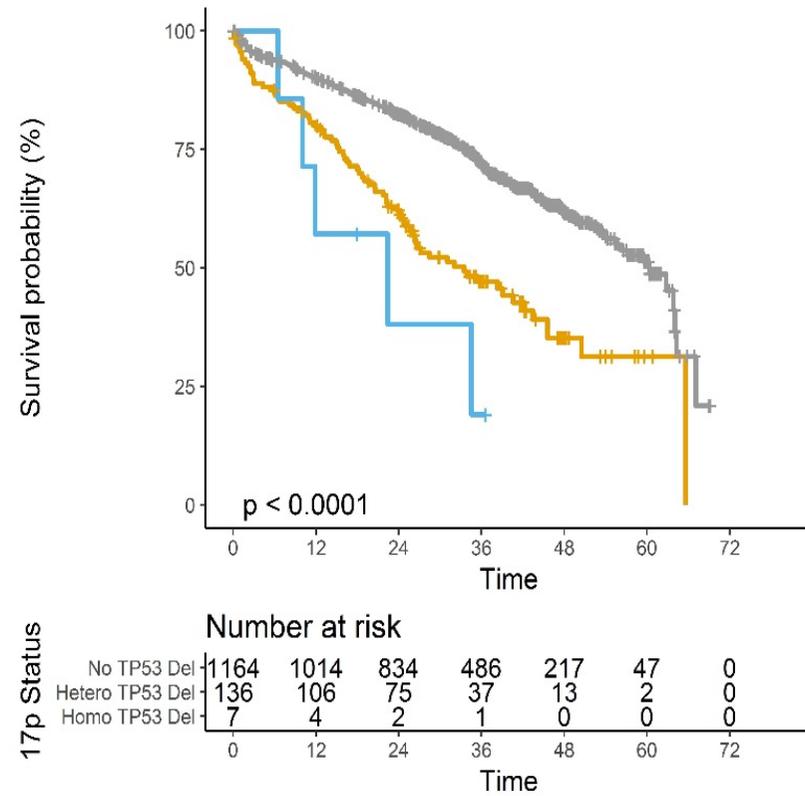
	OS	
	HR (95% CI)	<i>P</i>
Heterozygous <i>TP53</i> deletion	2.13 (1.58-2.86)	5.14x10 ⁻⁰⁷
Homozygous <i>TP53</i> deletion	2.98 (1.22-7.26)	0.017
Gain(1q21)	1.37 (1.09-1.73)	0.0061
Adverse Translocations	1.47 (1.12-1.92)	0.0048
ISS	1.8 (1.53-2.13)	2.5x10 ⁻¹²
LDH	1.001 (1.00-1.001)	0.0013

a) PFS



17p Status — No TP53 Del — Hetero TP53 Del — Homo TP53 Del

b) OS



17p Status — No TP53 Del — Hetero TP53 Del — Homo TP53 Del

Figure 46:Kaplan-Meier survival curves a) PFS and b) OS of patients who showed homozygous *TP53* deletion, heterozygous *TP53* deletion and no deletion among 1307 patients in the Myeloma XI trial

4.3.7 *TP53* mutations and deletions combined

As stated earlier, mutations in *TP53* have been reported in a smaller subset of NDMM patients compared with deletions. These mutations are reported to affect between 3% and 5% of patients (37, 53, 125, 126, 304). The majority of *TP53* mutations that occur in MM are missense mutations. Around 10% of *TP53* mutations are nonsense mutations that result in truncation of the p53 protein. The presence of *TP53* mutations is associated with poor prognosis in MM (41). The frequency of *TP53* mutations increases with disease progression, as is the case for deletions; this suggests that the gene is an important driver of MM progression (114, 131, 132, 304). Bi-allelic inactivation that is caused as a result of combinations of deletions and/or mutations is thought to be an important driver of poor prognosis. We therefore used the overlapping datasets of copy number and whole exome sequencing from this trial to investigate this further.

As previously reported (53), 14/463 (3.0%) of patients who underwent WES had a detectable *TP53* mutation (5). All mutations were located within the DNA binding region of *TP53* between exons 4 and 8. Two tumours carried two mutations each within the *TP53* gene. One of these patients had a variant allele fraction of 93% for one mutation and 38% for the other mutation, which suggested that the second mutation followed the first as a sub-clonal event. This patient also had a heterozygous *TP53* deletion that was shown by MLPA. The second patient had similar variant allele fractions in the two mutations of 44% and 52% and did not have a *TP53* deletion that was detectable by MLPA.

Of the 463 exome-sequenced tumours, 422 showed matched MLPA data with the XO73 and P425 probesets and were included in an integrated analysis of deletions and mutations together. Of these, three tumours had a homozygous deletion in at least one exon within *TP53*. Mutations and homozygous deletions were not detected in the same patients. Of the 12 patients with a mutation of *TP53*, six had a detectable deletion by MLPA and one patient had two concurrent mutations with no deletion, as mentioned above. The normalised MLPA copy number value for these deletions was between 0.46 and 0.76. In two of the six tumours without bi-allelic inactivation of *TP53*,

normalised MLPA copy number values approached the cut-off of 0.8, which suggested the presence of a sub-clonal *TP53* deletion.

Bi-allelic loss of *TP53* defined by heterozygous deletion and mutation or homozygous deletion was found in 2.4% (n=10) and mono-allelic loss in 11.1% (n=47) of tumours. We investigated the association with survival in these groups. Both bi- and mono-allelic *TP53* loss were associated with inferior survival rates, with median OS of 16.2 months (HR 5.54; CI: 2.79-11.03; $P=1.07 \times 10^{-6}$) for bi-allelic loss vs. 39.1 months for mono-allelic loss (2.06; CI: 1.37-3.1, $P=0.0005$). This compared with 60.4 months in the group with normal *TP53* status (Table 46a, Figure 47). Multivariable analysis confirmed the prognostic significance of both mono-allelic and bi-allelic loss of *TP53* (Table 46b Table 46).

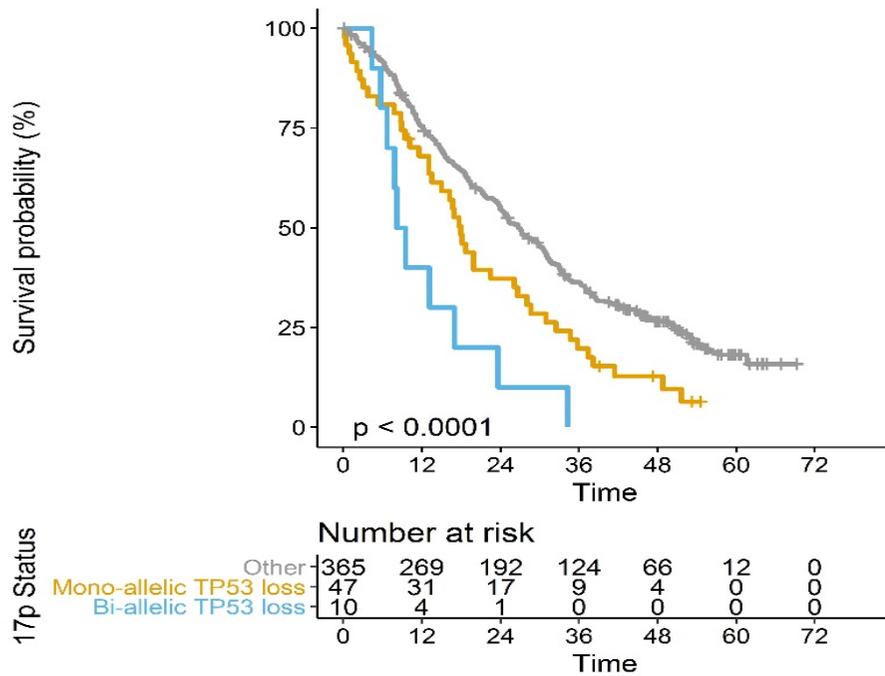
The observation that both mutations in *TP53* and bi-allelic deletions and mutations mostly occur in the context of heterozygous deletion at *TP53* provides a potential mechanism by which heterozygous deletions confer a poor prognosis over time, as further DNA damage occurs that causes bi-allelic aberrations. This is in line with the observation that bi-allelic *TP53* inactivation evolves in advanced tumours (109). Although studies with matched samples are limited, development of bi-allelic lesions from a mono-allelic lesion has been previously shown in myeloma (304).

Table 46: a) Univariate survival and Cox proportional hazard ratios for heterozygous *TP53* deletion, homozygous *TP53* deletion, *TP53* mutation, bi-allelic *TP53* alteration and mono-allelic *TP53* alteration in 422 patients. b) Multivariate analysis for overall survival of mono-allelic and bi-allelic *TP53* loss with known clinical and genetic risk features in 343 patients respectively on the Myeloma XI trial. Adverse translocations include t(4;14), t(14;16) and t(14;20)

a)	PFS				OS			
	Median months (95% CI)	<i>P</i>	HR(95% CI)	<i>P</i>	Median months (95% CI)	<i>P</i>	HR(95% CI)	<i>P</i>
No <i>TP53</i> mutation/deletion	26.7 (23.9-30.6)	n/a	1.0	n/a	60.4 (57.1-NR)	n/a	1.0	n/a
<i>TP53</i> mutation	13.3 (9.4-NR)	0.0024	2.4 (1.3-4.3)	0.0033	22.2 (16.8-NR)	0.00029	3.27 (1.7-6.4)	0.00062
Heterozygous <i>TP53</i> deletion	17.0 (13.0-26.6)	0.0017	1.7 (1.2-2.3)	0.0019	38.3 (19.7-NR)	8.8x10 ⁻⁵	2.2 (1.5-3.2)	0.00013
Homozygous <i>TP53</i> deletion	6.67 (4.3-NR)	0.018	3.6 (1.2-11.4)	0.027	10.1 (6.5-NR)	0.00056	5.9 (1.9-18.7)	0.0024
Bi-allelic <i>TP53</i> alteration	8.8 (6.7-NR)	0.00022	3.1 (1.7-5.9)	0.00047	16.3 (9.9-NR)	2.9x10 ⁻⁷	5.01 (2.5-9.9)	3.9x10 ⁻⁶
Mono-allelic <i>TP53</i> alteration	17.9 (13.5-26.6)	0.0051	1.6 (1.2-2.2)	0.0055	39.1 (23.8-NR)	0.0011	1.95 (1.3-2.9)	0.0013

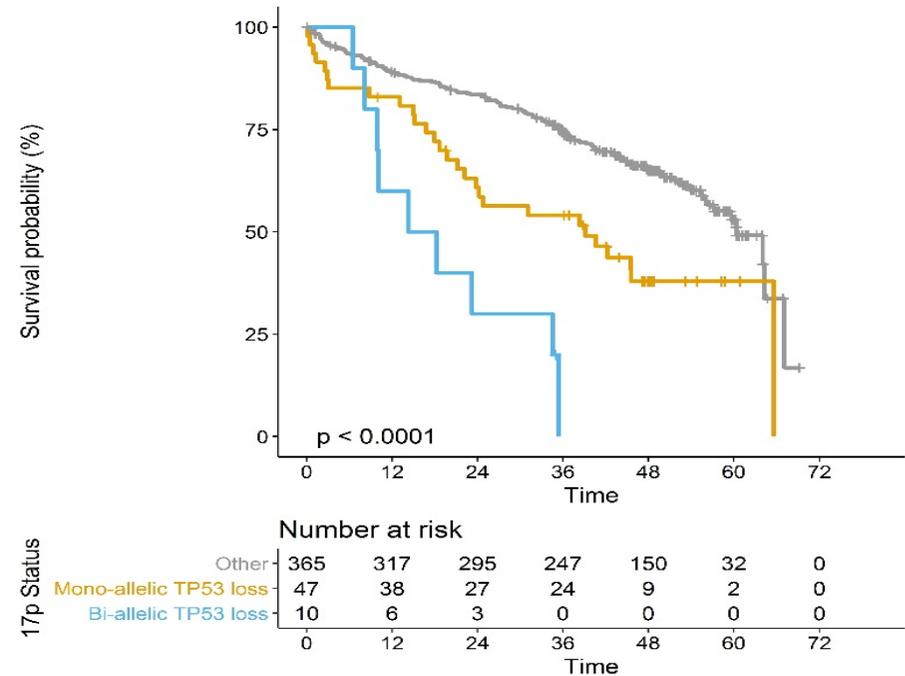
b)	OS	
	HR (95% CI)	<i>P</i>
Bi-allelic <i>TP53</i> loss	2.18 (1.35-3.52)	0.0014
Mono-allelic <i>TP53</i> loss	4.31 (2.03-9.18)	0.00015
Gain(1q21)	1.58 (1.12-2.23)	0.0098
Adverse Translocations	1.64 (1.08-2.49)	0.02
ISS	1.45 (1.15-1.84)	0.0019
LDH	1.001(1.0001-1.002)	0.029

a) PFS



17p Status — Other — Mono-allelic TP53 loss — Bi-allelic TP53 loss

b) OS



17p Status — Other — Mono-allelic TP53 loss — Bi-allelic TP53 loss

Figure 47: Kaplan-Meier survival curves: a) PFS and b) OS of bi-allelic and mono-allelic *TP53* loss in 422 patients

As *TP53* is associated with DNA repair and the total number of mutations in a tumour sample have been found to be associated with prognosis in MM (131), we went on to investigate whether the total number of mutations per tumour sample was affected by the patient's *TP53* status. Although the number of samples was relatively small (n=422), we found a statistically significant difference in patients with no *TP53* alteration vs. patients with bi-allelic alterations in line with p53's known role as a DNA repair protein, although this was lost when multiple testing was taken into account. We found no difference in the total number of mutations in patients with no *TP53* alteration vs. patients with a mono-allelic alteration in *TP53* (Figure 48).

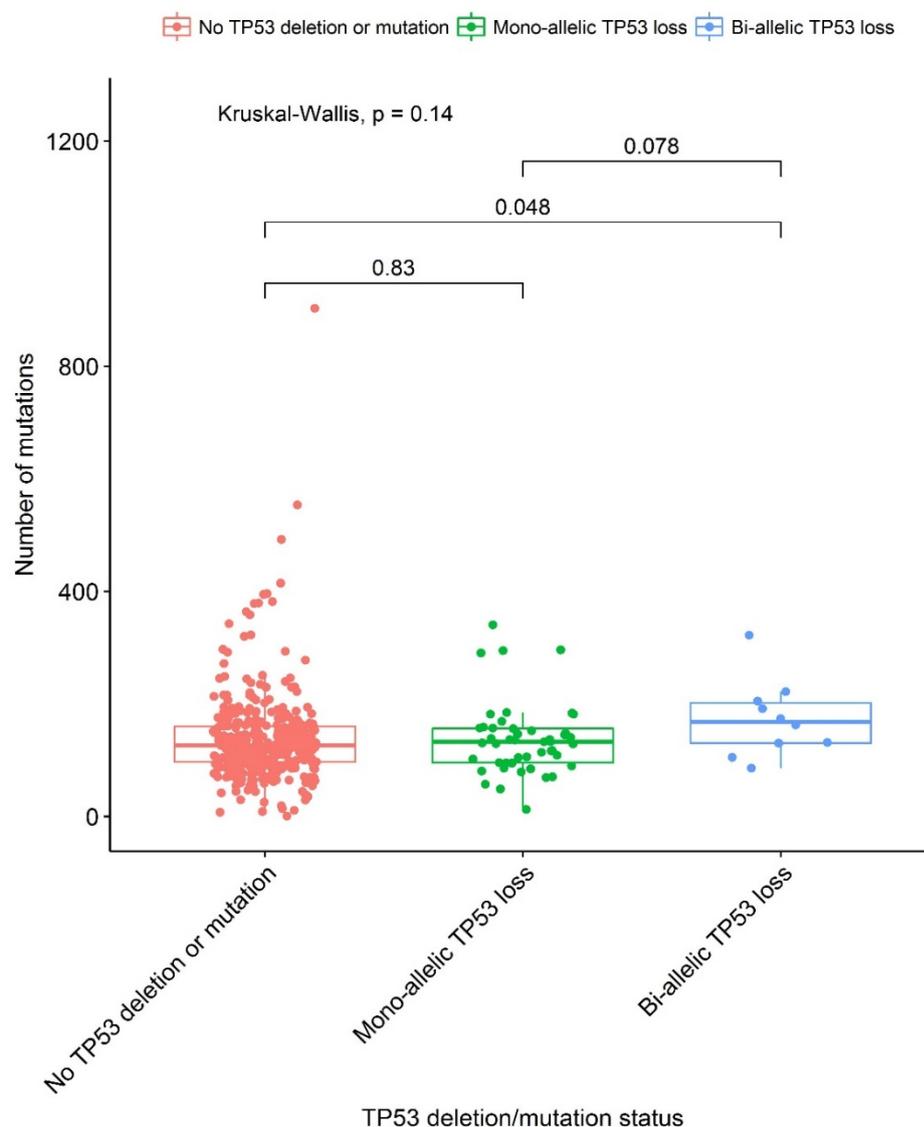


Figure 48: Box plot with individual data points showing total number of mutations in any gene per tumour sample within mono-allelic, bi-allelic and no *TP53* alterations that were found during whole exome sequencing

4.4 Discussion

To assess the prognostic importance of clonal and sub-clonal *TP53* CNAs, we profiled tumours of 1777 NDMM trial patients through use of MLPA. The UK NCRI Myeloma XI trial included multi-staged randomisation of therapy, which therefore enabled the study of this lesion in the context of novel therapies at different stages of MM therapy in a homogeneously treated group of patients. We identified sub-clonal deletions to be of independent prognostic relevance by applying several methods to confirm the cut-off that showed the most prognostic significance. Methods for evaluation of optimal cut-points including time-dependent area under the ROC curve analysis, minimal *P*-value survival analysis using Kaplan-Meier based log rank analysis, and Cox regression as well as C statistics in relation to overall survival, were applied. Additionally, subclonal *TP53* deletion was found to be associated with poor survival in both intensively and non-intensively treated patients. These findings are consistent with some studies in NDMM (136) and in other cancers such as chronic lymphocytic leukaemia (332, 333); yet they contradict the findings of other studies (62, 134, 316).

The advantages of this study over most studies that have been previously reported include the techniques that were used in this analysis. MLPA is a largely automated method with little inter-operator variability in results (320). Previous studies have shown that MLPA can be used to detect sub-clonal copy number deletions that are as low as 10-20% of tumour cells through use of serial dilution studies (320). Previously, reporting of copy number quantitation by iFISH has been shown to be potentially unreliable (318). A laboratory exercise was performed in which two cases of del(17p) were sent to 20 laboratories for testing of deletion percentages. A central laboratory had reported 38% del(17p). The results that were reported by the 20 test laboratories ranged from 17% to 80%. Similarly, a del(17p) case that was reported to show 92% deletion by a central laboratory was reported to show a range of 69-95% by testing laboratories. Additionally, three laboratories had false-positive results for del(17p) (318). The examples above demonstrate the poor reliability of quantitation in iFISH; in contrast, MLPA may partially overcome this.

Limitations of our findings include the fact that we did not have a validation cohort. However, there is currently no other sufficiently large, uniformly profiled, controlled clinical trial dataset for myeloma. Of our trial dataset of 1,777 patients, it was found that 3.7% of tumours had a sub-clonal *TP53* deletion. Based on the data presented here, a uniformly profiled validation trial dataset of approximately equivalent size that could provide informative results does not exist.

Another limitation was concerned with treatments that were offered within the trial. These were largely IMiD-based with limited exposure to proteasome inhibitors. As shown in this chapter, patients with *del(TP53)* who were exposed to a proteasome inhibitor had a statistically improved outcome compared with those who did not. However, within this study, randomisation of this cohort was only present in patients who achieved a PR or MR in terms of treatment response. The findings do suggest that studies that involve triplet regimens that contain a proteasome inhibitor may produce different results to ours. This has clinical relevance as triplet-based regimens are now offered as standard across the world for those who are fit enough to tolerate them.

Within the trial, we show that the prognostic impact of *TP53* deletion is not abrogated by IMiD-based treatment such as CRD or CTD, and there is no statistical difference between the two regimes in the context of this lesion. Similarly, transplantation did not abrogate the higher associated risk; however, as this arm of the trial was not randomised, we could not conclude whether application of HDM-ASCT treatment offered any benefit in this set of patients. Maintenance IMiD (lenalidomide monotherapy) similarly did not confer a survival advantage compared with no maintenance in the context of this lesion.

The only randomisation that appeared to be associated with improved survival for patients with a *TP53* deletion was with therapy that was based on proteasome inhibitors. However, this treatment was only delivered during consolidation to a subset of patients for a short amount of time in our trial. While it appeared to be associated with improved survival for those who were randomised to the consolidation arm, it did not abrogate the risk completely. In findings similar to these, patients who were treated with bortezomib at induction as well as in maintenance in the context of 17p

deletion in the HOVON/GMMG-HD4 study showed improved survival rates, but the treatment did not completely abrogate the association with poor survival during long term follow up of patients(89).

Despite strong evidence of the poor prognostic impact of *TP53* aberrations or del(17p), clinical trials do not report the effect of these mutations in isolation, since outcomes for patients with high-risk disease are grouped by most studies and del(17p) is combined with t(4;14), t(14;16) +/- gain(1q) within these analyses. This has led to difficulty in the assessment of the effect of this lesion in the context of novel therapy (147). As a result of this scarcity of evidence, adaptation of therapy for MM according to cytogenetics has not been widely brought into standard clinical practice (148, 149). While this practice may allow broader appeal for a drug and increased sales, it does not allow selection of drugs that are more likely to be beneficial to a patient. This study adds to evidence that is available regarding the delivery of novel therapies in a step-wise randomised fashion, which has enabled the evaluation of each therapy in the context of *TP53* alteration at different stages of treatment.

We explored clinical correlation that was associated with *TP53* deletion in this large cohort of patients. Overall, patients with *TP53* deletion had a poorer WHO performance status, suggesting an inter-relationship with genetic and clinical features that are normally thought of as patient-related rather than disease-related. Interestingly, however, no association was found between age and *TP53* deletion, which suggests that this is not a confounding factor. Patients on the intensive and non-intensive arms of the study were also balanced, which suggests that the WHO performance status did not appear to influence physician choice in terms of which arm of the trial was best for each patient. A poor performance status secondary to genetic lesions has potential impact on the ability to deliver intensive therapy. Our data therefore suggests that the treatment of high-risk patients who have this lesion earlier in the evolution of the disease may prevent the worsening of the performance score and therefore enable the delivery of more intensive or prolonged treatment. It is important to assess this hypothesis in trials of asymptomatic myeloma in the context of *TP53* deletion for this reason.

Comparing clinical characteristics, patients with clonal rather than sub-clonal *TP53* deletions were found to be associated with markers of high proliferation rate and/or high disease burden such as elevated LDH levels and reduced platelet counts. This association was more marked in patients with bi-allelic alterations of *TP53*. Given that LDH is part of the R-ISS, this suggests that clinical markers that are used in the prognostic score capture data that were not previously captured in terms of high-risk genetic lesions due to the lack of routine sequencing or evaluation of homozygous deletional status of *TP53* of tumours in MM.

Our results also demonstrate an association between shortened survival periods and both heterozygous and homozygous deletions of del(17p) in 1307 patients. We confirm the extremely poor prognosis that is associated with bi-allelic alterations in *TP53* but also confirm an independently poor prognosis that is associated with mono-allelic alterations using 422 tumours with copy number and WES data.

TP53 has not been routinely sequenced in most previous clinical trials. Data are therefore limited in terms of whether mono-allelic alterations have a prognostic impact. Recently, some groups have suggested that only bi-allelic alterations have a prognostic impact, but these studies involve limited follow up (100, 136). Our findings are supported by the finding that most bi-allelic alterations of *TP53* are focal deletions, which are likely to follow mono-allelic alteration given that the remaining probes in *TP53* demonstrate mono-allelic deletion as described in the results section. Studies have shown that patients with mono-allelic *TP53* aberrations can acquire bi-allelic mutations and deletions at relapse (91, 304, 308) and that these patients have an associated poor overall survival at relapse (91). This study provides potential mechanisms that show that mono-allelic aberrations have a prognostic impact.

It is also possible that *TP53* demonstrates haplo-insufficiency, as suggested by some in-vitro studies (141, 334-337). Additionally, it has been demonstrated that certain *TP53* mutations exert a dominant negative effect on cancer cells and may have a prognostic association through this mechanism. A further possibility is that inactivation of the other allele may occur by alternative means such as post-translational modifications and epigenetic mechanisms. *TP53* promoter hypermethylation has been reported in MM cell lines as well as in NDMM patients

(334). Post-translational modifications include alterations in proteins that interact with *TP53*, such as MDM2; these have also been demonstrated in MM samples (338, 339).

Interestingly, tumours with clonal *TP53* deletions frequently carry del(13q) and del(1p). This association is particularly strong in the case of del(13q); increasing clonality is associated with increasing *TP53*-deleted clone size, which raises the possibility of late co-evolution of these two lesions due to a possible competitive advantage. Our data suggests that del(13q) is the dominant lesion, which is consistent with its early occurrence in the evolutionary development of MM. Co-occurrence of deletion (17p), however, appears to allow this lesion to become fully clonal. This suggests synergy between the two lesions. While the association of del(17p) with del(13q) has previously been demonstrated (125, 137, 138, 145), its relationship with clone size in MM has not.

The RB1 tumour suppressor gene at chromosome 13q has been implicated in cancer due to its synergy with *TP53* aberrations. D-type cyclins elicit activation from CDK4 and CDK6 through production of oncogenic or mitogenic signals. This process initiates phosphorylation and inactivation of RB1, which leads to progression of downstream pathways and cell-cycle continuation in the cancer cell (340). Additional mechanisms of synergy between the two genes enable cell lineage plasticity (327, 328). Single-cell analyses, which were beyond the scope of this analysis, are required to test for the presence of additional del(13q) or del(1p) within individual, *TP53*-deleted sub-clones. Such a study could potentially provide further insights into clonal evolutionary dynamics in MM.

In summary, this study has demonstrated an independent association between subclonal *TP53* deletions and MM outcomes. We also demonstrate independent association of mono-allelic *TP53* alterations with outcomes, taking into account bi-allelic alterations of *TP53*. We confirm the poor prognosis that is associated with *TP53* deletions through analysis of a variety of treatments that were followed under the multi-stage randomisation method that was used in this trial. In addition, we demonstrate interesting clinical and genetic associations with *TP53* alterations.

5 Results: Gain 1q

5.1 Introduction

Chromosome 1 is the largest human chromosome. It codes for approximately 2000 proteins (341). Rearrangements, mutations and duplications of this chromosome are prevalent in cancer (290, 342-345). As detailed in the introductory chapter, gain or amplification of chromosome 1q copy number is a frequent event in myeloma. It has a prevalence of 30-50% at diagnosis of symptomatic MM (81-86).

The incidence of gain(1q), which is defined as three copies of chromosome 1q, increases between the MGUS and the NDMM stages of MM (72, 87, 88). The incidence continues to rise at relapse and shows an incidence of up to 70% (90, 91). Amplification of 1q, which is defined in this study as ≥ 4 copies of chromosome 1q, has been reported to be between 5% and 18% among newly diagnosed patients (86, 88, 89) and frequency rises at relapse (88). The combination of these findings suggests that this lesion is important for progression of MM (86).

Gains of chromosome 1q have been delineated by global SNP-based mapping (79, 346). Most studies show that the gain is of the whole arm (318). These studies have also identified a minimally amplified region between 1q21.1-1q23.3 that contains 679 genes (76), among which myeloid cell leukaemia 1 (*MCL1*), interleukin 6 receptor (*IL6R*), B cell leukaemia 9 (*BCL9*), potassium calcium-activated channel subfamily N member 3 (*KCNN3*), acidic nuclear phosphoprotein 32 family member E (*ANP32E*), interleukin enhancer binding factor 2 (*ILF2*), RNA-specific adenosine deaminase 1 (*ADARI*), proteasome 26S subunit, non-adenosine triphosphatase (*PSMD4*) and CDC28 protein kinase regulatory subunit 1B (*CKS1B*) are all implicated (76, 94, 347). *CKS1B* is implicated in cell-cycle transition (348); *ILF2* has been implicated in DNA repair (349).

Gene expression profiling has found that 1q21 gain shows significantly altered expression of proteins that are involved in the unfolded protein response (UPR). This response includes upregulation of chaperone gene ceroid-lipofuscinosis neuronal 3 (*CLN3*), ubiquitin-associated protein 2 like (*UBAP2L*), ubiquitin conjugating enzyme

E2 Q1 (UBE2Q1), the proteasome degradation gene PSMD4 and caspase4 (CASP4), which are involved in UPR-induced apoptosis (350). Because UPR-induced apoptosis plays an important role in the sensitivity of malignant cells to bortezomib, overexpression of genes on chromosome 1q may be associated with bortezomib resistance (273, 286, 351).

One key target of gain(1q) appears to be associated with *CKS1B*, which is a highly conserved member of the CKS1/suc1 gene family that plays an important role in cell-cycle control through interaction with cyclin-dependent kinase (CDK) and skp, Cullin, F-box-containing (SCF) complexes (352). Expression of CDK regulatory protein CDKS1B is negatively associated with survival in both breast and hepatocellular cancer (353, 354). It is important to note, however, that gene amplification, per se, does not indicate whether *CKS1B* is a driver or passenger gene (355) in oncogenesis as multiple genes are included in copy number gain in the 1q21 region (356). *CKS1B* has a role in the promotion of both G1-S and G2-M stages of the cell-cycle transition (357). Excess of *CKS1B* results in inhibited CDK and delayed mitosis because dephosphorylation of the regulatory tyrosine residue is inhibited. It also results in phosphorylation of CDK regulators CDC25, Myt1 and Wee1 (348). Together these regulatory events promote entry into mitosis, but this effect is context dependent. *CKS1B* also has important interactions with the proteasome. It targets SCF-SKP2 ubiquitin ligase towards CDK-inhibitor p27KIP/CDKN1B to promote degradation and allow entry into the S phase (349).

Several reports have suggested that 1q gain is associated with chromosomal instability and drives amplification at 1q but also deletions in other non-homologous chromosomal locations. Abnormalities such as balanced translocations, amplification and jumping translocations are thought to be the mechanism that leads to increased copy numbers of genes at this locus as shown in Figure 49(358, 359). These events appear to be secondary to de-condensation of the pericentromeric chromatin of chromosome 1 and endo-duplication of 1q, which are followed by fusion of the gained 1q region with either the centromeric or the telomeric end of another chromosome.

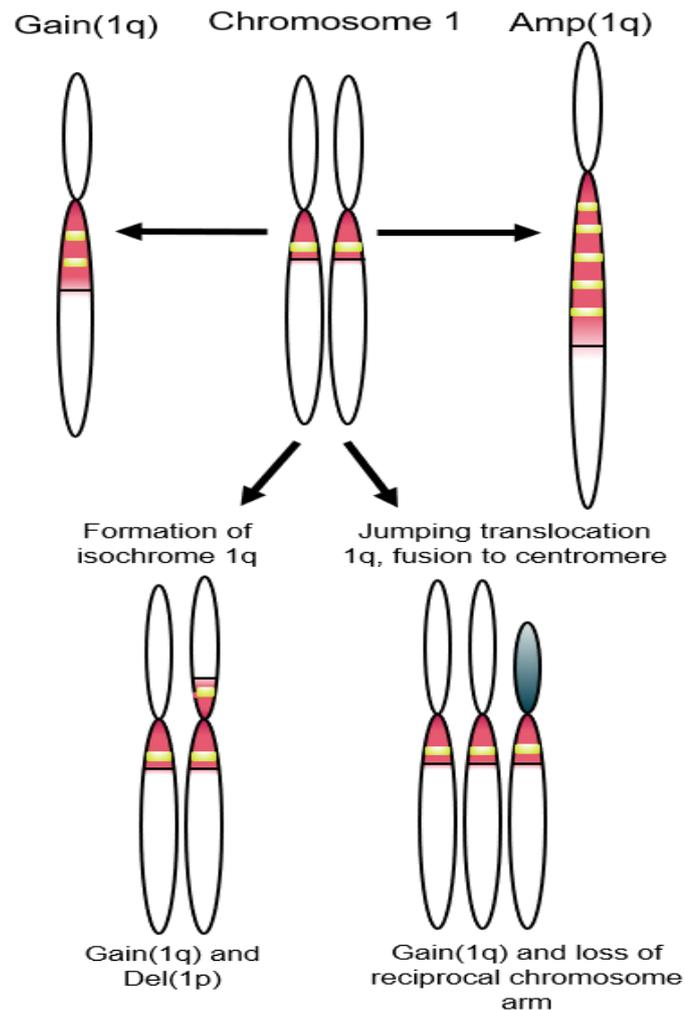


Figure 49: Diagram demonstrating mechanisms of gain or amplification of 1q21, formation of isochromosome 1q and jumping translocations at 1q. Red part of chromosome represents pericentromeric heterochromatin. Yellow band represents the frequently focally amplified 1q21 region in chromosome 1 (Adapted from (360))

From the clinical standpoint, current data conflict regarding the role of gain(1q) as an independent prognostic factor (67, 92-96). Some studies have demonstrated an association between gain(1q) and shortened survival (84, 85, 88, 97-99). This has been shown in the context of thalidomide induction (67, 88), autologous stem cell transplant (84, 361) and bortezomib-based chemotherapy (89, 362). Gain(1q) has also been associated with bortezomib resistance (90) as well as resistance to triplet (proteasome inhibitor and IMiD-based) induction (362). However, other studies have provided evidence that gain(1q) does not have a negative prognostic impact after IMiD therapy (84, 86).

The number of 1q duplications that are associated with poor prognosis also remains controversial. Some studies have shown that amplification of chromosome 1q (amp(1q)) has a worse prognosis than gain(1q) (53, 88). As a result of this controversy, gain(1q) has often not been included in prognostic scoring or reported in clinical trials, so as a consequence there is limited evidence of its role in treatment resistance, especially in the context of recent therapies (104, 238). Interestingly, one recent study suggests that only amplification of 1q, bi-allelic *TP53* deletion and a score of ISS III are prognostic in myeloma out of hundreds of genetic and clinical factors (100).

Historically, the main method that has been used to examine gain 1q has been iFISH. Modern methods of discovery of bulk tumour sample copy number status, such as MLPA, SNP arrays and whole exome/genome sequencing assays, cannot be used to gain information regarding clonality status as they are used to evaluate the copy number status of the whole tumour rather than at the single-cell level. Due to the disadvantages of bulk DNA methods, it is therefore more challenging to evaluate tumour heterogeneity compared with single-cell methods. We therefore first examined results of 1q status that were recorded in the Myeloma IX database by use of iFISH to examine this region in terms of subclonal gains and amplifications. This comes with the caveat that iFISH can be unreliable with regards to quantification of subclones as detailed in the previous chapter.

As the main aim of this study was to examine gain(1q) in the context of amp(1q), these lesions were examined first in terms of their inter-relationship. We examined them when they occurred in isolation and in combination with regard to their clonal status as well as their association with survival.

The effect of gain(1q) in combination with amp(1q) is important because discussion about survival revolves around which of these clones is prognostic. Since bulk tumour copy number assays are likely, in some cases, to contain a mix of the two lesions, as is demonstrated later in this chapter, it is important to delineate whether the prognosis of patients with a predominantly gain 1q lesion is in fact driven by a small 1q amplified subclone. Investigation of this possibility can only be performed through use of single-cell methods such as iFISH or single-cell sequencing methods.

Once the impact of clonality was established, the study continued with examination of survival with a set cut-off that was informed by the above studies to consider the association of clinical, genetic and survival associations in patients with gain(1q) and amp(1q).

I applied the knowledge gained through examination of iFISH data in the Myeloma IX trial to study gain 1q status by use of MLPA in the Myeloma XI trial. As mentioned, MLPA examines bulk tumour status rather than individual subclones, but it is more reliable in the provision of a quantitative result compared with iFISH. Additionally, the clonal burden of both gain and amplification at the 1q21 site was evaluated in order to study patients with gain 1q in the context of therapy that was delivered within the Myeloma XI trial. Through use of both methods and comparison of them, we were able to provide a comprehensive analysis of gain 1q in terms of its association with survival in the newly diagnosed setting.

5.2 Aims of this study

- To investigate the optimal subclonal cut-off point to measure the association of survival with 1q copy number gain and amplification;
- To investigate amp(1q) and gain(1q) and their independence in terms of prognostic association;
- To investigate clinical and genetic associations of both gain and amp 1q;
- To characterise the clinical impact of 1q copy number in the various treatment contexts within the trials.

5.3 Results

5.3.1 Examination of iFISH data on clonal and subclonal gain and amplification 1q in the Myeloma IX trial

The Myeloma IX trial was evaluated in terms of chromosomal aberrations through use of the iFISH method. This allows evaluation of the 1q region at the single cell level in terms of clonality and its association with survival. Previously, it has only been examined by using a dichotomous result of gain(1q21) vs. no gain(1q21) if more than 20% of CD138 selected cells had ≥ 3 copies of chromosome 1q (67). However, at the time, data were recorded with regard to the number of 1q copies that were seen per cell, as well as the percentage of cells with each gain/amplification. This rich dataset was therefore evaluated to inform the current areas of debate with regard to chromosome 1q, as detailed in the introduction.

First, we examined percentage gain, amp and diploid 1q status within the Myeloma IX dataset to examine clonal status at diagnosis. When a 20% threshold was applied for copy number calling, gain 1q21 accounted for 29.2% of samples and amp 1q accounted for 11% of samples. The remaining samples had a diploid copy number. We repeated this evaluation with no percentage threshold for calling gain or amp 1q21. Using no threshold, gain and amp 1q21 accounted for 49% of samples (Figure 50).

Subclonal and clonal gain(1q) deletions, which have been demonstrated above, suggest that bulk-sequencing or array-based methods that call samples as gain or amplification at 1q21 may instead find a mixture of the two, or that a subclonal amplification may also be mistaken as a gain 1q21. Values above certain thresholds may be reliably called amplification, but intermediate values are likely to be subclonal mixtures that are not easily studied using these methods. This is further demonstrated in this chapter with a direct comparison of gain(1q) and MLPA copy number calling results.

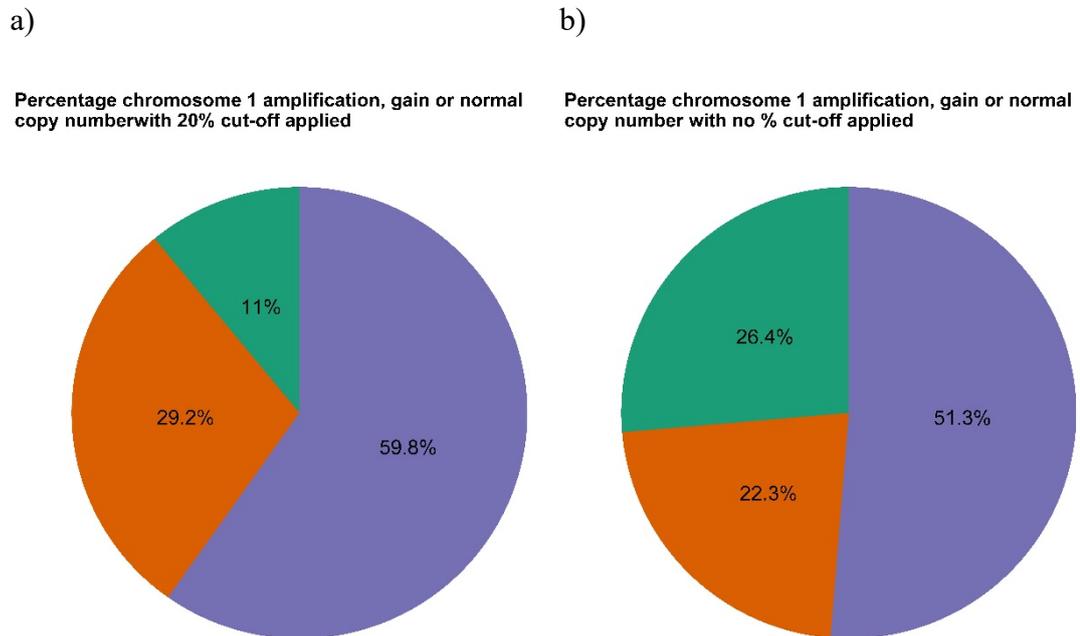


Figure 50: Pie chart showing the percentage of patients with amp 1q/gain1q/diploid 1q copy number with a) 20% threshold applied for calling copy number; b) no percentage threshold for calling copy number. Orange: gain 1q; Green: amplification >3 copies 1q; Purple: diploid copy number

We show percentage gain data at the individual level in Figure 51, which displays percentage gain or amp (>3 copies) of 1q in all samples that contain a deletion. The mean gain(1q) percentage by iFISH was 85.7%, which was consistent with other studies that show that gain 1q has a high clonal burden at the time of diagnosis of symptomatic myeloma (363).

The frequency of concurrent amplification that was found within these patients was 26.8%. Results from iFISH analysis show that patients with gain and amplification can have several different subclones in terms of 1q copy number (up to five in our data set). Due to the difficulty of distinguishing between, for example, four and five copies, the clonal data may not be as reliable as methods such as single cell sequencing analysis, but their examination remains useful.

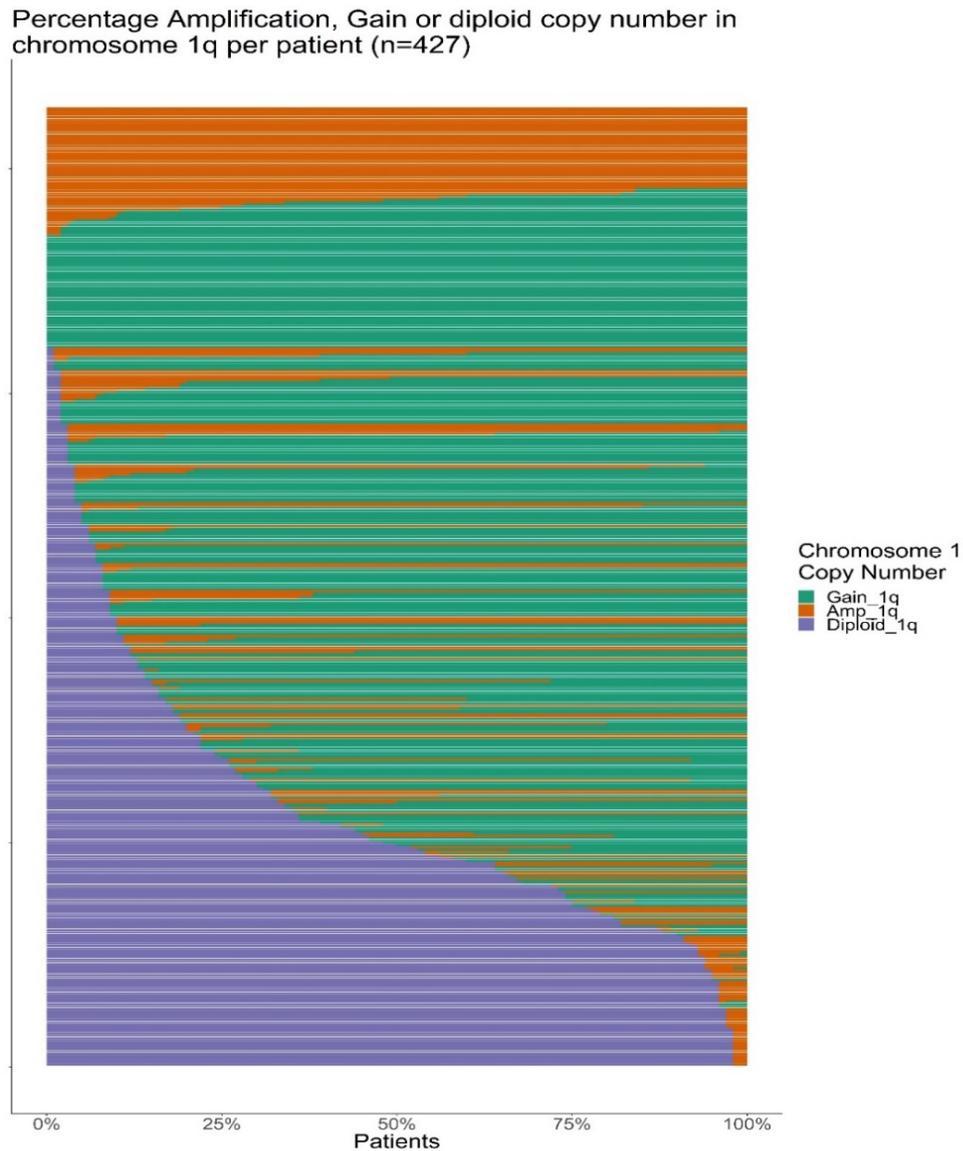
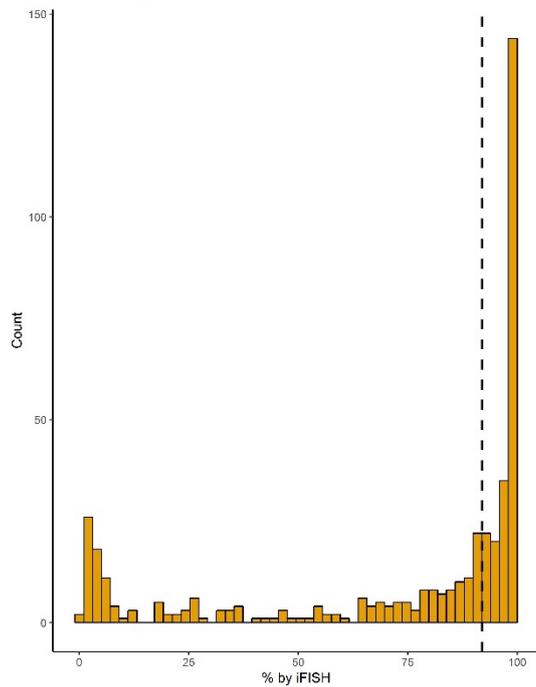


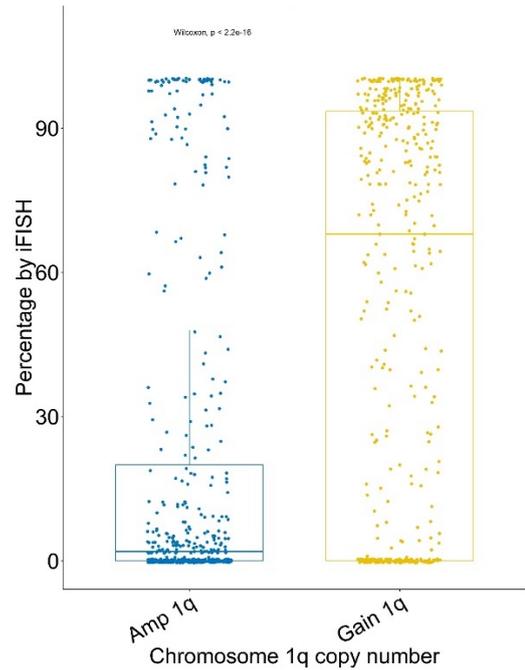
Figure 51: Bar chart with percentage amplification (>3 copies), gain (3 copies) or diploid (2 copies) copy number at chromosome 1q21 per patient in the Myeloma IX trial

Figure 52 demonstrate clonality data for gain and amplification 1q together (, a and b). Figure 52 c demonstrates the clone size of gain vs. amplification 1q when these clones are present in the same sample. Figure 52d demonstrates clonality of gain or amplification 1q when the clones are present in isolation. Overall, gain 1q was found to be at a higher clonal percentage than amplification 1q.

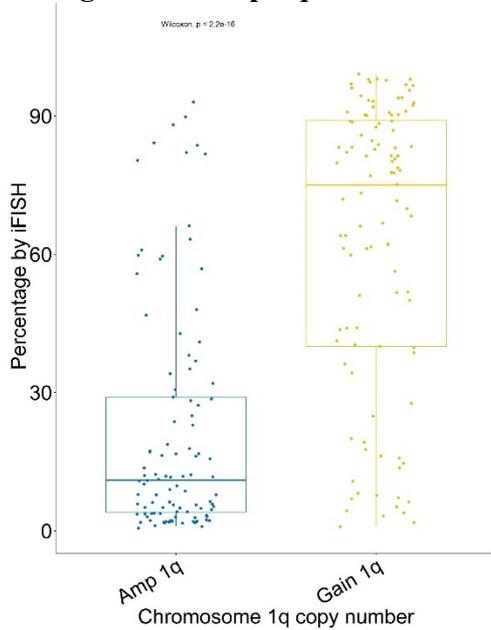
a) Histogram showing iFISH based clonal percentage of amp or gain 1q21



b) Gain vs. amp 1q clonal percentage in patients with either gain 1q or amp 1q21 or both



c) Gain vs. amp 1q clonal percentage in patients with both gain and amp 1q21



d) Gain vs. amp 1q in patients with either gain 1q or amp1q21 exclusively

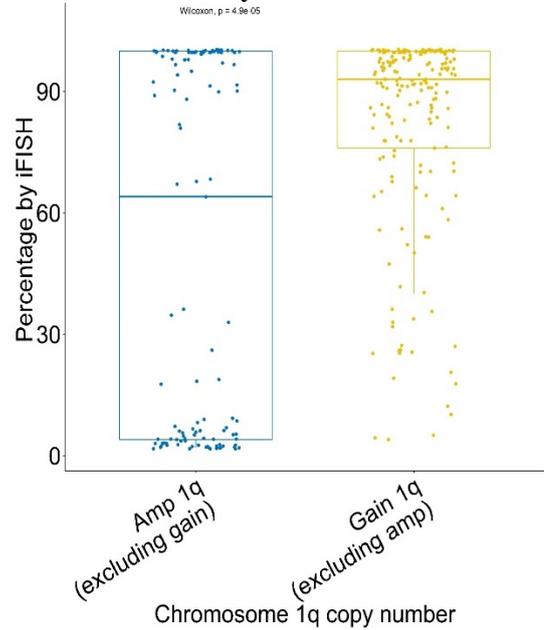


Figure 52: Clonal percentages for patients with gain or amplification of chromosome 1q by iFISH

Next, we explored the significance of the number of gain(1q) in terms of clonality to firmly establish whether clonality within gain(1q21) and/or amp(1q21) was significantly associated with survival. We explained various methods of optimal cut-point analysis in the *TP53* chapter of this thesis. In order to understand the association with gain(1q) and survival, we used the same methods as well as additional methods such as the standardised log-rank statistic to evaluate cut-points in the context of gain(1q).

We evaluated percentage gain or amplification of 1q in the context of survival in terms of clonality. First, we examined the optimal cut-point for gain 1q calling in all samples with gain 1q21 only (without amp(1q)) as shown graphically in Figure 53a. Gain 1q in the absence of any amp(1q) clone had a maximum log-rank statistic of 18%. Next, we examined amplification 1q when it was seen without gain1q. This had a maximum log-rank statistic at the 9% threshold. Finally, we examined tumour samples with both gain(1q) and/or amp(1q). Among these samples, the standardised log-rank statistics demonstrated a cut-point percentage threshold of 17%. When these lesions were evaluated individually, the highest standardised log-rank statistic was observed when both gain and amplification 1q were evaluated together with a standardised log-rank statistic of 5.97 vs. 4.57 for gain 1q only and 3.72 for amp 1q only. These results suggest improved association with survival of both aberrations together. It must be acknowledged, however, that iFISH is generally considered to be unreliable below a cut-point of 10-20%, so the true lower cut-point is likely to be within this range rather than an absolute value.

We validated the above findings through examination of step-wise dichotomous cut-points using the minimal *P*-method to evaluate the optimal cut-point for survival association with samples with gain and/or amplification 1q. Use of both Kaplan-Meier-based log-rank testing (Table 47) and Cox proportional hazards-based modelling (Table 48), we found that the $\geq 10\%$ threshold had the lowest log-rank *P*-value associated with this cut-off. Samples could not be cut further due to the limited number of samples in each group for this evaluation as well as the relative inaccuracy of the exact percentage that was called by iFISH.

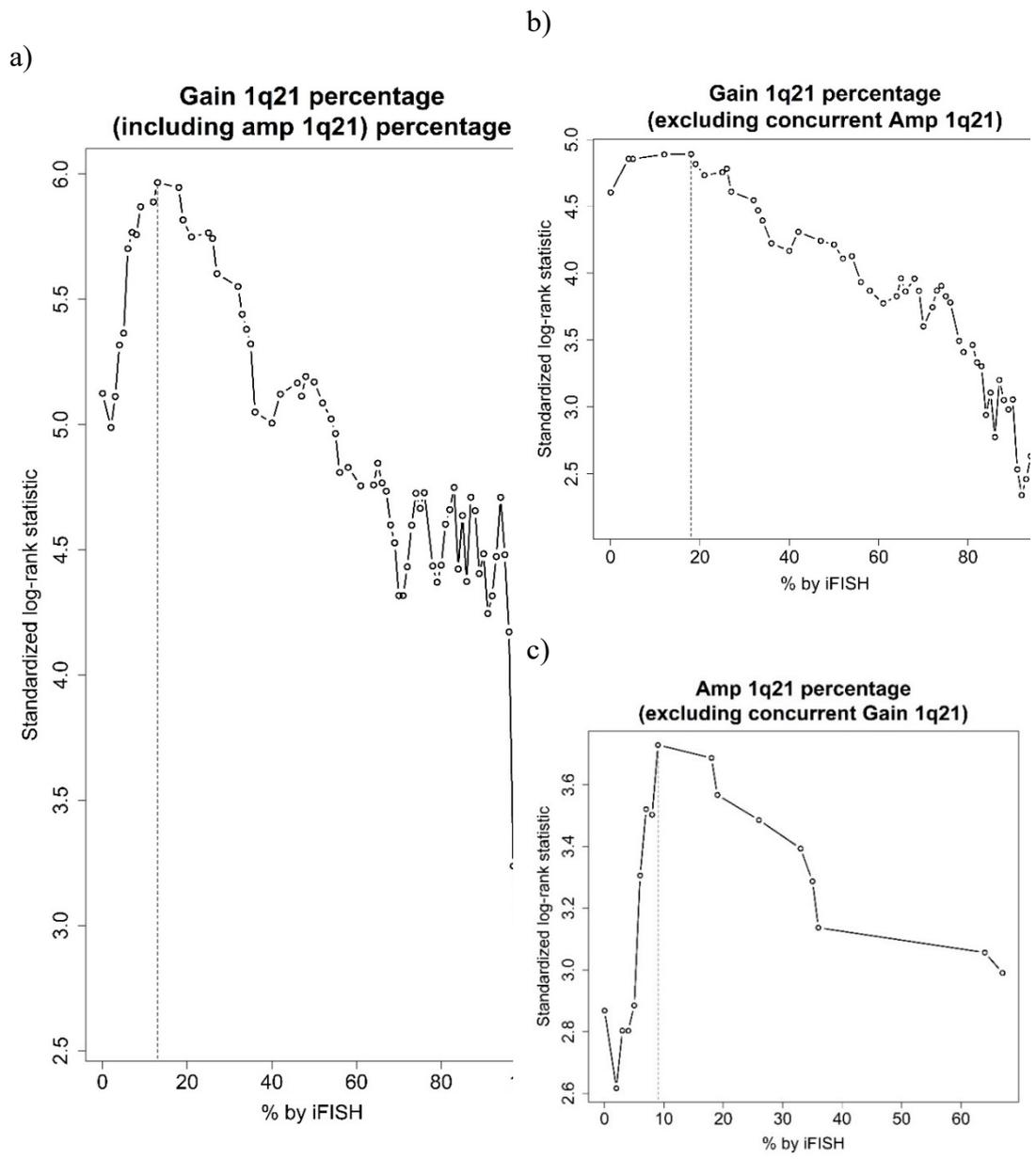


Figure 53: Maximally ranked statistics of optimal gain(1q) percentage in terms of its association with survival. a) All patients with combined gain and amplification percentage; b) Gain percentage only in patients with no amp(1q); c) Amp 1q percentage in patients without gain 1q

Table 47: Survival statistics for different gain 1q21 (including amplification 1q) cuts by iFISH using univariate Kaplan-Meier-based overall survival in months in the Myeloma IX trial

OS	Median (95% CI) months Gain 1q21	Median (95% CI) months No Gain 1q21	Log-rank <i>P</i>
Gain 1q ≥1%	36.9 (31-43.7)	57.6 (50.9-66.1)	2.15E-07
Gain 1q ≥5%	35.1 (29-42.3)	57.1 (50.4-64.5)	3.77E-08
Gain 1q ≥10%	33.3 (28.6-40.9)	58.9 (50.9-65.2)	1.28E-09
Gain 1q ≥20%	32.9 (28-39.7)	58.9 (50.9-64.5)	1.62E-09
Gain 1q ≥30%	32.9 (28.2-40.9)	57.1 (50.4-64.2)	6.12E-09
Gain 1q ≥40%	35.1 (29-41.9)	55.3 (49.7-62.6)	2.21E-07
Gain 1q ≥50%	33.7 (28.9-41.8)	55.3 (49.7-62.6)	7.97E-08
Gain 1q ≥60%	35.3 (29-42.4)	54.4 (49-62)	5.63E-07
Gain 1q ≥70%	35.7 (29-43.5)	52.1 (47.7-60.6)	7.86E-06
Gain 1q ≥80%	35.7 (29-43.5)	51.9 (47.6-60.6)	3.80E-06
Gain 1q ≥90%	35.3 (28.2-43.5)	51.4 (46.8-59.1)	2.28E-06
Gain 1q ≥100%	36.9 (31-43.7)	57.6 (50.9-66.1)	2.15E-07

Table 48: Survival statistics for different gain 1q21 (including amp(1q21)) percentage cuts by iFISH: univariate overall survival by Cox-based proportional hazards modelling in the Myeloma IX trial

OS	HR	Wald <i>P</i>
Gain 1q ≥1%	1.56 (1.32-1.84)	2.67E-07
Gain 1q ≥5%	1.6 (1.35-1.89)	4.99E-08
Gain 1q ≥10%	1.68 (1.42-1.99)	1.93E-09
Gain 1q ≥20%	1.67 (1.41-1.98)	2.43E-09
Gain 1q ≥30%	1.64 (1.39-1.95)	8.71E-09
Gain 1q ≥40%	1.56 (1.32-1.85)	2.78E-07
Gain 1q ≥50%	1.59 (1.34-1.88)	1.04E-07
Gain 1q ≥60%	1.54 (1.3-1.83)	6.90E-07
Gain 1q ≥70%	1.48 (1.24-1.76)	9.00E-06
Gain 1q ≥80%	1.51 (1.26-1.79)	4.44E-06
Gain 1q ≥90%	1.55 (1.29-1.85)	2.73E-06
Gain 1q ≥100%	1.56 (1.32-1.84)	2.67E-07

The area under the ROC curve (AUC) was evaluated over time as shown in Figure 54. Each point represented the calculated AUC at the time of each event (death in this case). The highest AUC through time within the graph showed that the 10% and 20% cut off were very similar to each other and showed the highest predictive ability. AUCi comparison between different cuts of gain 1q21 by iFISH showed that the 20% cut had the highest predictive power and was statistically better than the 10% cut by AUCi ($P=1.8 \times 10^{-31}$).

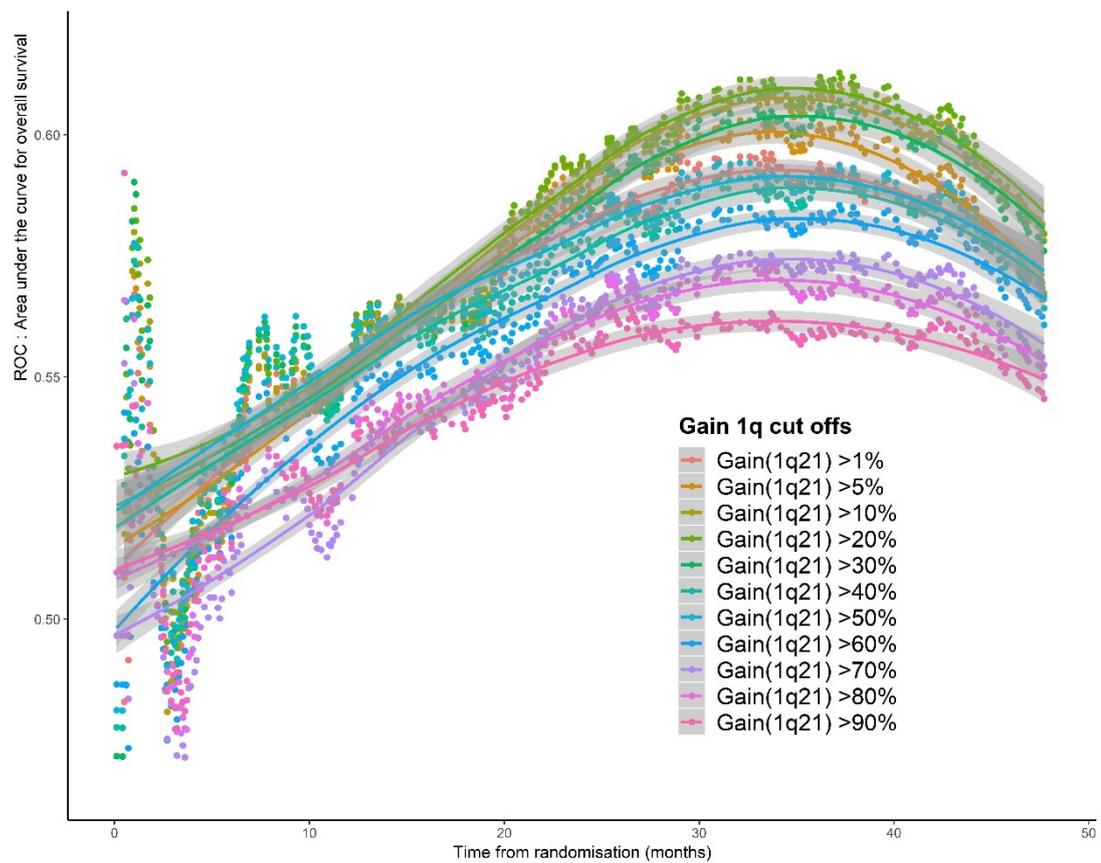


Figure 54: Area under the ROC curve plotted over time along different cut-points of the 1q21 probe with polynomial regression applied to each cut-point

Table 49: Cox-model-based multivariable analysis of overall survival including different clonal cuts of 1q21 and/or amp12q1 and known adverse prognostic markers of myeloma (n=821, events 532)

Genetic Variables	HR(95% CI)	Wald P
Gain 1q $\geq 1 - 5\%$	1.17(0.79-1.74)	0.437
Gain 1q 6 – 10%	0.59(0.28-1.26)	0.171
Gain 1q 11 – 20%	1.23(0.46-3.31)	0.678
Gain 1q 21 – 50%	1.82(1.15-2.89)	0.011
Gain 1q 51 – 80%	1.53(1.07-2.21)	0.0211
Gain 1q 81 – 100%	1.5(1.23-1.82)	6.48E-05
High-risk translocation	1.43(1.14-1.79)	0.00182
Deletion 17p	2.07(1.59-2.69)	6.70E-08

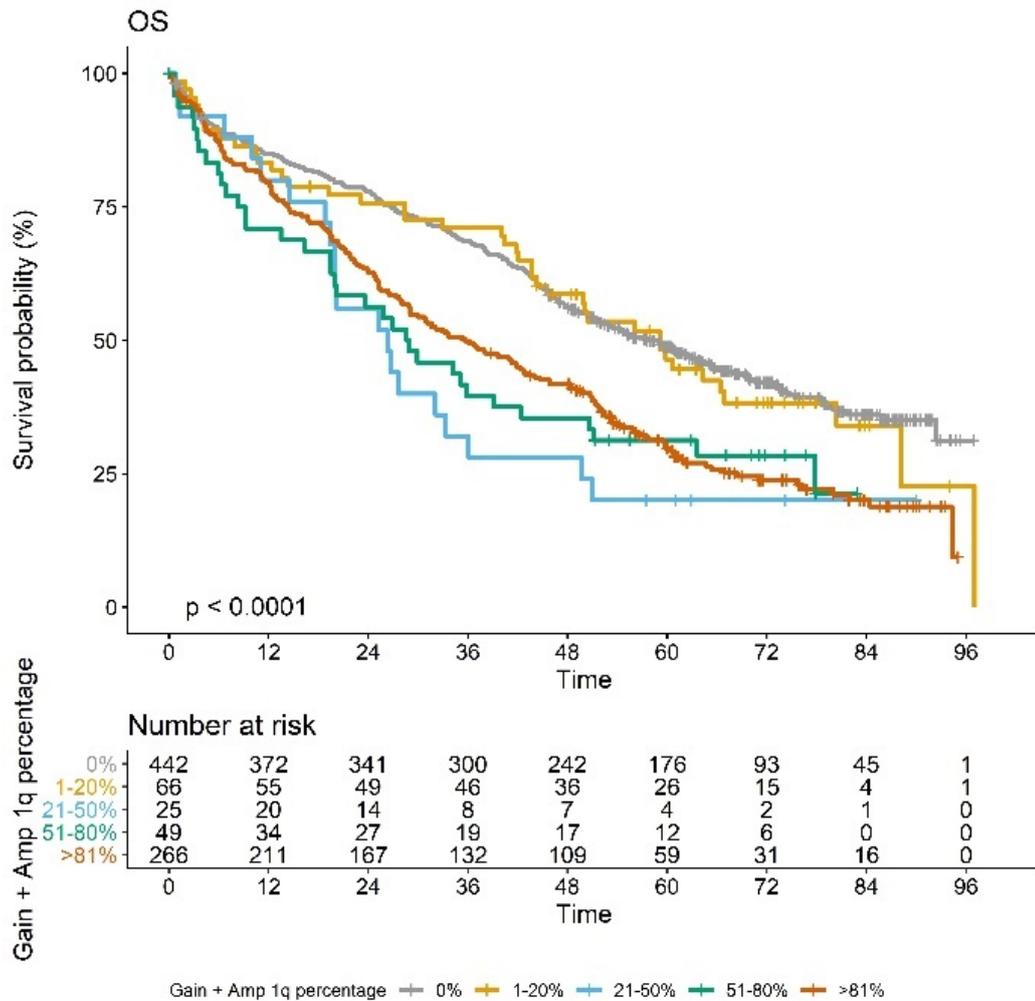


Figure 55: Kaplan-Meier survival curves of overall survival based on different cuts for gain and/or amp 1q21

The above studies were repeated for gain 1q21 without concurrent amplification 1q21 as well as for amplification 1q21 in the absence of gain 1q21. For both these studies, the 20% threshold was found to be superior for survival cut-off calling (data not shown). We also ran a Cox-based multivariable analysis (Table 49) with different 1q21 cut-offs and with other high-risk genetic lesions. The significance of gain1q21 was retained in cut-points from $\geq 21\%$ in this analysis. This is shown graphically in Figure 55 with different cut-points of gain(1q) and/or amp(1q) percentage.

The true difference between the 10% cut and the 20% cut, however, is difficult to differentiate by iFISH, as several laboratories have shown that the accuracy of calling gain or deletion around this percentage can be low. This is in line with results from log-rank testing, maximally ranked statistics and Cox-based statistics, which show that a cut off of between 10% and 20% is optimal in the setting of iFISH. Considering the cumulative results from the above analyses, we chose a 20% cut-off for evaluation of gain1q21 by iFISH for further analysis.

5.3.2 Association of survival with gain and amplification of 1q21 in NDMM

The above examination of the association of gain(1q21) and amp(1q21) suggests both these lesions are associated with prognosis. However, as they had not been directly compared, we examined whether gain or amplification 1q21 had an association with survival in the overall subset of patients in the Myeloma IX trial. In this analysis, the definition of gain(1q) was defined as ≥ 3 copies 1q21 in more than 20% of cells and amp(1q21) defined as ≥ 4 copies in more than 20% of tumour cells for this analysis due to the analysis in the above section. In any analysis where both gain(1q) and amp(1q) have been analysed together, gain(1q) is defined as 3 copies and amp 1q id defined as >3 copies with the exception of Figure 59 where amp(1q) is defined as >4 copies.

Kaplan-Meier survival analysis and multivariable testing for overall survival demonstrated that both gain 1q21 (HR 1.58, 95% CI 1.3-1.91; $P=3.7 \times 10^{-6}$) and amp (1q21) (HR 1.38, 95% CI 1.04-1.83, $P=0.02$) (Figure 56) were associated with shortened overall survival. This was also found to be the case when landmarked from the time of HDM-ASCT (Figure 57). Additionally, both gain(1q21) and amp(1q21) demonstrated independence in terms of their association with shortened survival in a multivariable analysis with other high risk genetic lesions (Table 50).

Table 50: Cox proportional hazards ratio multivariable analysis for overall survival (n=821, events 532)

	HR(95% CI)	Wald P
Gain 1q21	1.49(1.2-1.84)	0.000255
Amplification 1q21	1.35(1.09-1.67)	0.00586
Deletion 17p	2.03(1.56-2.63)	1.41E-07
High-risk translocation	1.49(1.19-1.86)	0.000463

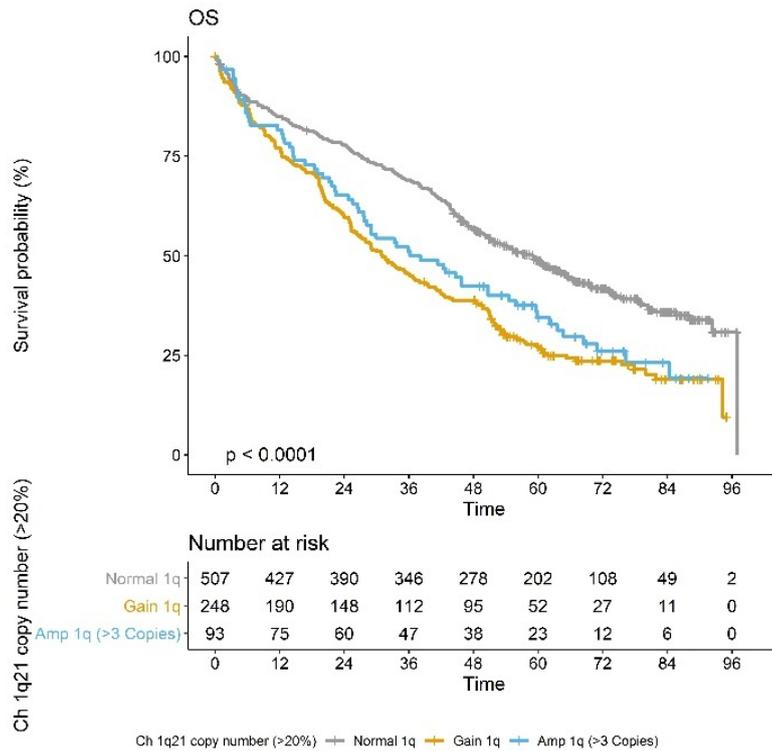


Figure 56: Kaplan-Meier survival curves for overall survival in patients with gain or amplification 1q21 landmarked from time of induction randomisation

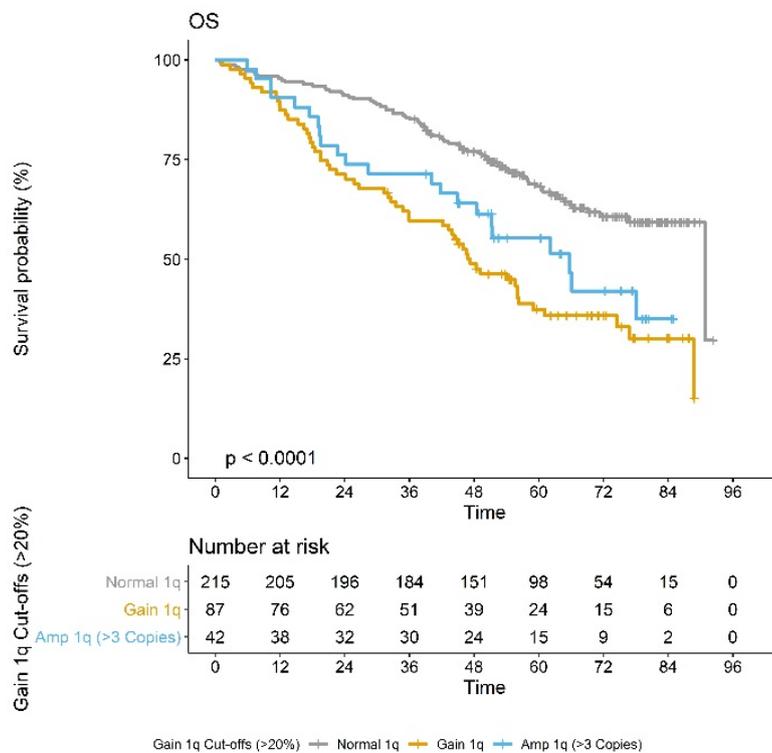


Figure 57: Kaplan-Meier survival curves for overall survival in patients with gain or amplification 1q21 landmarked from time of HDM and ASCT

We also investigated whether ≥ 5 copies of 1q had a much higher association with survival as has been suggested by certain research groups. Figure 58 shows a Kaplan-Meier survival analysis of gain 1q21 (defined as ≥ 4 copies 1q21) vs. amplification 1q21 (≥ 5 copies of 1q21). This analysis demonstrates no difference in overall survival in patients with this lesion. The numbers of patients with ≥ 6 copies of chromosome 1q were too small for investigation. Overall, our data suggested that patients with gain or amplification of 1q both demonstrate an association with shortened survival with no difference between the two lesions.

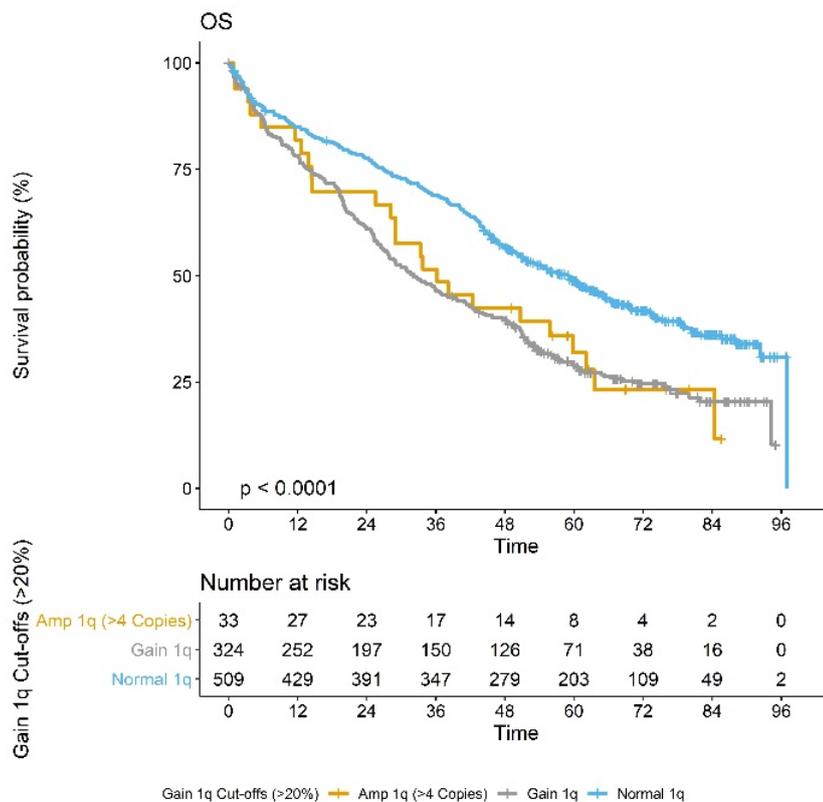


Figure 58: Kaplan-Meier survival curves of overall survival in patients with gain (3 or 4 copies) vs. ≥ 5 copies of 1q21 from time of induction randomisation

5.3.3 Genetic associations and gain or amp 1q21

We went on to investigate whether there were genetic or clinical associations with regards to the number of 1q21 copies within tumours as shown in Table 51. Through this analysis, we observed a progressive decrease in the frequency of associated primary cytogenetic lesions, such as hyperdiploid karyotype and t(11;14), with increasing 1q21 copy number, along with a concurrent rise in the number of all high-risk translocations. We also observed a much higher incidence of deletion 13q in patients with amplification 1q21 compared with gain 1q21. No statistical differences were observed between increased copies of 1q21 and deletion 1p32 or deletion 17p. As a result of the associations of amplification of 1q21 described above, amplification 1q21 was associated with a higher incidence of ‘double-hit’, which was largely due to the increased incidence of high-risk translocations.

Table 51: Genetic associations with amp vs. gain 1q21 by iFISH in the Myeloma IX trial. Gain 1q baseline characteristics

	Normal 1q21 (n=507)	Gain 1q21 (n=248)	Amplification 1q21 (n=93)	P
Hyperdiploidy (%)	329 (67.1)	135 (56.0)	30 (32.3)	<0.001
t(4;14) (%)	26 (5.1)	51 (20.6)	27 (29.0)	<0.001
t(11;14) (%)	89 (17.6)	26 (10.5)	4 (4.3)	0.001
t(14;16) or t(14;20) (%)	9 (1.8)	13 (5.3)	14 (15.2)	<0.001
t(6;14) (%)	4 (0.8)	2 (0.8)	1 (1.1)	0.963
High-risk translocation (%)	35 (7.0)	64 (26.0)	41 (44.6)	<0.001
Del(1p32.3) (%)	45 (9.5)	24 (10.3)	14 (16.3)	0.169
Del(22q) (%)	53 (12.3)	34 (14.7)	8 (9.4)	0.417
Del(13q) (%)	176 (34.9)	142 (58.2)	67 (75.3)	<0.001
Del(16) (%)	81 (17.2)	46 (19.2)	21 (23.1)	0.396
Del(17p) (%)	37 (7.5)	28 (11.5)	8 (8.9)	0.199
Number of high-risk lesions (%)				<0.001
0	421 (85.9)	0 (0.0)	0 (0.0)	
1	67 (13.7)	163 (67.4)	46 (51.7)	
2	2 (0.4)	69 (28.5)	38 (42.7)	
3	0 (0.0)	10 (4.1)	5 (5.6)	

5.3.4 Clinical associations of gain or amp 1q21

There was no statistical difference in patient demographics with increasing 1q21 copy number (Table 52). In terms of haematological parameters, patients with increased copy number at 1q21 had a statistically significant increase in rates of thrombocytopenia at diagnosis compared with those without this increased copy number (29% vs. 14.9%) and 10.3% in amplification, gain and diploid 1q copy number ($P<0.001$). There was also a statistically significant fall in haemoglobin concentration with increasing 1q21 copy number ($P=0.001$) There was no difference in mean white cell count (Table 53).

In terms of biochemical parameters, patients with amplification 1q21 were associated with a lower albumin level. There were no associated differences in lactate dehydrogenase, CRP or corrected calcium levels or in renal function. Patients with amplification and gain 1q21 were associated with lower overall bone-related morbidity including a lower incidence of bone disease in general ($P=0.005$) and lower incidence of bone pain ($P=0.002$) (Table 53).

Table 52: Differences in baseline demographics between gain(1q) and amp(1q)

Demographics	Normal 1q21 (n=507)	Gain 1q21 (n=248)	Amplification 1q21 (n=93)	<i>P</i>
Gender				
Female (%)	177 (34.9)	102 (41.1)	42 (45.2)	0.078
Age (mean (sd))	63.83 (10.30)	65.05 (9.44)	63.14 (10.58)	0.183
WHO (%)				0.383
0	146 (29.0)	53 (21.5)	29 (31.2)	
1	217 (43.1)	111 (45.1)	38 (40.9)	
2	87 (17.3)	55 (22.4)	16 (17.2)	
3	50 (9.9)	25 (10.2)	8 (8.6)	
4	4 (0.8)	2 (0.8)	2 (2.2)	

Table 53: Haematological, biochemical and clinical parameters at diagnosis in patients in the Myeloma IX trial with comparison of amplification vs. gain and diploid 1q21 status

Variable	Normal 1q21 (n=507)	Gain 1q21 (n=248)	Amplification 1q21 (n=93)	P
Albumin <35g/l (%)	236 (47.0)	140 (56.7)	49 (52.7)	0.041
B2M>5.5 (%)	131 (36.3)	74 (41.8)	33 (44.6)	0.262
LDH (median [IQR])	328.00 [251.75, 406.75]	313.00 [239.00, 411.00]	375.00 [243.00, 480.00]	0.266
CRP (median [IQR])	7.00 [4.00, 18.00]	7.05 [5.00, 17.98]	6.00 [3.00, 11.75]	0.334
Calcium (median [IQR])	2.40 [2.30, 2.50]	2.40 [2.30, 2.50]	2.40 [2.26, 2.50]	0.915
Creatinine (median [IQR])	100.00 [84.00, 125.00]	101.00 [84.25, 127.50]	100.00 [81.00, 143.50]	0.982
eGFR (mean (sd))	62.21 (23.21)	61.59 (25.14)	60.39 (26.28)	0.79
eGFR <30mls/min (%)	45 (9.3)	27 (11.2)	10 (10.9)	0.69
Urea (mean (sd))	7.17 (3.90)	7.35 (4.23)	7.66 (4.95)	0.544
ISS (%)				0.099
1	85 (23.5)	25 (14.1)	13 (17.6)	
2	145 (40.2)	78 (44.1)	28 (37.8)	
3	131 (36.3)	74 (41.8)	33 (44.6)	
Haemoglobin g/l (mean (sd))	106.84 (18.99)	102.73 (19.02)	100.62 (17.42)	0.001
White blood count (mean (sd))	6.44 (3.20)	6.39 (2.62)	6.58 (3.79)	0.885
Platelets <150 ⁹ /l (%)	52 (10.3)	37 (14.9)	27 (29.0)	<0.001
Bone pain (%)	373 (74.6)	174 (71.0)	54 (58.1)	0.005
Bone disease	364 (72.2)	165 (67.9)	50 (53.8)	0.002

5.3.5 Examination of MLPA data in the Myeloma XI trial in the context of chromosome 1q

As described in the introduction, the area of interest within chromosome 1q appears to be 1q21 in terms of its role as a focal region of amplification and increased gene expression. The Myeloma IX trial iFISH probe was performed within this region and analysis was therefore restricted to this region. In Myeloma XI, MLPA was used for copy number calling. In this trial, the investigation of different regions for copy number within the chromosome 1q arm was possible with study of the different regions of 1q simultaneously. Also, the Myeloma XI trial incorporated the application of more novel agents than Myeloma IX, and it is therefore important to study the effect of this lesion in the context of these therapies.

The MLPA P425 employs seven probes along chromosome 1q. We examined copy number frequency through use of the previously defined cut-offs for MLPA at each probe in chromosome 1q. As shown in Figure 59, deletion at chromosome 1q was rare (0.25-0.45% of all samples). Gain and amplification events were frequent across all probes (30-36%). The highest proportion of patients with amplification (defined as MLPA normalised ratio of >1.6) along the chromosome 1q probes was at 1q21.3, and these accounted for 9.1% of samples.

Overall, patients with gain(1q21) demonstrated gains in all 1q probes within the MLPA P425 probeset. Similarly, the density distribution of copy number of these probes (Figure 60) shows an overlapping pattern of MLPA values with a bi-modal peak. The major peak demonstrates samples with a diploid copy number around the relative ratio of 1. A minor secondary peak is associated with increased copy number. These data, along with previous SNP array data (79, 346) from previous studies, show arm-level events in terms of copy number gains on chromosome 1q with amplification within the whole arm, as well as a higher frequency of focal amplifications at 1q21.

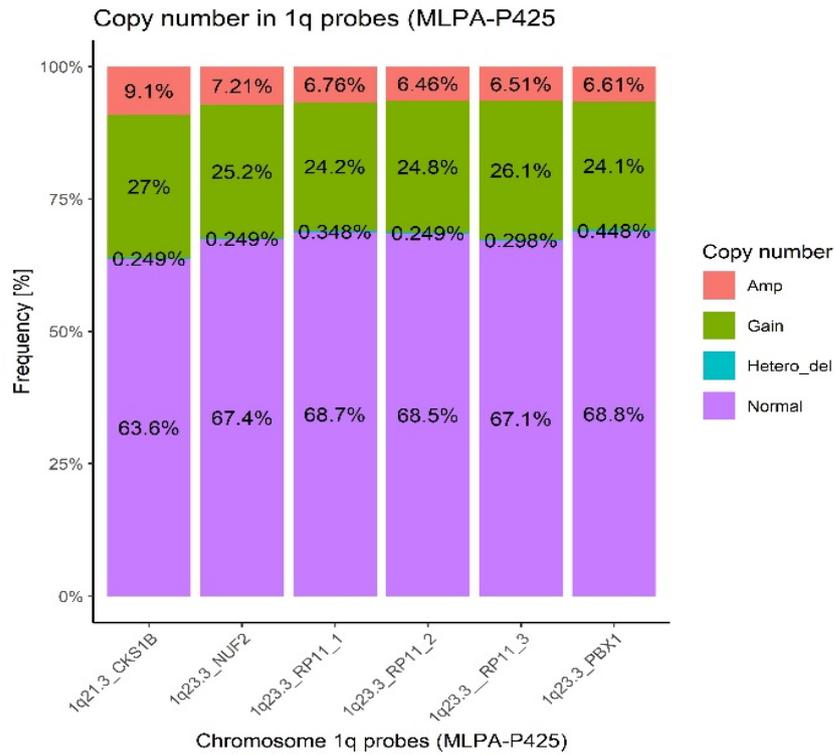


Figure 59: Bar chart with copy number by MLPA along probes found on chromosome 1q on the P425 probeset. Hetero-del = heterozygous deletion; Amp = amplification; Normal = diploid copy number

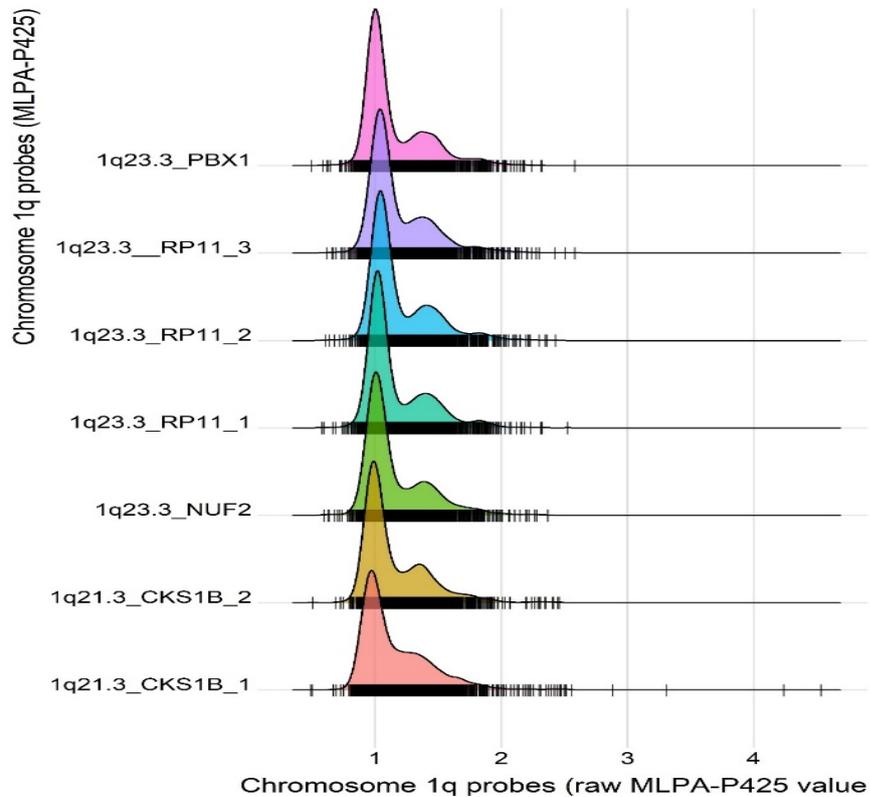


Figure 60: Density plots of relative MLPA values for each probe along chromosome 1q on the P425 MLPA probeset.

5.3.5.1 iFISH and MLPA values for calling gain or amplification

To add context to MLPA data from the Myeloma XI trial to our findings from iFISH from the previous chapter we compared the two methods. We examined the relationship of iFISH within the Myeloma IX trial with samples that had undergone MLPA as well, as shown in Figure 61. Samples were not handled by the same centres in terms of CD138 separation and the original intention of this analysis was to give a dichotomous gain/no gain answer for iFISH. Hence the reliability of these results may not be the same as for those samples that were handled in the same way with quality control measures for percentage copy number calling. The normalised relative MLPA ratios show that, while samples with diploid copy number had a normal MLPA ratio (<1.2) in all but one case, gain and amplification could both have a normal MLPA ratio. Further analysis of samples for gain or amplification of 1q21 by iFISH showed that most samples with a normal MLPA ratio had subclonal gains or amplifications.

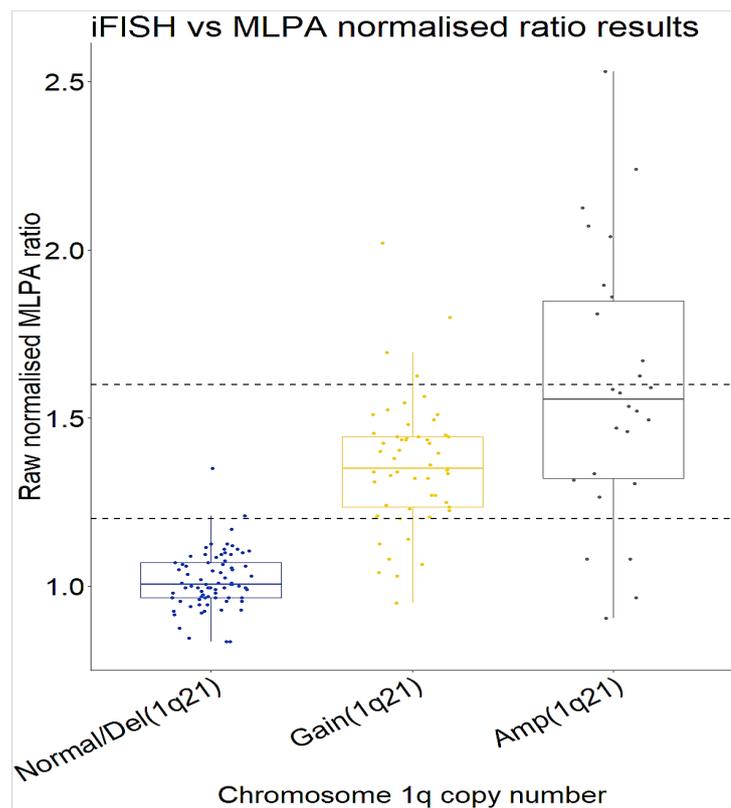


Figure 61: iFISH percentage vs. MLPA normalised values in the Myeloma IX trial

5.3.5.2 Cut-point optimisation for association with survival

As with Myeloma IX, due to the debate within the myeloma community with regard to whether gain or amplification of chromosome 1q is prognostic, there has been a resultant non-uniform reporting of results. Some trials have reported gain and amplification of 1q in the context of treatment and others have reported amplification of 1q only. We therefore investigated the optimum cut point in terms of survival estimation as the most clinically relevant endpoint. While iFISH enabled examination of cells at the single-cell level in the Myeloma IX trial, MLPA could be used to consider the fraction of cells with gain where a ratio was given and average cell copy number within the tumour sample could be calculated.

A variety of methods was used to evaluate the optimal cut-point for survival estimation which are described in this section. For each probe, maximally ranked log-rank testing (shown in Figure 62) demonstrated variability in the maximally ranked statistic that ranged from 1.2 to 1.44. Unlike iFISH data, the maximally ranked statistics in MLPA-associated survival reached a plateau at around 1.15-1.2 and then fell at around 1.45 for each probe. Interpretation of these results is therefore more difficult than interpretation of iFISH data.

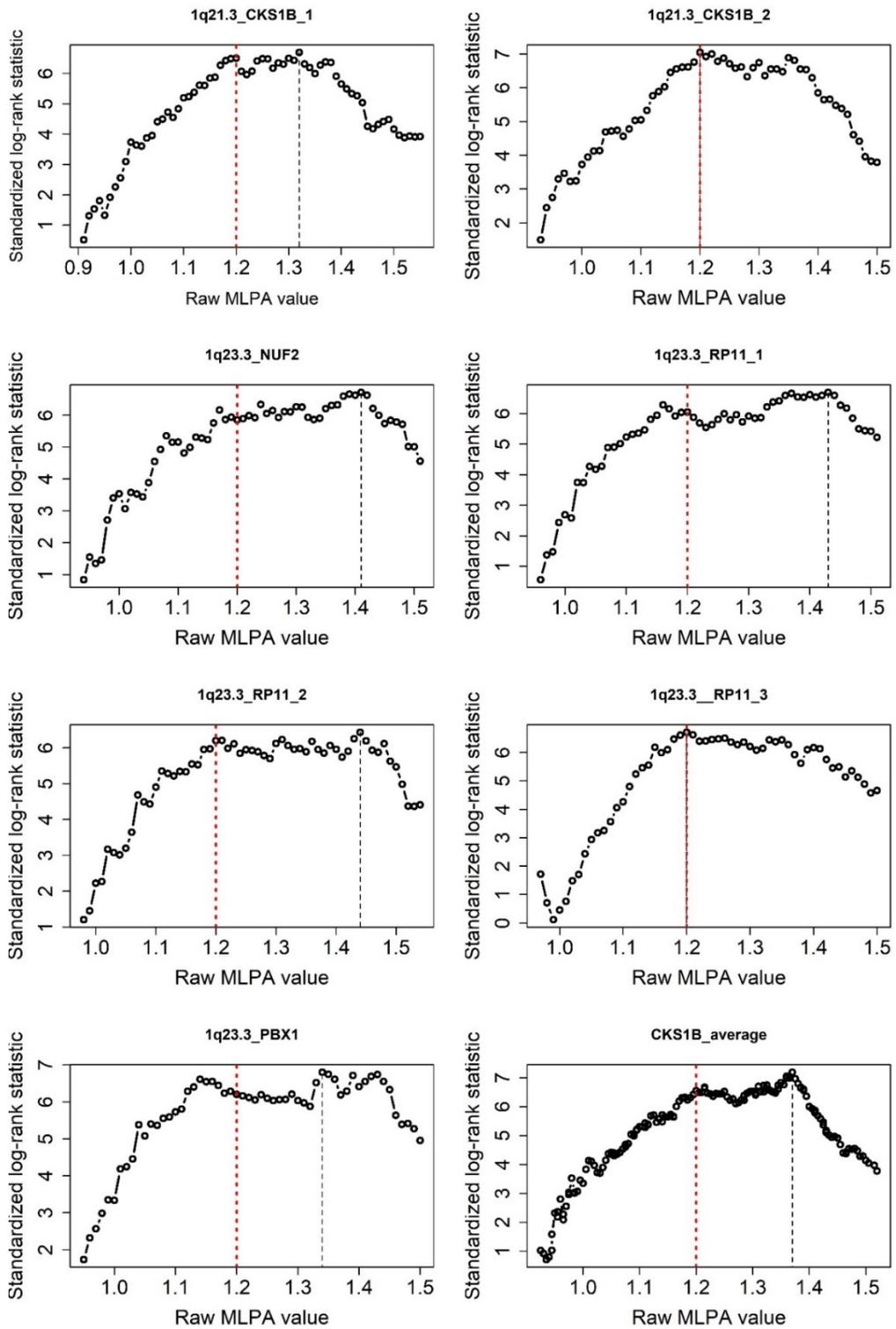


Figure 62: Maximally ranked statistics to define optimal cut-point for survival across seven probes along 1q21 as well as the average of the two CKS1B probes at 1q21. Dashed black line denotes highest maximally ranked statistic for each probe. Red dashed line is the current cut-point for calling gain at chromosome 1q by MLPA

AUCi statistics per probe, which were calculated for several cut points per probe, showed that, for five of seven probes, 1.2 was the optimal cut-point for overall survival (Figure 63). A plot of AUC over time demonstrated similar curves for the 1.2 and 1.25 cut-points (Figure 64). Minimal P methods with serial log-rank testing and Cox-based regression also found that the 1.2-1.3 cut point had the lowest *P*-value (data not shown). Multivariable Cox-based survival analysis with different cut-points at the 1q21 probe showed an independent prognostic association of cut-points from 1.2 onwards (Table 54). This data were also shown as Kaplan-Meier survival curves for different cut-points to detail this data graphically (Figure 65).

As the majority of tests for the evaluation of optimal cut-points for gain(1q21) agreed on 1.2 as the optimal cut point, we chose a cut point of 1.2 for calling gain 1q21 (the average value of the two 1q21 for further analyses). In addition, this figure was chosen for calling amplification as it appears almost exclusively to contain amplification 1q within this subgroup, as shown in Figure 61. As previously stated, there were subclonal amplification 1q samples under this cut-point, but these cannot not be separated using bulk DNA methods as we did for iFISH data.

Table 54: Cox-based multivariable regression analysis of different cut-offs for chromosome 1q21 and association of cut-offs with overall survival

Overall Survival	Multivariate Analysis	
	HR(95% CI)	Wald P
1q21.3 (CKS1B) probe cut-point 1.0-1.09	1.01 (0.81-1.26)	0.947
1q21.3 (CKS1B) probe cut-point 1.1-1.19	0.92 (0.68-1.26)	0.617
1q21.3 (CKS1B) probe cut-point 1.2-1.29	1.31 (1.01-1.71)	0.0427
1q21.3 (CKS1B) probe cut-point 1.3-1.39	1.59 (1.23-2.06)	0.00041
1q21.3 (CKS1B) probe cut-point 1.4-1.49	1.9 (1.43-2.52)	9.25E-06
1q21.3 (CKS1B) probe cut-point 1.5-1.59	1.94 (1.37-2.74)	0.000169
1q21.3 (CKS1B) probe cut-point >=1.6	1.69 (1.31-2.18)	5.13E-05

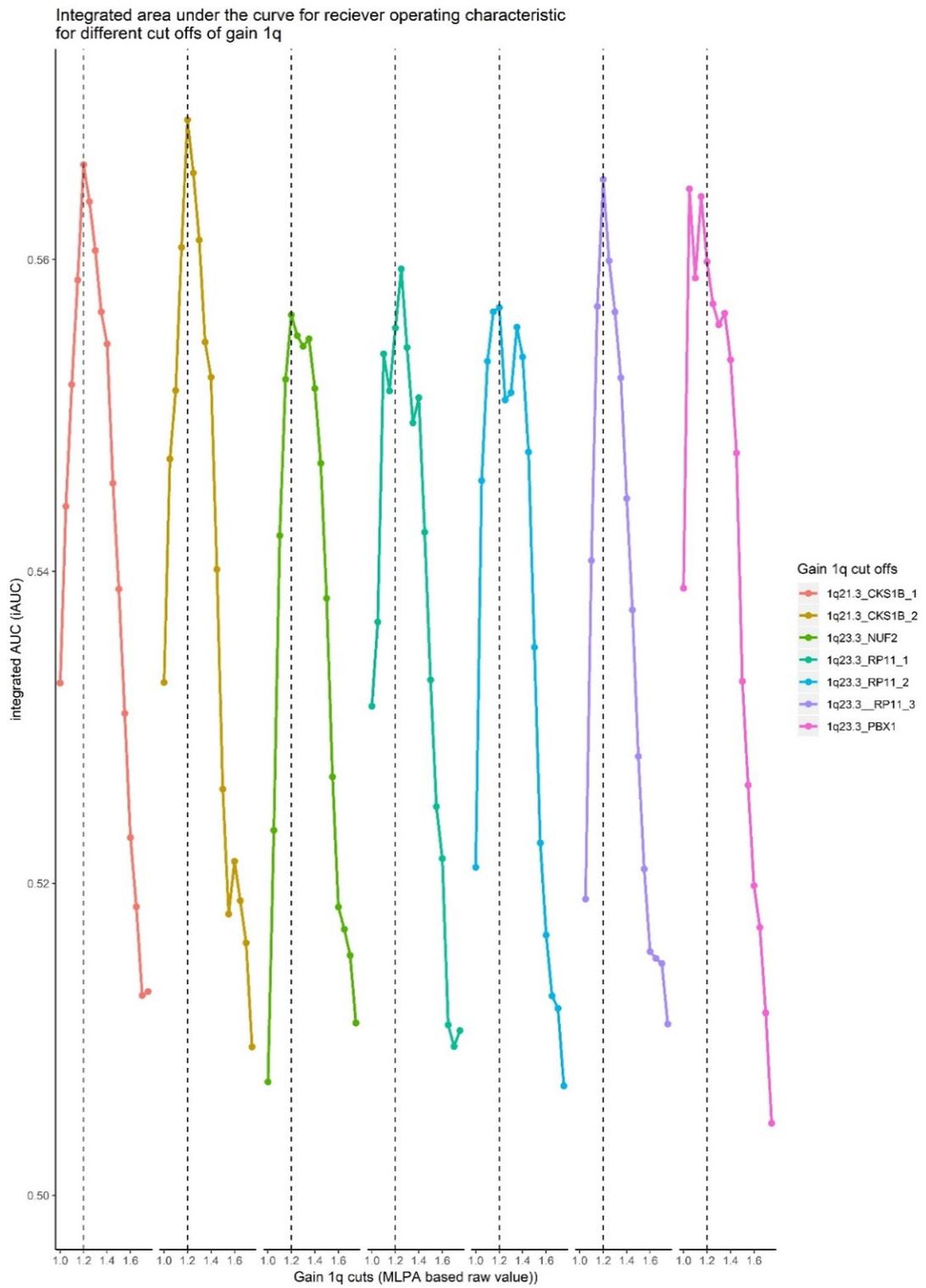


Figure 63: AUCi over different cut points for different probes along chromosome 1q

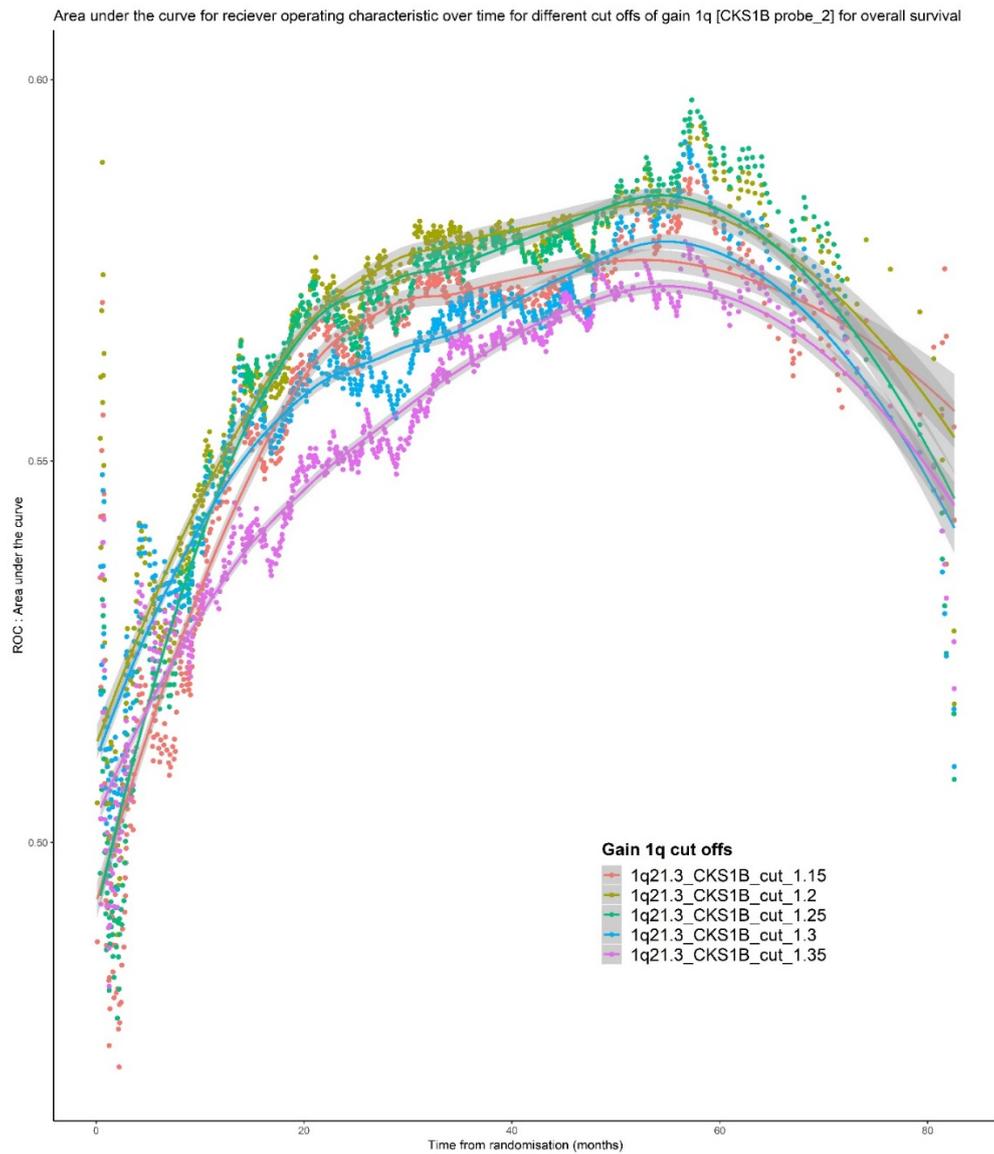


Figure 64: Area under the ROC curve plotted over time along different cut-points of the CKS1B probe with polynomial regression applied to each cut-point

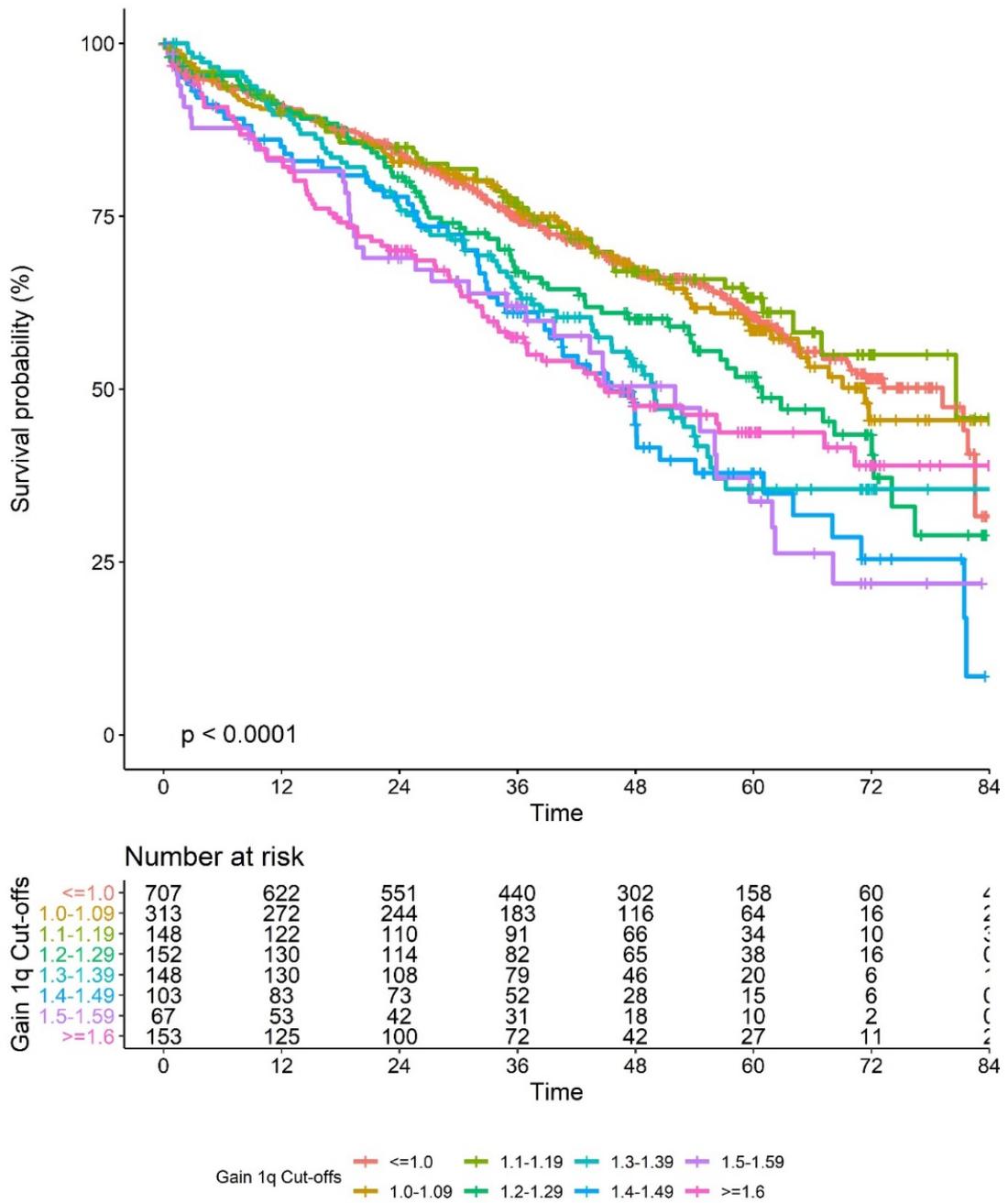


Figure 65: Kaplan-Meier survival curves for different cut points for chromosome 1q21

5.3.6 Comparison of survival rates with gain or amplification at chromosome 1q21

In the previous section, we showed that a demonstrating that a cut-point of 1.2 by MLPA relative ratio is prognostic in myeloma, thereby also suggesting that both gain(1q) as well as amp(1q) are prognostic in MM.

We went on to compare gain(1q21), and amp(1q21) in terms of survival association as this is an area of debate within the myeloma community using the 1.2 relative ratio as to define gain(1q) and 1.6 to define amp(1q). We demonstrate no survival difference ($P=0.35$) between the two cut-points (Figure 66) similar to our findings using iFISH in the Myeloma IX trial in the previous section. Since the comparison of iFISH with MLPA demonstrated that very few gain(1q) samples had an MLPA ratio >1.6 , we used this 1.6 ratio as our definition for calling amp(1q) by MLPA. We acknowledge that some tumours with subclonal amp(1q) will have an MLPA normalised ratio of ≤ 1.6 . We use this to further delineate amp(1q) and gain(1q) in terms of survival as well as clinical and genetic baseline associations in the next section.

We also show that both gain and amplification of 1q21 exhibit statistical independence in their association with overall survival with other genetic variables, through use of Cox-based multivariable analysis (Table 55). However, the independence of amplification 1q21 was lost when ISS and LDH scores were added into a multivariable analysis. The next section describes the demonstration in this study of a positive association with ISS and LDH that increases step-wise from gain(1q) to amp(1q21). It is therefore likely that this co-association explains why amplification does not show prognostic association when LDH and ISS are evaluated with other genetic lesions in a multivariable analysis.

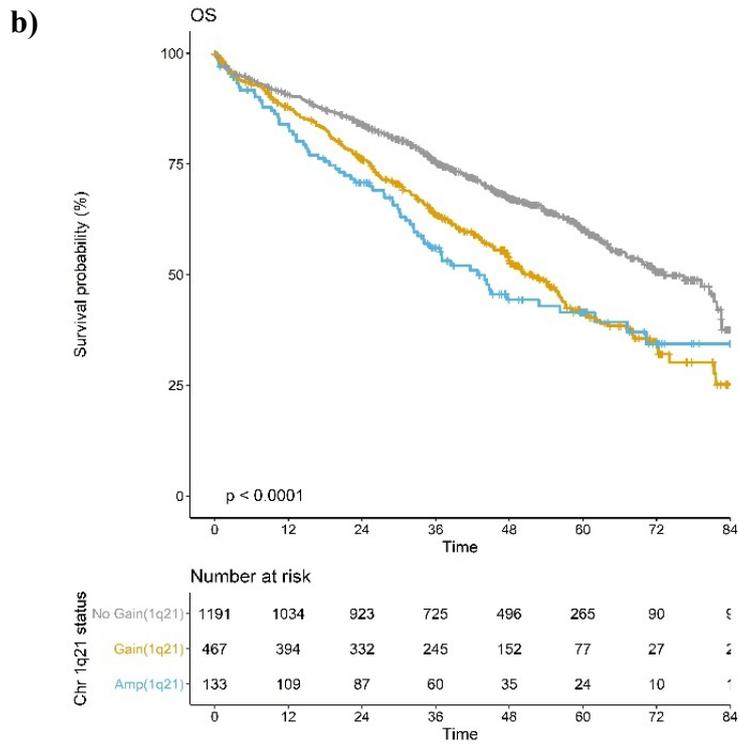
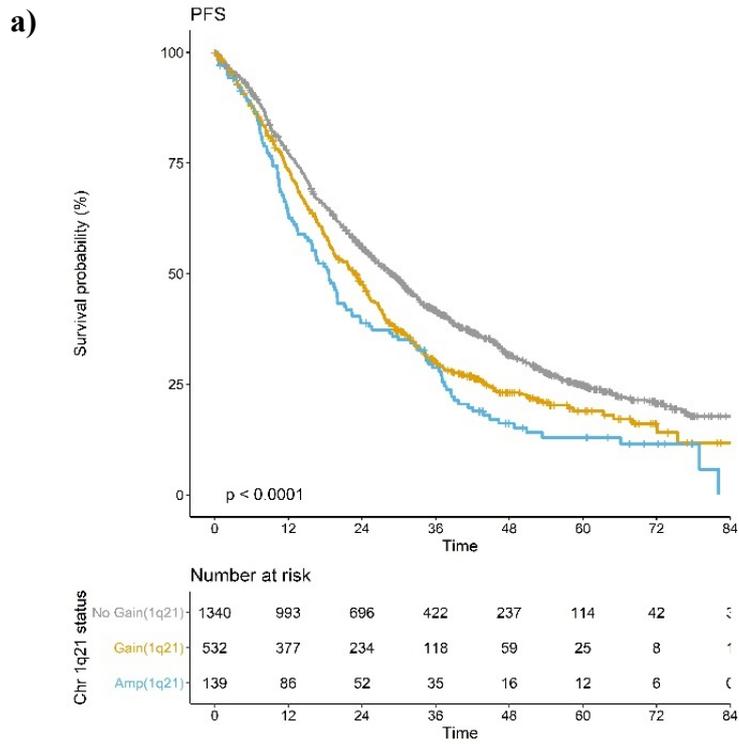


Figure 66: Kaplan-Meier curves that compare a) progression-free survival and b) overall survival in patients with diploid, gain and amplification 1q21

Table 55: Cox-based multivariable analysis of genetic and clinical factors at diagnosis

	OS		PFS	
	HR(95% CI)	Wald P	HR(95% CI)	Wald P
Gain 1q21	1.37 (1.16-1.62)	0.000256	1.17 (1.03-1.33)	0.0144
Amplification 1q21	1.5 (1.14-1.97)	0.0038	1.27 (1.03-1.57)	0.0254
t(4;14)	1.54 (1.22-1.95)	0.000314	1.65 (1.38-1.97)	3.55E-08
MAF translocations	1.73 (1.26-2.36)	0.000599	1.25 (0.95-1.64)	0.104
Deletion 1p32	1.56 (1.24-1.95)	0.000129	1.17 (0.97-1.4)	0.093
Deletion 17p	2.34 (1.87-2.91)	5.48E-14	1.53 (1.27-1.85)	8.18E-06
Hyperdiploid	0.84 (0.71-0.99)	0.0371	0.94 (0.83-1.06)	0.306
Age	1.05 (1.04-1.06)	2.20E-25	1.05 (1.04-1.05)	4.89E-46

	OS		PFS	
	HR(95% CI)	Wald P	HR(95% CI)	Wald P
Gain 1q21	1.21 (1-1.46)	0.0444	1.11 (0.97-1.28)	0.131
Amplification 1q21	1.27 (0.94-1.72)	0.114	1.13 (0.89-1.43)	0.301
t(4;14)	1.54 (1.18-1.99)	0.00123	1.63 (1.34-1.99)	8.62E-07
MAF translocations	1.51 (1.06-2.14)	0.0226	1.26 (0.93-1.71)	0.137
Deletion 1p32	1.57 (1.23-2)	0.000329	1.13 (0.93-1.38)	0.216
Deletion 17p	2.22 (1.74-2.83)	1.69E-10	1.51 (1.23-1.85)	6.72E-05
Hyperdiploid	0.77 (0.64-0.92)	0.004	0.91 (0.8-1.03)	0.135
Age	1.05 (1.04-1.06)	7.50E-21	1.05 (1.04-1.05)	4.56E-37
ISS staging	1.5 (1.34-1.68)	5.47E-12	1.31 (1.21-1.42)	1.42E-10
LDH (U/L)	1 (1-1)	4.99E-07	1 (1-1)	0.000196

5.3.7 Association of gain and amplification of 1q21 with other genetic abnormalities

As shown in Table 56 we compared genetic associations of gain(1q) and amp(1q) as it may point towards genetic dependencies within these subgroups. It was found that there was a positive association of amplification(1q) compared to gain 1q21 in tumours with high-risk translocations ($P<0.001$). Conversely there was a with a negative association in hyperdiploid and t(11;14) tumours and amp(1q) as well as gain(1q). These results were in line with data from the Myeloma IX trial analysed in the previous section of this chapter. In terms of hyperdiploidy, the most significant negative association was observed with gain of chromosome 5 ($P<0.001$). As studies have shown this to be the hyperdiploid chromosomal gain that offers the best prognosis, it is important to verify that this is not due to its negative association with chromosome 1q. We found no association with deletion 17p. Deletion 13q was the most significantly associated copy number abnormality that was associated with gain 1q and with an even higher incidence in amplification 1q21 (24% vs. 48% vs. 62%; $P<0.001$). There was a positive association of deletion 1p and gain 1q but frequency of this did not rise with the development of amplification 1q21 (6.6% vs. 15.6% vs. 11.5%; $P<0.001$). Mutational analysis of available whole exome data demonstrated an association between DIS3 mutations and increasing 1q21 copy number, possibly as a result of the co-association with deletion 13q. No other recurrent mutations had a statistically significant association with 1q21 gain.

Table 56: Genetic associations with gain and amplification of 1q21 as defined by MLPA at diagnosis

	Diploid 1q21 N=1340	Gain 1q21 N=532	Amplification 1q21 N=139	<i>P</i>
t(11;14) (%)	248 (21.0)	61 (12.6)	7 (5.6)	<0.001
t(4;14) (%)	93 (7.9)	79 (16.3)	40 (32.0)	<0.001
MAF translocation (%)	34 (2.9)	30 (6.2)	10 (8.0)	0.001
High-risk translocation (%)	127 (10.8)	109 (22.5)	50 (40.0)	<0.001
Hyperdiploidy (%)	652 (48.7)	229 (43.0)	46 (33.1)	0.001
Deletion 17p (%)	112 (8.4)	41 (7.7)	13 (9.4)	0.798
Deletion 1p32 (%)	88 (6.6)	83 (15.6)	16 (11.5)	<0.001
Gain 5 (%)	631 (47.1)	212 (39.8)	45 (32.4)	<0.001
Gain 9 (%)	630 (47.0)	242 (45.5)	48 (34.5)	0.019
Gain 15 (%)	669 (49.9)	226 (42.5)	52 (37.4)	0.001
Deletion 1p12 (%)	259 (19.3)	123 (23.1)	21 (15.1)	0.058
Deletion 12p (%)	92 (6.9)	30 (5.6)	11 (7.9)	0.513
Deletion 13q (%)	323 (24.1)	255 (47.9)	86 (61.9)	<0.001
Deletion 14q (%)	141 (10.5)	82 (15.4)	18 (12.9)	0.012
Deletion CYLD (%)	187 (14.0)	100 (18.8)	20 (14.4)	0.03
Deletion WWOX (%)	186 (13.9)	101 (19.0)	19 (13.7)	0.019

5.3.8 Association of gain and amplification of 1q21 with demographics and haematological and biochemical features at diagnosis

Next, we examined how blood and clinical parameters changed between this diploid, gain and amplification at 1q21. The results are shown in Table 57. There was no significant difference in patient ages or genders between groups. Patient performance status was also similar between groups.

Patients with gain 1q21 showed a significant decrease in haemoglobin concentration overall and there was a subsequent rise in the percentage of patients who presented with anaemia at diagnosis ($P<0.001$). Similarly, thrombocytopenia also increased in a stepwise fashion with increasing 1q21 copy number ($P<0.001$).

Recent reports have stated that only bi-allelic 17p deletion, amplification 1q gain and ISS III scores are significant in prognostication in MM (100). In light of this, it is important to state that this study found a significant stepwise increasing association between β 2M and 1q21 copy number (β 2M ≥ 5.5 ; 26% vs. 33% vs. 40%; $P<0.001$). Similarly, it was found that low albumin levels occurred with increasing 1q21 copy number (albumin ≤ 35 g/l, 32% vs. 35% vs. 48%; $P<0.001$). As a result, gain 1q21 was associated with a significant increase in ISS score. These findings lead to questions regarding the earlier study, as gain 1q may have been removed from statistical significance because ISS III was in the model, rather than because it was not a true prognostic marker. Further evaluation of this model is warranted so that genetic markers with true significant associations are not removed from analysis in the future.

Overall, patients also showed worse renal function with a lower estimated glomerular filtration rate (eGFR) ($P=0.001$), increased incidence of hypercalcaemia ($P<0.001$) and a higher LDH ($P=0.01$) at presentation with increasing 1q21 copy number. There was a lower incidence of bone pain and bone disease with increasing 1q21 copy number.

Table 57: Clinical, haematological and biochemical associations with gain 1q21 defined by MLPA at diagnosis

	Diploid 1q21 N=1340	Gain 1q21 N=532	Amplification 1q21 N=139	P
Age (mean (sd))	65.32 (10.05)	66.40 (10.16)	66.34 (8.90)	0.077
Male (%)	813 (60.7)	320 (60.2)	86 (61.9)	0.932
WHO PS ≥2 (%)	263 (20.4)	114 (22.5)	35 (26.5)	0.207
Anaemia (%)	621 (46.3)	278 (52.3)	98 (70.5)	<0.001
Thrombocytopenia (%)	119 (8.9)	77 (14.5)	41 (29.5)	<0.001
WCC x10⁹/l (mean (sd))	6.92 (4.67)	7.13 (5.60)	6.77 (4.17)	0.62
B2M (%)				<0.001
<3.5	574 (43.4)	185 (35.3)	42 (30.4)	
3.5-5.4	404 (30.5)	166 (31.7)	40 (29.0)	
≥5.5	346 (26.1)	173 (33.0)	56 (40.6)	
Albumin <35g/l (%)	430 (32.1)	190 (35.8)	68 (48.9)	<0.001
CRP (median [IQR])	3.50 [1.30, 10.60]	4.00 [1.22, 11.00]	3.30 [1.00, 9.30]	0.396
eGFR (mean (sd))	73.57 (26.55)	70.45 (28.52)	65.01 (25.44)	0.001
LDH U/L (mean (sd))	302.14 (183.15)	301.81 (215.06)	358.48 (235.91)	0.011
LDH >300 U/l (%)	475 (42.2)	177 (41.1)	58 (50.0)	0.215
Calcium >2.6 mmol/l(%)	198 (14.8)	120 (22.6)	33 (23.9)	<0.001
ISS (%)				<0.001
1	453 (34.2)	142 (27.1)	26 (18.8)	
2	525 (39.7)	209 (39.9)	56 (40.6)	
3	346 (26.1)	173 (33.0)	56 (40.6)	
Bone pain (%)	332 (73.8)	110 (59.8)	21 (61.8)	0.002
Bone disease (%)	985 (80.3)	352 (75.4)	88 (69.3)	0.004

5.3.9 Gain 1q21 and survival in the context of other high-risk chromosomal aberrations

This work has demonstrated independence of gain(1q) in the context of other high-risk genetic markers as shown in Table 55. However, given the strong association that was observed with gain(1q), amplification(1q) and other high-risk genetic lesions such as t(4;14) and MAF translocations in the previous section, we explored survival further in the context of high-risk lesions. This was particularly pertinent as our and other groups have observed that ‘double-hit’ (the presence of ≥ 1 high-risk cytogenetic lesion) is associated with a particularly poor survival. Hence, we went on to analyse the inter-relationship of these lesions in the context of survival.

We show that the combination of other high-risk lesions with gain(1q21) is associated with shorter overall survival rates and that the combination of three lesions is associated with the worst prognosis. Although a combination of ≥ 1 high-risk cytogenetic risk factor is associated with a further increase in the risk of early death, we show that even outside the co-association of these risk factors, gain(1q) is still prognostic (Table 58a, Figure 67). We also demonstrate that there is no statistical evidence of interaction between these high-risk genetic features (Table 58b).

Table 58: Cox-based multivariable analysis of overall survival of combined high-risk cytogenetic risk lesions: a) combination of genetic lesions with gain(1q); and b) individual high-risk genetic lesions with interaction terms

a)	HR(95% CI)	Wald P
Gain(1q)	1.38 (1.16-1.65)	0.000337
Gain(1q) & high-risk translocation	2.03 (1.59-2.59)	1.06E-08
Gain(1q) & del(17p)	3.12 (2.07-4.71)	6.26E-08
Gain(1q) & del(17p) & high-risk translocation	6.06 (3.61-10.17)	8.67E-12

b)	HR(95% CI)	Wald P
Gain (1q)	1.52 (1.26-1.83)	9.42E-06
Del(17p)	2.19 (1.61-2.97)	4.74E-07
High-risk translocation	1.46 (1.08-1.99)	0.0155
Interaction of gain(1q):del(17p)	1.09 (0.69-1.71)	0.716
Interaction: gain(1q):high-risk translocation	1.03 (0.7-1.51)	0.885
Interaction: del(17p):high-risk translocation	1.15 (0.69-1.91)	0.594

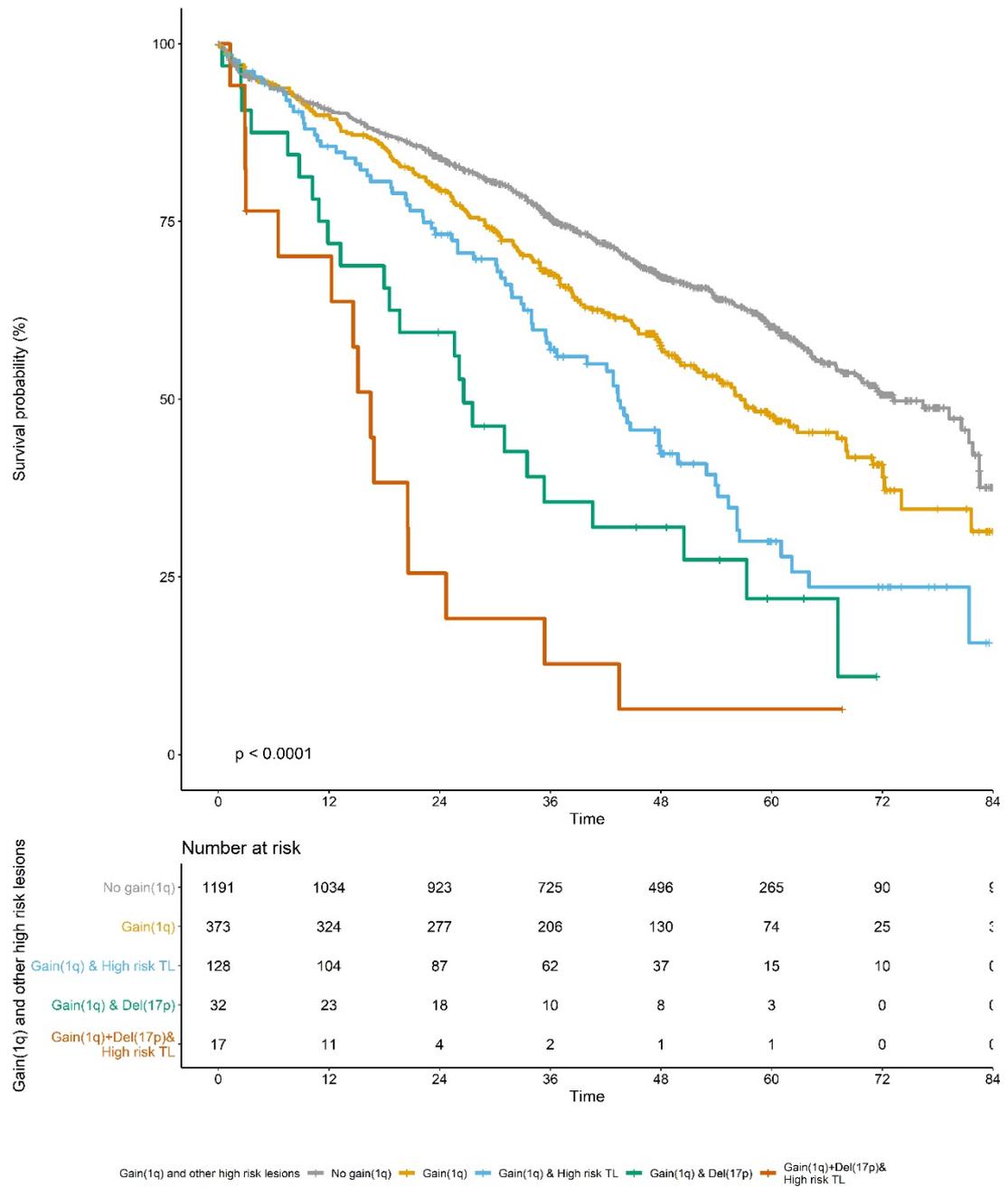


Figure 67: Kaplan-Meier curves demonstrating overall survival of gain(1q) in the context of other high-risk cytogenetic lesions

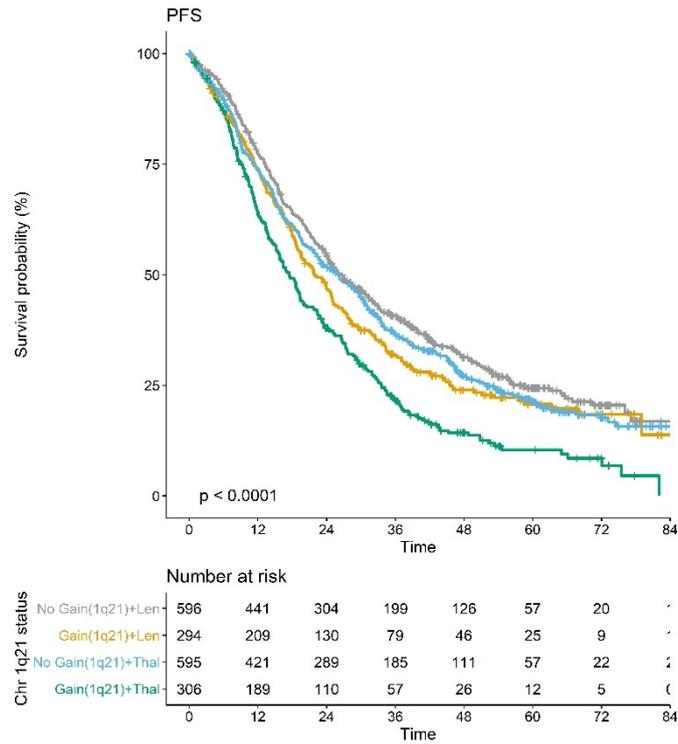
5.3.10 Survival in the context of therapy and gain 1q21

Our analysis shows the strongest association of survival with gain 1q21 with a cut-off of 1.2 by use of MLPA. Therefore, we used this cut-off for the evaluation of therapies that were used in this trial. Figure 68 shows Kaplan-Meier survival curves for the initial induction randomisation between lenalidomide-based (CRD) and thalidomide-based (CTD) induction. For both induction randomisations, gain 1q21 was associated with a shortened overall survival period. Patients who were randomised to thalidomide treatment at induction had a median OS of 44.9 months vs. 55.3 months for patients who were randomised to lenalidomide ($P=0.008$).

In contrast, patients with gain 1q21 who achieved a suboptimal response to induction (PR or MR) and who were randomised to Velcade, cyclophosphamide and dexamethasone (VCD) intensification vs. no intensification (Figure 69) had no difference in overall survival ($P=0.39$).

Maintenance lenalidomide randomisation vs. no maintenance appeared to show benefit in terms of progression-free survival with a median PFS of 34.3 months vs. 25 months ($P=0.003$). However, this treatment did not result in any benefit in overall survival for patients with gain 1q who were randomised to no maintenance therapy ($P=0.8$) (Figure 70).

a)



b)

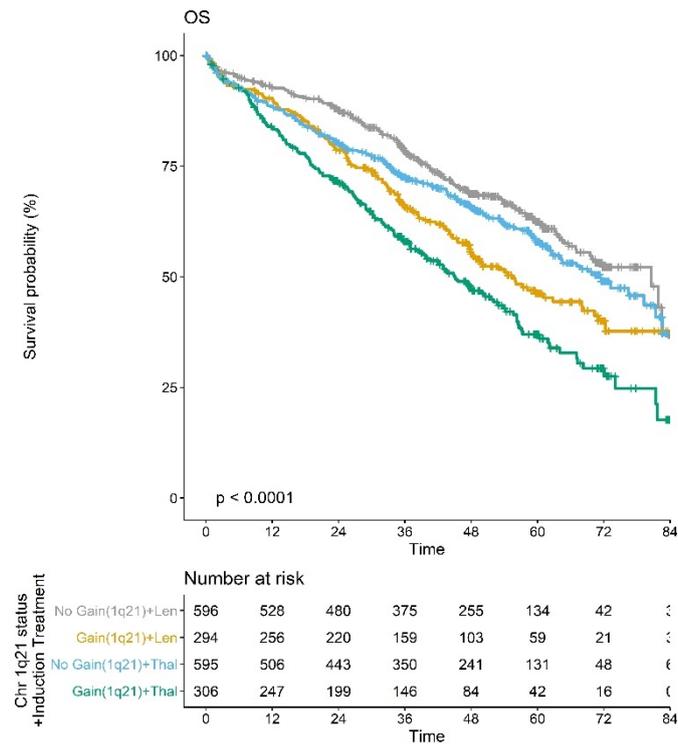
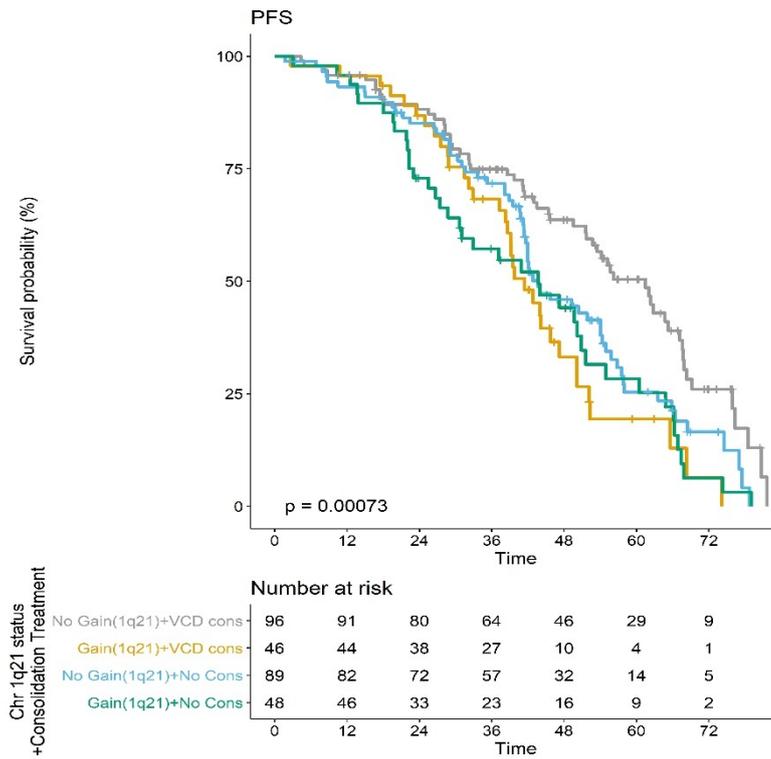


Figure 68: Kaplan-Meier survival curves of a) progression-free survival and b) overall survival of patients treated with either lenalidomide- or thalidomide-based induction treatment in the context of gain 1q21 copy number status

a)



b)

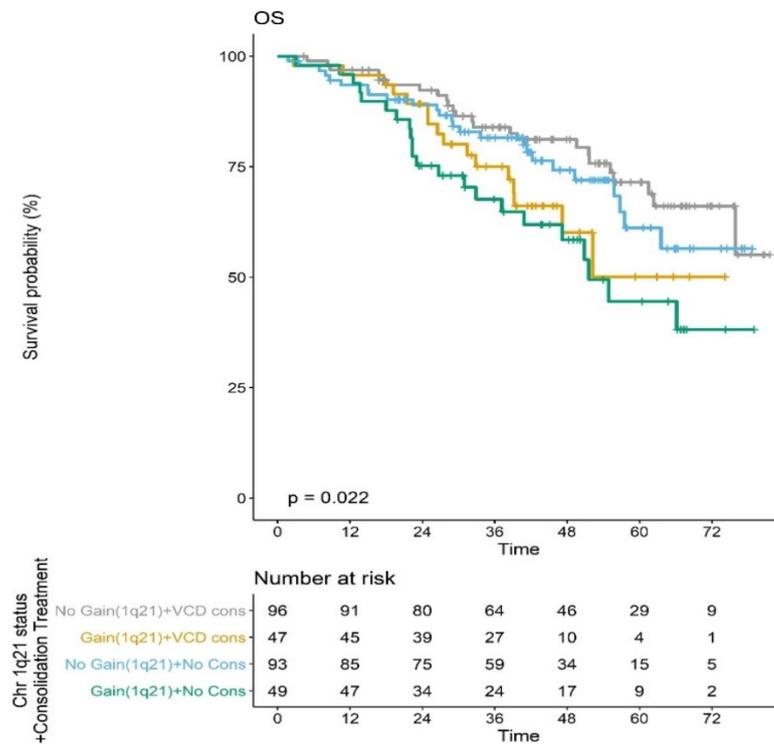
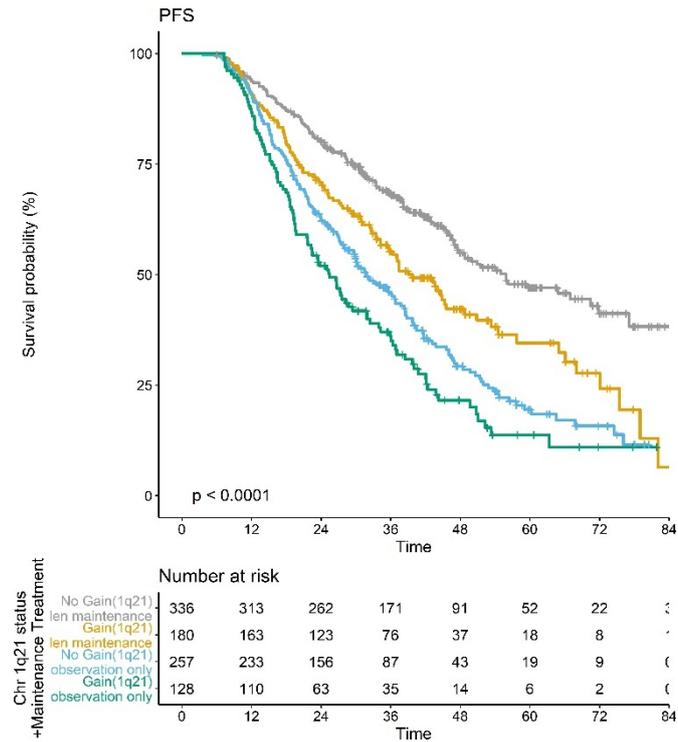


Figure 69: Kaplan-Meier survival curves, landmarked from consolidation randomisation, of a) progression-free survival and b) overall survival of patients who achieved only PR or MR response and who were randomised to VCD intensification or no intensification in the context of gain 1q21 copy number status

a)



b)

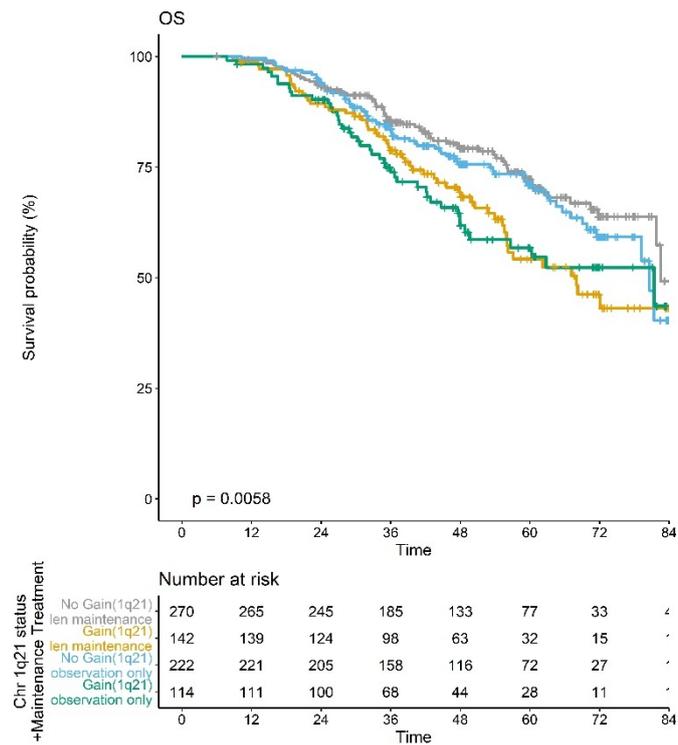


Figure 70: Kaplan-Meier survival curves, landmarked from maintenance randomisation, showing a) progression-free survival and b) overall survival of patients randomised to lenalidomide maintenance or no maintenance in the context of gain 1q21 copy number status

5.4 Discussion

We have investigated copy number of chromosome 1q through different methods including single-cell-based methods (iFISH) and bulk-DNA-based methods (MLPA) to investigate whether gain or amplification 1q has prognostic association. Through our analysis of 2,859 patients within the Myeloma IX and Myeloma XI trials, we highlight the limitations of each method regarding reliability of iFISH analysis in terms of clonal percentage calling and loss of information regarding clonal architecture through bulk DNA methods that are employed by many laboratories at present.

Through analysis of the iFISH data in Myeloma IX, we demonstrate that subclonal amplification at 1q is frequently present in tumours with a predominantly gain 1q phenotype. Additionally, amplification 1q can be clonal or subclonal within tumours without gain 1q. This suggests that amplification events can evolve in isolation or as progressive events after an initial gain (3 copies) at 1q, as has been shown by another group (364, 365). The mechanism by which this occurs is thought to be secondary to whole-arm translocations, partial duplications or jumping translocations of chromosome 1q (364). Pericentromeric instability and jumping translocations are thought to lead to progressive copy number changes through the tumour genome, and to tumour progression (365). This is also suggested by the finding that chromosome 1q has been found to be the site of progressive structural change in longitudinal studies with paired analyses in MM (366).

The analysis that was performed as part of the current study demonstrates that the association with survival of these subclones is valid at low-gain percentages (around 10-20%) although due to limitations in the number of samples that were used in this analysis at the lower subclonal tumour samples, validation of these findings would be important. Additionally, studies of matched samples would be helpful to study clonal dynamics with treatment pressures.

By use of both trials and analyses in the context of therapy, we show that gain 1q21 is associated with poor prognosis and is independent of amplification 1q. Both lesions are associated with shorter overall survival rates independent of other genetic high-

risk lesions. Although this study does not combine IMiD and proteasome inhibitor therapy upfront, the findings from this study are in line with those of another study that included patients with IMiD and PI therapy upfront (287), although the data from this latter study were limited to progression-free survival. The same study also examined results from the CoMMpass study, which based copy number on whole genome sequencing. This examination demonstrated poorer overall survival among patients with both gain and amplification of chromosome 1q21. Another iFISH-based dataset confirms the significance of both gain(1q) and amp(1q) with overall survival in the context of novel therapies (367).

Our study adds to the findings of the above studies through the evaluation of the evolution of this lesion by examining gain and amplification of chromosome 1q21 separately in terms of genetic and clinical characteristics. It shows progressive changes in terms of association with high-risk primary genetic lesions as well as other secondary genetic abnormalities. We also demonstrate that certain clinical markers show progressively increased association with this genetic abnormality. Examples of these clinical markers are increased levels of LDH, lower albumin and higher β 2M levels, all of which are used as prognostic markers in MM. These findings suggest that these clinical markers may be surrogates for genetic baseline tumour genetic abnormalities and are not independent.

Our analysis of therapies that were used in the Myeloma XI trial found that lenalidomide-based induction was associated with an improved overall survival rate compared with thalidomide in the context of gain 1q lesions. As this is a sub-analysis of the study, this result requires validation by other trials. The reasons behind improvement in overall survival with lenalidomide-based induction over thalidomide-based treatment require elucidation with further clinical data from the trial.

In the Myeloma IX trial, thalidomide maintenance was associated with a poorer survival in patients with gain 1q. Although lenalidomide maintenance did not result in an OS benefit, it did not appear to be associated with worse OS. The reason behind the lack of OS benefit in this group despite improved PFS would need further investigation to discover whether this result is related to toxicity of continued therapy or due to

application of selective pressure in MM and the development of more resistant clones to future therapy.

Velcade-based intensification in poor responders did not appear to improve OS rates in patients with gain 1q. This result was in line with those of other studies, which have suggested that gain 1q confers resistance to proteasome inhibitor therapy (90). However, as this randomisation was limited to a small number of patients who showed poor response to IMiD-based therapy, it is not conclusive. We may be able to clarify this further when overall survival results of the carfilzomib, Revlimid, cyclophosphamide and dexamethasone arm at induction are released.

Overall, in the context of therapies that were trialled in both Myeloma IX and Myeloma XI, none of the above therapies was associated with abrogation of risk that was associated with gain 1q. This finding was consistent with several other trials.

In conclusion, our study has added granularity to the data regarding survival in the context of additional copies of chromosome 1q. We demonstrate the effects of clonality and increasing copy number through use of different methods and have enabled the advantages of each method to be utilised for the study of this lesion.

6 Gene expression profiling in Myeloma

6.1 Introduction

Biological markers such as genetic and gene expression profiling (GEP) markers are increasingly seen to have predictive potential in precision medicine (147, 368, 369), in the same way that clinical classifiers such as the ISS do, we have described chromosomal aberrations that are associated with poor survival extensively in the introductory chapters. The focus of this chapter is the commercially available SKY92 GEP signature.

The introductory chapter described the use of unsupervised cluster-based methods to identify subsets of myeloma after the development of gene expression arrays technology. While these approaches were useful to characterise tumours biologically in several cancers, it was found that underlying characteristics of the tumour such as specific translocations, differentiation and the presence of oncogenes had the most impact on classification and therefore unsupervised clustering was less useful as a tool for prognosis (370). This was also the case in MM, as has been described in the introduction chapter. However, some subsets do show correlation with survival, including the subgroups that were based on gene expression: the proliferation (PR) group, the MS group (over-represented by $t(4;14)$) and the MF group (over-represented by MAF translocations) (48, 56, 152).

Therefore, another method was developed for prognostication that employed supervised approaches to identify biological phenomena that were associated with poor prognosis based on a hypothesis. Such approaches have been used to prognosticate survival in several cancers including MM by identification of cell survival and proliferation signatures (371, 372) and chromosomal integrity (373, 374), but these rely on the hypotheses for such signatures being accurate surrogate endpoints for the clinical endpoint of interest.

A further approach to the prediction of response or survival has been the development of a predictive signature in terms of response to specific therapy. There are difficulties with this approach, especially in the context of MM. As explained in the introduction

chapter, treatment of MM involves the use of multiple different therapeutic agents at different stages. The treatment of MM is also rapidly advancing with development of new drugs. Another challenge with this approach is the choice of endpoint with regard to making a predictive signature. The immediate response to treatment through paraprotein/light-chain measurement appears to be a poor predictive factor as demonstrated by others and work performed for this study such as that with *TP53* and gain(1q). Although both *TP53* and gain(1q) showed no statistically different results with regard to treatment at induction, they were independently related to risk. Finally, through the knowledge gained from all the signatures that have been developed in cancer, it appears that prognosis is associated with underlying tumour characteristics, which seems to be a stronger predictor of overall survival than specific response to treatment. Failure to characterise these underlying characteristics while solely focusing on predictive factors would therefore limit their usefulness. Having said this, there are some predictive signatures that have been developed in MM for response to bortezomib and IMiDs to date and their validation within studies will be useful in the future (375, 376).

The final, most commonly used and validated approach in MM is the so-called ‘bottom-up’ approach. This uses endpoints such as the occurrence of a studied event studied, e.g. metastasis or survival, to create expression signatures. The first such signatures were developed for cancers in the early 2000s (377, 378). In MM, several of the approaches that have been described above have been utilised and have resulted in the creation of several risk signatures (272, 277, 347, 372, 379-386).

Major gene-expression signatures that have been developed in MM include the UAMS-GEP70, the UAMS-80, the MRC-MMIX-based signature, the IFM-15 signature, the millennium signature that was based on relapsed patients, and the proliferation index-based signature. Other signatures have been based on cell lines (277) or on cytogenetic abnormalities (275). There is little overlap in the probes or genes that are identified through use of these signatures. This is likely to be due to the different methodologies and treatments that are used in the signatures, as well as statistical issues with high-dimensional data evaluation (387). Commonly used signatures are summarised in Table 59.

Table 59: Gene-expression signature characteristics and overlap of involved genes

Gene expression survival-based risk signature	Number of genes	Number of genes in common with UAMS GEP-70 score	Number of genes in common with EMC92 score	Ref
UAMS GEP 70	70 genes	--	2 genes	(347)
EMC92	92 genes	2 genes	--	(272)
IFM 15	15 genes	0 genes	1 gene (FAM49A)	(383)
Chromosome instability signature	214 genes	7 genes	15 genes	(374)
Centrosome index signature CNTI	4 genes	0 genes	0 genes	(388)
Cell death signature implicated by homozygous deletion (HMDCD)	6 genes	0 genes	0 genes	(275)
Human myeloma cell lines (HMCL)	7 genes	0 genes	0 genes	(277)
Proliferation signature	50 genes	3 genes (BIRC5, ASPM, CKS1B)	6 genes (ESPL1, MCM6, NCAPG, SPAG5, ZWINT, BIRC5)	(372)

As detailed above, although multiple GEP risk signatures have been developed for MM, only the EMC92 and UAMS GEP70 signatures have been developed within the commercial space and are available as diagnostic tests. The diagnostic tests are known as SKY92 MMprofiler and MyPRS, respectively, and have been used in clinical settings (272, 275-277, 375, 389, 390). In the work that was performed for this thesis, we focused on the two main, commercially available prognostic scores, as these could be applied in the diagnostic setting. The development of these two signatures is described in the next section.

The UAMS-70 signature was one of the first gene expression-based signatures that was developed in MM. It was created through application of a training set of 351 gene-expression arrays with normal, MGUS and symptomatic NDMM as well as relapsed patients' plasma cells. Patients who needed treatment samples came from the TT2

(thalidomide-based vs. no thalidomide) and TT3 trials (Velcade and thalidomide-based). Treatments included induction followed by autologous transplantation and maintenance treatment. The gene expression microarray platform that was used to develop this signature employed the Affymetrix U133 plus2 (347).

Risk prediction was performed by log₂ conversion and MAS5 normalisation of Cel file data. Univariate log-rank tests for event-free survival (EFS) and OS were performed using four quartiles of each gene expression. A false discovery rate of 2.5% was found (e.g. Q1 vs. Q2, Q3, Q4, Q2 vs. Q3, Q4, Q3 vs. Q4). The process yielded 19 under-expressed and 51 over-expressed probe sets. A step-wise multiple linear discriminant analysis was used to select 70 probes that were then used in the signature. To simplify this signature into dichotomous variables, k-means clustering was applied to divide gene expression of these 70 genes into three groups. The cut-off setting that was chosen for the highest-risk group was 0.66 (347).

The EMC92 signature was first developed by use of the HOVON65/GMMG-HD4 data as a training set. This multicentre trial compared the efficacy of bortezomib (PAD) to standard (chemotherapy-based) treatment (VAD) in newly diagnosed patients. Patients were randomised to induction treatment with three VAD or PAD cycles. For a total of 290 patients, both follow-up and Affymetrix U133 plus2 gene expression microarrays were available. In this study, PFS was used for the supervised analysis. Probes were filtered using univariate Cox-based regression with a false discovery rate (FDR) of <10% for probe sets and survival data. This set was put into a supervised principal component analysis framework in combination with simulated annealing. The analysis resulted in a 92 probe-set signature, termed the EMC-92 signature. Although this signature was initially issued as a continuous risk score, the strong desire for a “high” or “low” discrete risk score resulted in high-risk disease being defined as the proportion of patients with an overall survival of less than two years in the training set with a cut-off of 0.827 (272).

A large number of genes within the 70-gene UAMS-GEP70 signature show high expression in chromosome 1q and low expression in chromosome 1p. Clinical and cytogenetic correlation with a high GEP70 gene expression score reveal correlations with higher levels of β 2M, CRP, creatinine, LDH and del(13q). There were also a

higher proportion of MMSET/FGFR3 subgroups in high-risk cohorts that reflected the correlation of the GEP70 signature with known high-risk cytogenetic markers (347). Similarly, during the evaluation of the EMC92 signature, del(17p), albumin or β 2M levels were used as independent predictors of survival outside gene expression in different studies. However, a lack of data led to an inability to perform comprehensive analysis of other known risk predictors that have been described previously in this thesis (272). There was also correlation between the MS, MF and PR gene-expression subsets and the EMC92 ‘high risk’ classification.

As described in the previous chapters of this thesis, markers that are associated with risk such as translocations in tumours that are associated with poor outcome – adverse translocations (t(4;14), t(14;16), t(14;20)), CNAs gain(1q) and del(17p) as well as the co-occurrence of these lesions, termed double-hit and which typify especially aggressive disease (391-395) – have not been evaluated comprehensively in the context of high gene expression risk. This is also the case for clinical risk markers such as the ISS score and LDH levels due to limitations in the comprehensiveness of the annotation of databases. It is therefore important to evaluate comprehensively the value of this risk predictor in the context of known lesions to evaluate whether it adds any prognostic value. This is especially important outside the research setting as this test is resource intensive and expensive.

Validation of gene expression signatures is important for scientific and statistical reasons. Many signatures cannot be validated as they are created ‘in-silico’ and bioinformatics modelling can result in high false-positive development of risk signatures in the absence of real biological differences and/or with little clinical significance. Studies based on small sample sizes can produce an FDR so high that predictive signatures may have no validity in the real world. This is demonstrated by researchers who have validated 47 published breast-cancer datasets that show that 1,000 randomly generated signatures in each dataset are almost always statistically significant and sometimes more strongly so than the original published signature (396). Uptake of gene-expression signatures into routine clinical practice has been limited due to this limitation of evidence in GEP-based survival prediction. Validation of gene-expression signatures is therefore important if they are to reach the clinic as a routine diagnostic procedure.

In order to validate the SKY92 risk signature, we profiled 329 NDMM patients from the UK NCRI Myeloma XI trial. We also explored its independence in the context of known genetic and clinical risk markers through this comprehensively annotated dataset. We used the continuous marker on which the SKY92 dichotomous risk signature is based, EMC92 to perform further exploratory analyses in the context of other risk signatures and genetic and clinical risk markers to explore further the underlying basis of this gene signature.

6.2 Aims

- To validate the commonly used gene-expression risk signatures in the Myeloma XI dataset;
- To explore correlation between risk signatures in the Myeloma XI dataset;
- To confirm independence of survival association in the context of known genetic and clinical risk markers;
- To explore EMC92 continuous score and extreme copy numbers as well as clinical risk markers.

6.3 Results

We studied 329 NDMM patients who took part in the Myeloma XI trial and who were placed on the transplant-eligible treatment pathway. Patients were selected by the Clinical Trials Research Unit (CTRU) at the University of Leeds based on age, sex, induction treatment and maintenance treatment to reflect the overall intensively treated trial population. Baseline characteristics of these 329 patients were checked against the overall intensively treated trial population by Leeds CTRU (Table 60). This was to confirm that they were reflective of the remaining 1,713 intensively treated population to ensure that confounders with regard to sample availability due to RNA quality would not cause bias in our results and that these results would be generalisable.

The baseline table demonstrates that there were no major differences in terms of baseline values in any parameter. This result suggests that the study is likely to be reflective of the overall intensively treated population.

Table 60: Baseline characteristics of representative transplant-eligible patients included in this analysis (left column), those not included (middle column) and all transplant-eligible trial patients (right column) from NCRI Myeloma XI trial

	Current study (n=329)	Others (n=1713)	Total (n=2042)
Male gender (%)	197 (59.9%)	1024 (59.8%)	1221 (59.8%)
Age at randomisation >65 years	81 (24.6%)	435 (25.4%)	516 (25.3%)
WHO performance status			
0	135 (41.0%)	729 (42.6%)	864 (42.3%)
1	124 (37.7%)	608 (35.5%)	732 (35.8%)
2	38 (11.6%)	219 (12.8%)	257 (12.6%)
3	16 (4.9%)	62 (3.6%)	78 (3.8%)
4	3 (0.9%)	7 (0.4%)	10 (0.5%)
Mean haemoglobin (g/l) (SD)	106.6 (19.01)	110.0 (20.41)	109.5 (20.23)
Platelets (*10/l) mean (SD)	248.3 (98.90)	252.4 (101.78)	251.7 (101.30)
β 2 microglobulin (mg/l) median (range)	3.8 (1.5, 41.0)	3.6 (0.6, 81.2)	3.6 (0.6, 81.2)
Calcium (mmol/l) median (range)	2.4 (1.8, 3.8)	2.4 (1.3, 4.6)	2.4 (1.3, 4.6)
Serum creatinine (μ mol/l)			
Mean (SD)	102.8 (58.97)	99.1 (62.45)	99.7 (61.91)
Median (range)	87.0 (37.0, 405.0)	83.0 (28.0, 897.0)	84.0 (28.0, 897.0)
Paraprotein type			
IgG	196 (59.6%)	1042 (60.8%)	1238 (60.6%)
IgA	83 (25.2%)	409 (23.9%)	492 (24.1%)
Light-chain type			
Lambda	97 (29.5%)	587 (34.3%)	684 (33.5%)
Kappa	231 (70.2%)	1106 (64.6%)	1337 (65.5%)
Serum albumin (g/l) mean (SD)	34.6 (7.13)	35.6 (6.88)	35.4 (6.93)
Lactate dehydrogenase (IU/l) median (range)	264.0 (<lower detection limit, 1042.0)	268.0 (<lower detection limit, 3550.0)	267.0 (<lower detection limit, 3550.0)
C-Reactive protein (mg/l) \geq 5	168 (51.1%)	880 (51.4%)	1048 (51.3%)

6.3.1 Correlation between risk-expression signatures

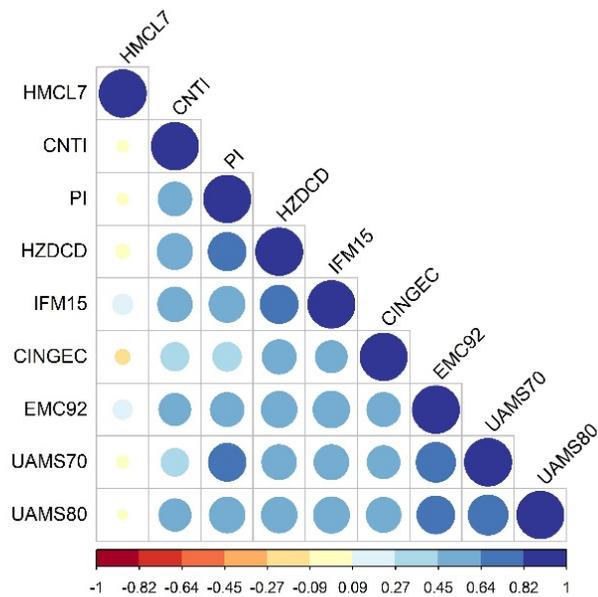
In this section we describe the evaluation of continuous risk scores, although there is a drive towards dichotomising risk to simplified ‘high’ vs. ‘standard’ risk in the setting of diagnostics to aid in the take-up of such diagnostic tests in clinical practice. Although commonplace in medicine, categorisation of continuous variables comes at the cost of loss of valuable information (397). It also increases the risk that positive results may be false (398) and leads to a decrease in the extent of variation in outcomes between groups. For research, it limits data exploration in terms of inter-relationships between variables as well as the variable and outcome. For research purposes, we use the continuous score for data exploration through application of published algorithms (399).

By using continuous scores, we found that the quantitative scores of most diagnostic signatures were highly correlated; in particular, the EMC92 and GEP70 signatures demonstrated the strongest and most statistically significant linear correlations ($r=0.79$; $P<0.001$) (Figure 71). The tight correlation between these two signatures despite the lack of significant correlation in the probes or genes that are used within the two signatures reflects the high likelihood that the same pathways are selected within the two signatures. However, as discussed in the introduction, the nature of probe-selection expression arrays does not necessarily enable directly targetable genes to be identified in this manner. The human myeloma cell lines (HMCL) signature, which is based on cell lines rather than patient samples, was not significantly correlated with most signatures. This finding is in line with the non-reflection of NDMM by HMCL. This has relevance as most drugs are first tested in vitro on signatures such as these. The lack of biological similarity may explain why many drugs that are tested in vitro fail in the clinical setting during trials.

The high correlation between EMC92 and GEP70 reflects the fact that on the whole the gene expression signatures find strong correlations in terms of underlying aggressive disease and resistance to IMiD, proteasome inhibitors, steroids and conventional chemotherapy, rather than treatment-specific resistance or sensitivity. This is also reflected by the fact that EMC92 was significantly correlated with the experimentally derived proliferation index ($r=0.64$, $P<0.001$), as were most other risk

signatures (Figure 71). The correlation also explains why many of these expression signatures are not independent in their risk association in multivariable analyses as shown in Table 61.

a)



b)

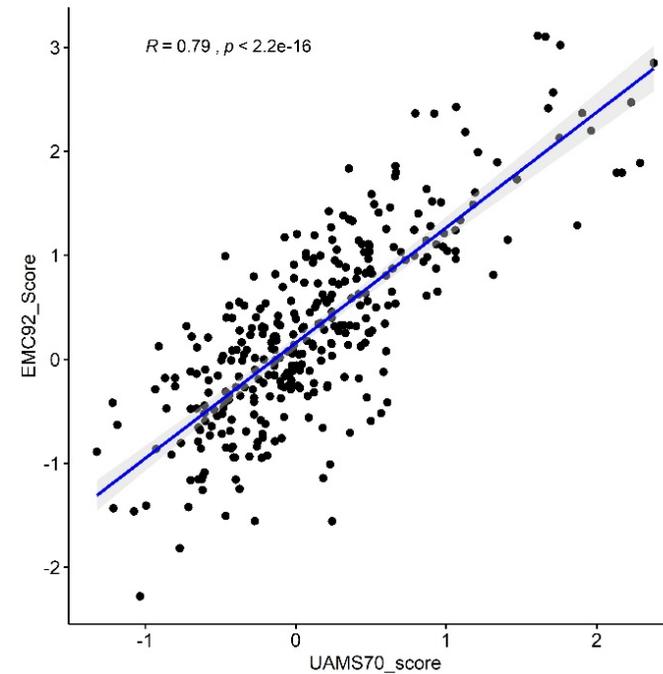


Figure 71: a) Correlation plot of quantitative scores for published risk and/or biological GEP signatures. Colour coding correlation coefficient. HMCL7: 7-gene prognostic signature from MM cell line study; CIGNECS: chromosome instability signature; CNTI: centrosome index; HZDCD: homozygous cell death signature (MIX signature); IFM-15: prognostic signature by IFM myeloma 99 clinical trial; PI: proliferation signature; UAMS70 and UAMS80: 70- and 80-gene signature by UAMS; EMC92: prognostic signature HOVON-65/GMMG-HD4. b) Dot plot of quantitative scores for EMC92 and UAMS GEP70 signatures.

6.3.2 Comparison of gene-expression signatures and their associations with survival in the Myeloma XI trial

Through use of the continuous scores that were generated for gene expression signatures, we compared expression signatures by first comparing commonly used gene expression scores in a multivariate analysis together. We found that the EMC92 and UAMS70 continuous scores retained a statistical association with overall survival (Table 61) and only EMC92 retained an independent association with short PFS. The remaining evaluated scores are likely to have lost significance due to correlation between the risk signatures that was demonstrated previously in this chapter.

Next, we compared the EMC92 and the UAMS70 continuous scores in terms of their independence with other known high-risk cytogenetic lesions (Table 61). We repeated multivariable Cox-based analysis of each score for the risk markers with other markers that were associated with poorer survival and demonstrated that all but the Myeloma IX signature and the millennium 100 risk signatures retained their independent prognostic association in the context of known high-risk cytogenetic lesions (Table 62).

Taken together, these data suggest that the EMC92 signature exhibits the strongest independent prognostic value within our trial data set. We therefore continued to explore this gene expression signature further.

Table 61: Multivariable Cox-based regression of different risk signatures as continuous variables and two most significant risk signatures (EMC92 and UAMS70) with adverse cytogenetic abnormalities from time of induction randomisation. N=329, events = 117. Cont score = continuous score

Overall Survival

	HR	Lower 95% CI	Upper CI (95%)	Wald P value
EMC92 Cont score	1.49	1.03	2.15	0.033587751
UAMS70 Cont score	1.66	1.01	2.73	0.043731921
IFM15 Cont score	1.06	0.89	1.27	0.482902818
MRCIX Cont score	0.95	0.66	1.36	0.77059199
Millennium100 Cont score	1	1	1	0.076977044

	HR	Lower 95% CI	Upper CI (95%)	Wald P value
EMC92 Cont score	1.44	1	2.05	0.047655512
UAMS70 Cont score	1.43	0.89	2.3	0.135848657
Del(17p)	2.08	1.23	3.5	0.006281561
Gain(1q)	1.32	0.87	2.01	0.191156667
Del(1p)	1.07	0.61	1.88	0.812906939
High-risk translocation	1.81	1.21	2.7	0.004019356

Progression Free Survival

	HR	Lower 95% CI	Upper CI (95%)	Wald P value
EMC92 Cont score	1.4	1.08	1.82	0.011114
UAMS70 Cont score	1.11	0.78	1.59	0.559367
IFM15 Cont score	0.98	0.87	1.1	0.685116
MRCIX Cont score	0.96	0.72	1.27	0.7622
Millennium100 Cont score	1	1	1	0.057333

	HR	Lower 95% CI	Upper CI (95%)	Wald P value
EMC92 Cont score	1.36	1.06	1.75	0.017283
UAMS70 Cont score	1.09	0.77	1.54	0.630087
Del(17p)	1.27	0.84	1.94	0.257488
Gain(1q)	0.99	0.73	1.36	0.974566
Del(1p)	0.92	0.58	1.45	0.707368
High-risk translocation	1.69	1.25	2.28	0.000713

Table 62: Multivariate Cox-based regression for overall survival of different risk signatures with adverse cytogenetics; n=155, events =40

Overall Survival	HR	Lower 95% CI	Upper CI (95%)	Wald P value
EMC92 Cont score	1.87	1.27	2.75	0.001646826
Del(17p)	4.16	1.85	9.37	0.000567053
Gain(1q)	2.35	1.1	5.04	0.027637291
Del(1p)	0.87	0.34	2.21	0.765895118
High-risk translocation	1.45	0.72	2.92	0.292290679
Induction IMiD	1.09	0.56	2.13	0.792245486
Maintenance treatment	0.48	0.24	0.97	0.042102468
UAMS70 Cont score	1.89	1.13	3.16	0.015719888
Del(17p)	3.96	1.78	8.81	0.000739032
Gain(1q)	2.27	1.06	4.88	0.034640475
Del(1p)	1.12	0.46	2.69	0.807471623
High-risk translocation	1.77	0.91	3.46	0.09498468
Induction IMiD	1.14	0.59	2.2	0.696441985
Maintenance treatment	0.54	0.27	1.07	0.078121482
IFM15 Cont score	1.42	1.06	1.91	0.020447251
Del(17p)	4.14	1.79	9.56	0.000869954
Gain(1q)	2.77	1.33	5.79	0.006637948
Del(1p)	1.03	0.4	2.64	0.945605932
High-risk translocation	1.9	0.97	3.71	0.062007814
Induction IMiD	1.3	0.67	2.51	0.432620651
Maintenance treatment	0.45	0.22	0.93	0.03122592
MRCIX Cont score	1.2	0.69	2.08	0.516054271
Del(17p)	3.97	1.79	8.81	0.000697956
Gain(1q)	2.77	1.33	5.8	0.006685646
Del(1p)	1.54	0.63	3.76	0.345473404
High-risk translocation	1.94	0.98	3.85	0.058277754
Induction IMiD	1.28	0.67	2.45	0.45350009
Maintenance treatment	0.52	0.26	1.03	0.060939244
Millennium100 Cont score	1	1	1.01	0.066097181
Del(17p)	3.36	1.45	7.78	0.004637225
Gain(1q)	2.68	1.29	5.55	0.008300648
Del(1p)	1.42	0.58	3.49	0.441918598
High-risk translocation	2.04	1.03	4.03	0.041209663
Induction IMiD	1.26	0.66	2.43	0.484089366
Maintenance treatment	0.55	0.27	1.11	0.09353442

6.3.3 Validation of expression-signature generation

The development of the EMC92 signature was described in the introduction to this chapter. EMC92 has been commercialised as the SKY92 signature, which is available worldwide as a diagnostic test; gene expression arrays are carried out locally but analysis is conducted centrally by the company in The Netherlands. Central analysis of the MMprofiler assay results by SkylineDx has identified 81/329 (24.6%) of patient tumours as high-risk.

As the EMC92 signature was found to be the most predictive within our data set, we went on to explore it further with regard to its genetic and clinical correlations. All gene-expression arrays were sent to SkylineDx for generation of a validated gene expression signature. The result of this analysis generated a ‘high-risk’ or ‘standard-risk’ label by the external laboratory. As explained in the previous section, for data exploration purposes, we were interested in the continuous value, which formed the basis of the risk cut-off, rather than just the binary value itself. We therefore generated the EMC92 signature as specified in the original study (272), which forms the basis of the SKY92 signature, but checked concordance with the central laboratory analysis to ensure the validity of our results.

We show that this resulted in 97.3% concordance with risk-labelling compared with the official SKY92 signature. The small difference in risk calling is likely to be a result of grouped batch correction rather than results for individual arrays that were used for diagnostic purposes in the SKY92 signature. Discrepant results from the generated data were close to the cut-off, which suggests that there were only minimal differences between the SKY92 and EMC92 risk calling (Figure 72).

Validation of EMC92 vs. SKY92 therefore enabled us to compare cytogenetic and clinical correlations as well as correlation with other risk signatures on a continuous scale as well as on a categorical one and therefore enabled more in-depth analysis around this area.

EMC92 score vs official SKY92 risk status by Skyline Dx

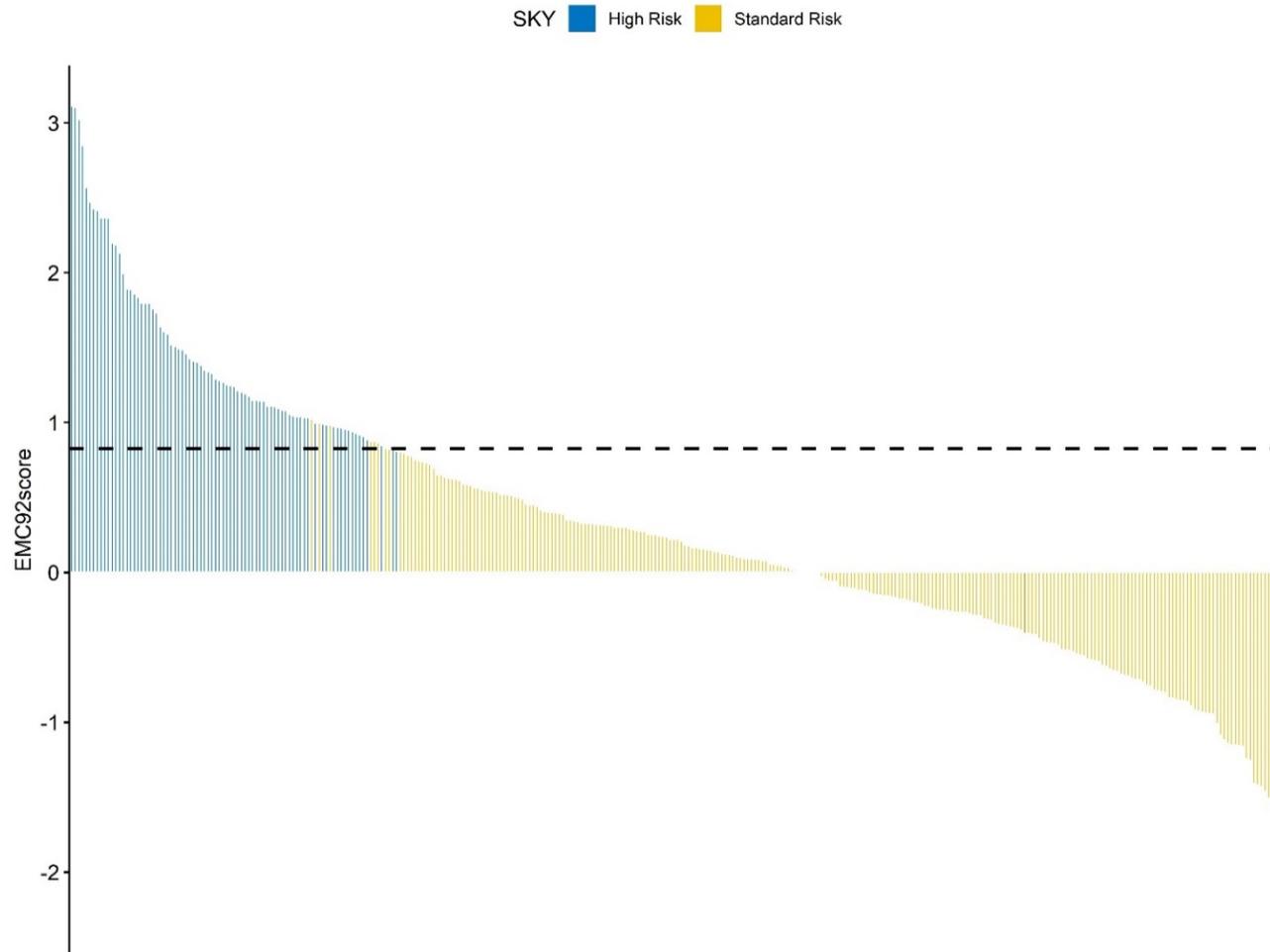


Figure 72: Continuous EMC92 risk score generated by methods described (272). Each bar represents an individual patient. The height of the bar represents the EMC92 continuous score. The horizontal dashed black line represents the cut-off for high-risk calling (0.827). Blue coloured bars represent those patients who were defined as high risk by SKY92. Yellow coloured bars represent those patients who were defined as standard risk by SKY92

6.3.4 Relationship between copy number changes, primary translocations and EMC92

As mentioned previously, high-risk GEP signatures have been found to be correlated with some high-risk cytogenetic abnormalities. As shown in Figure 73, there was partial but incomplete overlap between cases that were characterised by GEP and those characterised by genetic high-risk markers. However, we found that 6.1% (20/329) of all patients who were classified as SKY92 high risk showed no other high-risk cytogenetics such as adverse translocations, gain(1q) or del(17p). This represents a subgroup of patients who would otherwise be classified as standard risk MM. However, the majority of patients (61/81, 75%) of patients already had a high-risk cytogenetic risk marker.

Established high-risk cytogenetic lesions such as high-risk translocations (t(4;14) (P=0.001), t(14;16) & t(14;20) (P=0.016)) and gain(1q) (P<0.001) were present at a higher incidence in SKY92 high-risk patients than in standard-risk patients. Measures of del(17p) did not reach statistical significance but the deletion also occurred at a higher frequency in SKY92 high-risk patients (17% vs. 9%; P=0.08). There was also a strong positive correlation between del(1p) (p<0.001) and del(13q) (p<0.001). There was a negative correlation between t(11;14) and hyperdiploidy (Table 63).

‘Double-hit’ NDMM, which is defined as >1 established cytogenetic high-risk factor, has been described in the previous chapters of this thesis. It was found that 9.7% of patients who fell within the SKY92 high-risk group had overlapping ‘double hit’ in our cohort, but 16.4% of patients were double-hit but SKY92 standard-risk.

The above results show incomplete but overlapping measures of cytogenetic risk and SKY92 assignment. This is not surprising, since gene expression is the result of cytogenetic aberrations in most cancers. However, given that EMC92 has an independent association with survival in our dataset, these results also suggest that the current established high-risk cytogenetic markers do not fully account for the prognostic risk and further elucidation of these mechanisms is needed.

Table 63. Baseline cytogenetics of patients with tumours classified as SKY92 standard-risk or high-risk

	SKY92 standard-risk (N=248)	SKY92 high-risk (N=81)	<i>P</i>
Adverse translocation (%)	45 (18.1)	35 (43.2)	<0.001
t(4;14) (%)	40 (16.1)	28 (34.6)	0.001
t(14;16) or t(14;20) (%)	5 (2.0)	7 (8.6)	0.016
t(11;14) (%)	49 (19.8)	7 (8.6)	0.032
Hyperdiploid (%)	134 (54.0)	26 (32.1)	0.001
Gain(1q) [CKS1B] (%)	70 (28.2)	50 (61.7)	<0.001
Del(1p) [CDKN2C] (%)	17 (6.9)	19 (23.5)	<0.001
Del(17p) [TP53] (%)	21 (8.5)	13 (16.0)	0.083
Del(13q) (%)	89 (35.9)	54 (66.7)	<0.001

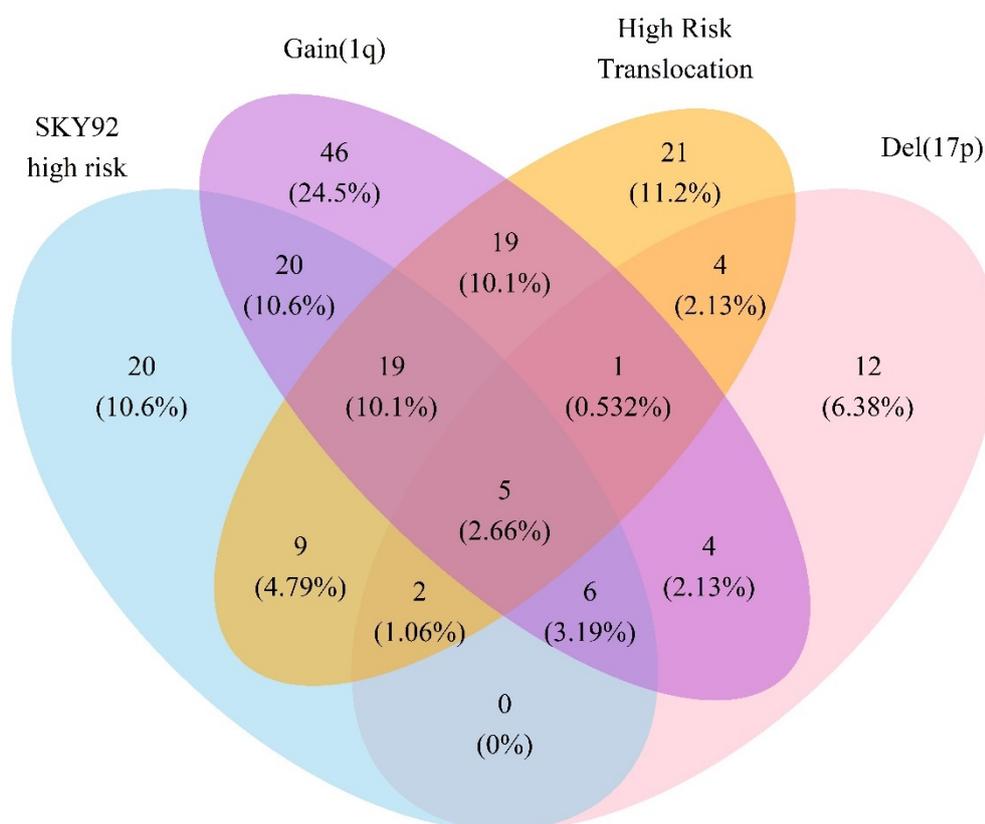


Figure 73: Venn diagram of patients with tumours that were positive for these validated genetic risk markers: adverse translocations, gain(1q), del(17p), SKY92 GEP high-risk. % shown is relative to other patients with high-risk lesions in the study

The next stage in the study was the exploration of the relationship of high-risk primary translocations with the continuous EMC92 signature, as shown in Figure 74, Figure 75 and Figure 76. Figure 74 demonstrates that, although there was a high degree of overlap between EMC92 high-risk patients and high-risk translocations, there were still a number of patients who had low EMC92 scores but high-risk translocations.

Next we examined copy number aberrations and their relationship to the EMC92 score (Figure 75). This examination demonstrated an association between higher EMC92 score and del(13q) and gain(1q), and showed that there appeared to be more of a continuous relationship with the copy number status and the continuous risk score. This suggests that some information may be lost when the gene expression score is binarised, and this may explain in part why cytogenetics retain some association with survival when multivariate analyses are performed. It also demonstrates the less robust relationship that is shown with patients who exhibit del(17p) and explains why del(17p) is the strongest cytogenetic factor to retain its prognostic association with poor survival in our multivariable analysis of this data.

Extreme CNAs (amplification/homozygous loss) have recently been described as drivers of high-risk behaviour. We therefore investigated the inter-relationship of quantitative CNAs with quantitative GEP risk scores. Median EMC92 scores were higher in tumours with gain(1q) vs. diploid 1q ($P=2.1 \times 10^{-8}$) but were not statistically different between tumours with gain (3 copies) or amplification (≥ 4 copies) 1q ($P=0.56$; Figure 76). The range of GEP scores in the amp(1q) group was wide. Some amp(1q) tumours showed GEP scores below the average, and demonstrated some non-overlapping features that were reflected by GEP and quantitative CNA profiling.

Tumours with deletion 17p had significantly higher EMC92 GEP scores than those without deletion ($P=0.008$), but homozygous del(17p) was very rare ($n=2$) in this cohort, and therefore formal comparison was not possible as shown in Figure 76. However, it is likely that homozygous deletion of 17p results in a significantly higher SKY92 score, given the high values of the data points that are shown in our results.

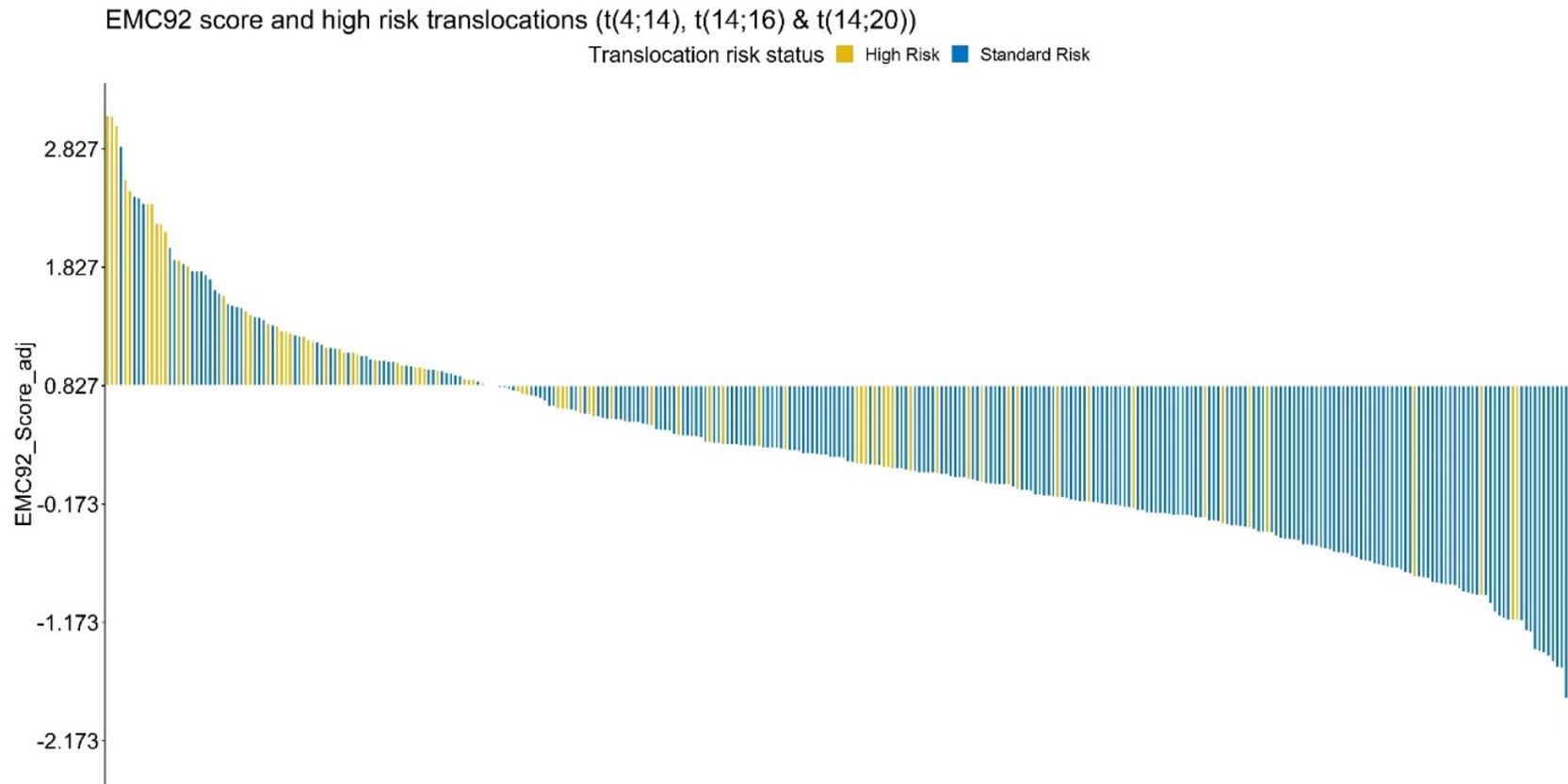
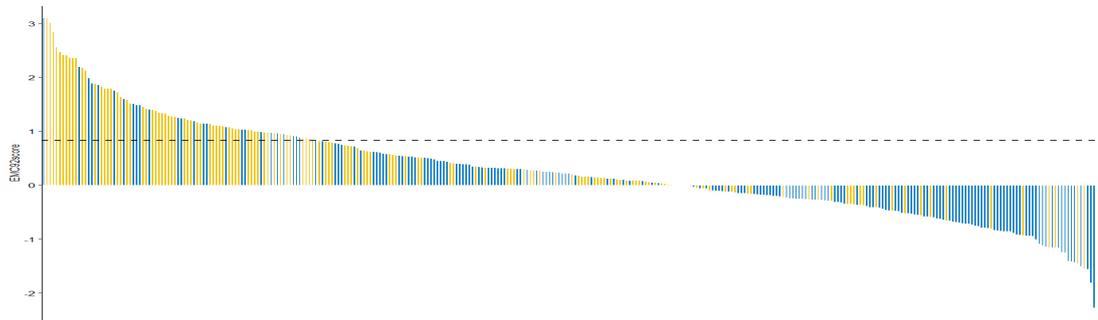
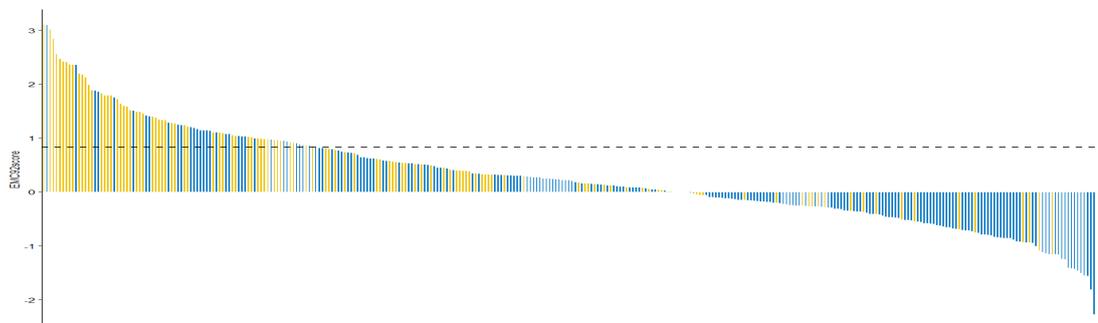


Figure 74: Bar plot of quantitative EMC92 continuous scores in quantitative order. Yellow bars represent positivity for the presence of a high-risk translocation (t(4;14), t(14;16) or t(14;20); blue bars represent patients who did not have these. All patients with a score above 0.827 were considered high risk as per EMC92 criteria

Deletion 13q status



Gain 1q status



Deletion 17p status

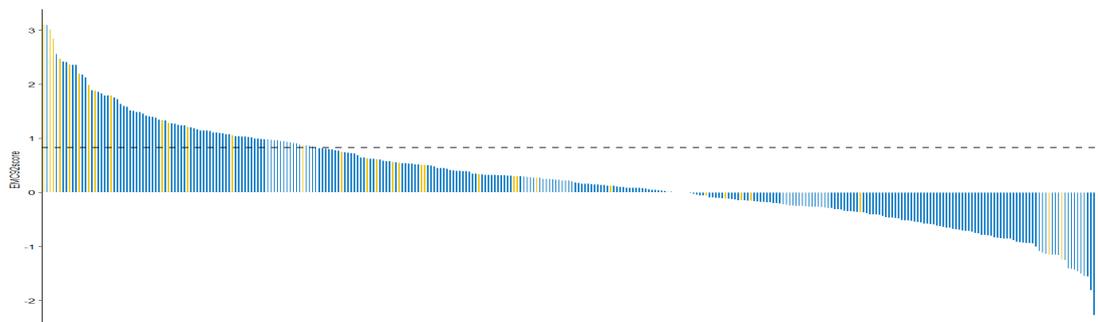


Figure 75: Bar plot of quantitative EMC92 continuous scores in quantitative order. Yellow bars represent positive samples for the presence of copy number changes (del(13q), gain(1q), del(1p) and del(17p)); blue bars represent samples that did not have these when tested by MLPA. All patients who scored above 0.827 were considered high risk as per EMC92 criteria represented by the horizontal dashed black line

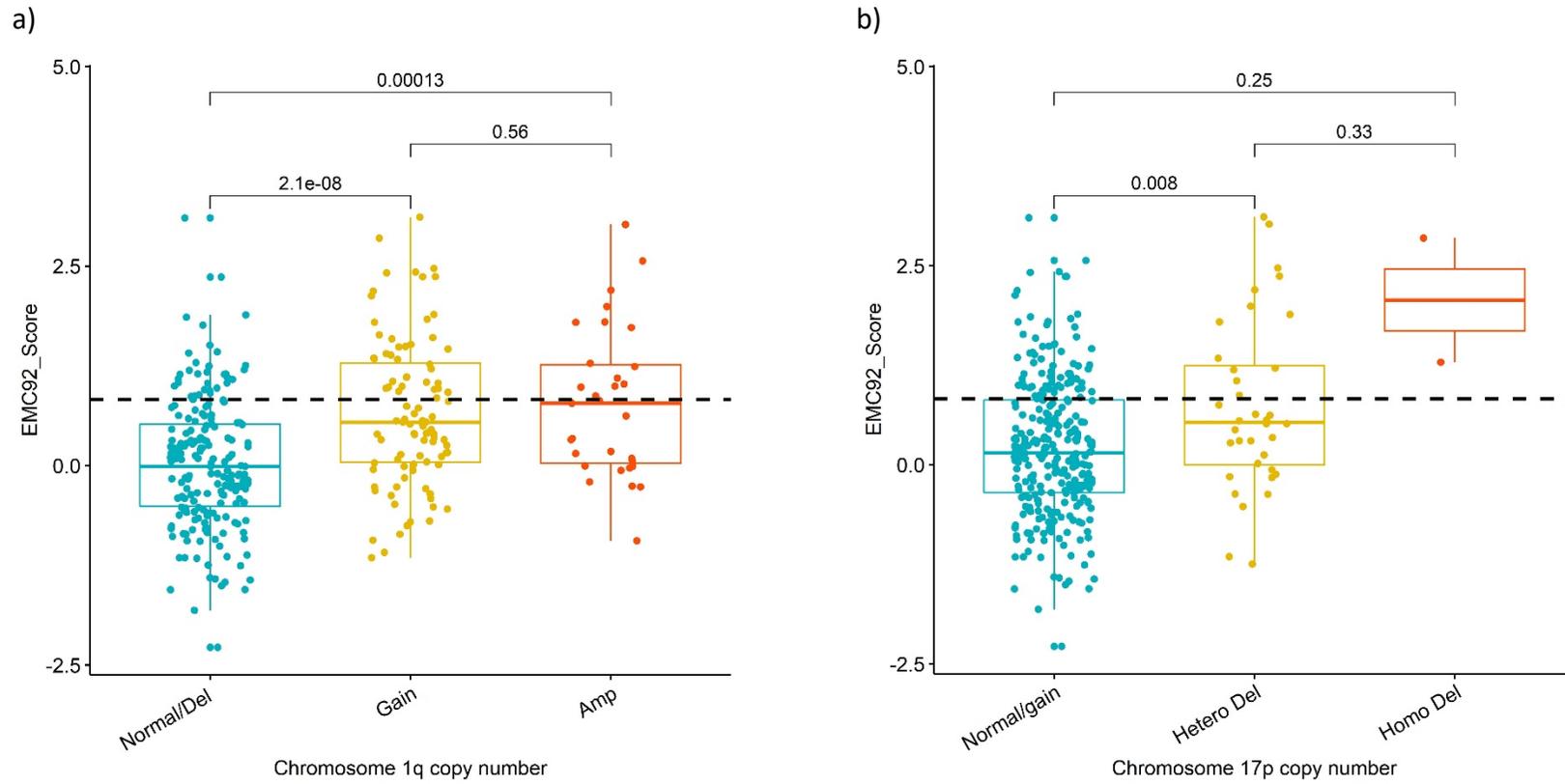


Figure 76: EMC92 quantitative score plotted for groups with a) normal, gained or amplified chromosome 1q; b) normal, heterozygous or homozygous deletion 17p

6.3.5 Association of clinical characteristics with gene expression signature

Baseline characteristics (Table 64) demonstrate the differences in patient characteristics within SKY92 high-risk vs. standard-risk patients. This study shows that baseline demographics such as age and gender were not significantly different between SKY92 standard-risk and high-risk patients. The group of patients who were allocated SKY92 high-risk scores had a higher proportion of patients with a WHO performance score ≥ 2 ($p=0.02$) than those who were allocated standard-risk scores. In the work that is discussed in the *TP53* chapter it had already been noted that genetic risk was associated with a poorer performance status in the case of patients with *TP53* deletion. These results corroborate our findings that tumour aggressiveness as defined by genetics can affect patient-related factors such as frailty.

Additionally, there are significant differences in baseline biochemical blood parameters that are associated with high disease burden, such as higher $\beta 2M$ ($P=0.002$) and LDH ($P<0.001$) levels in patients who are SKY92 high-risk. Consequently, the group of patients with SKY92 high-risk scores had a higher proportion of ISS stage III patients (38% vs. 21% in SKY92 standard-risk; $P=0.003$). The average platelet count ($P<0.001$) and haemoglobin concentration ($P<0.001$) were also reduced in SKY-92 high risk patients (Table 64; Figure 77, Figure 78).

The association between ISS and established high-risk cytogenetics also shows a step-wise, statistically significant increase with the number of cytogenetic aberrations that are present in terms of $\beta 2M$ level, ISS stage III score, LDH level and platelet count (Table 65, Figure 77; Figure 78).

Both these findings suggest the existence of an inter-relationship between baseline genetic abnormalities, gene expression, biochemical and haematological markers, and the patient's clinical status. In particular, the ISS score and LDH level are associated with genetic high risk and are likely to be surrogate markers of genetic high risk.

Table 64: Baseline demographics with clinical and laboratory characteristics of patients with tumours classified as SKY92 standard-risk or high-risk

	SKY92 Standard-risk (N=248)	SKY92 High-risk (N=81)	P
Demographics			
Age \geq 65 (mean(sd))	58 (23.4)	23 (28.4)	0.447
Male gender (%)	152 (61.3)	45 (55.6)	0.433
Clinical characteristics			
WHO performance score \geq 2 (%)	35 (14.9)	22 (27.2)	0.021
ISS score (%)			0.003
I	83 (33.6)	15 (18.5)	
II	112 (45.3)	35 (43.2)	
III	52 (21.1)	31 (38.3)	
β 2M mg/l (median [IQR])	3.60 [2.70, 4.95]	4.50 [3.30, 6.90]	0.002
Albumin <35g/dl (%)	86 (34.7)	38 (46.9)	0.07
CRP (median [IQR])	3.10 [1.20, 9.50]	5.20 [1.05, 13.50]	0.247
Serum creatinine (median [IQR])	85.50 [71.00, 103.25]	91.00 [74.00, 118.00]	0.111
Urea (median [IQR])	5.50 [4.60, 7.50]	5.90 [4.60, 8.60]	0.108
Haemoglobin g/l (mean (sd))	108.95 (18.21)	99.53 (19.75)	<0.001
Platelets (mean (sd))	261.95 (93.19)	206.68 (104.66)	<0.001
Lactate dehydrogenase units/l (mean (sd))	269.18 (135.26)	363.19 (222.35)	<0.001
Calcium mmol/l > 2.6 (%)	39 (15.8)	23 (28.4)	0.019
Bone disease (%)	163 (70.9)	47 (63.5)	0.295

Table 65: Baseline demographics with clinical and laboratory characteristics of patients with tumours with 0, 1 or ≥ 2 established high-risk cytogenetic aberrations

	No high-risk lesions (N=220)	1 high-risk lesion (n=77)	≥ 2 high-risk lesions (n=32)	P
Demographics				
Age ≥ 65 (mean(sd))	59.05 (7.91)	60.52 (6.71)	61.69 (5.76)	0.089
Male gender (%)	134 (60.9)	49 (63.6)	14 (43.8)	0.134
Clinical characteristics				
WHO performance score ≥ 2 (%)	34 (16.3)	16 (21.1)	7 (21.9)	0.552
ISS score (%)				0.028
I	75 (34.2)	18 (23.4)	5 (15.6)	
II	97 (44.3)	37 (48.1)	13 (40.6)	
III	47 (21.5)	22 (28.6)	14 (43.8)	
$\beta 2M$ mg/l (median [IQR])	3.60 [2.60, 4.95]	4.30 [3.30, 6.20]	4.65 [3.30, 6.93]	0.011
Albumin $< 35g/dl$ (%)	37.57 (6.60)	36.16 (7.02)	35.83 (4.83)	0.143
CRP (median [IQR])	3.10 [1.20, 9.60]	3.30 [1.00, 12.00]	6.65 [1.30, 12.78]	0.359
Serum creatinine (median [IQR])	85.00 [70.75, 101.25]	91.00 [76.00, 121.00]	86.50 [73.75, 104.00]	0.161
Urea (median [IQR])	5.60 [4.60, 7.40]	5.70 [4.50, 8.20]	5.75 [4.60, 7.75]	0.685
Haemoglobin g/l (mean (sd))	109.63 (17.93)	100.74 (20.81)	100.19 (17.27)	< 0.001
Platelets (mean (sd))	266.42 (93.91)	226.95 (102.12)	175.53 (81.78)	< 0.001
Lactate dehydrogenase units/l (mean (sd))	272.23 (136.59)	307.06 (189.09)	393.52 (238.89)	0.001
Calcium mmol/l > 2.6 (%)	34 (15.5)	18 (23.4)	10 (31.2)	0.055
Bone disease (%)	147 (72.4)	47 (66.2)	16 (53.3)	0.09

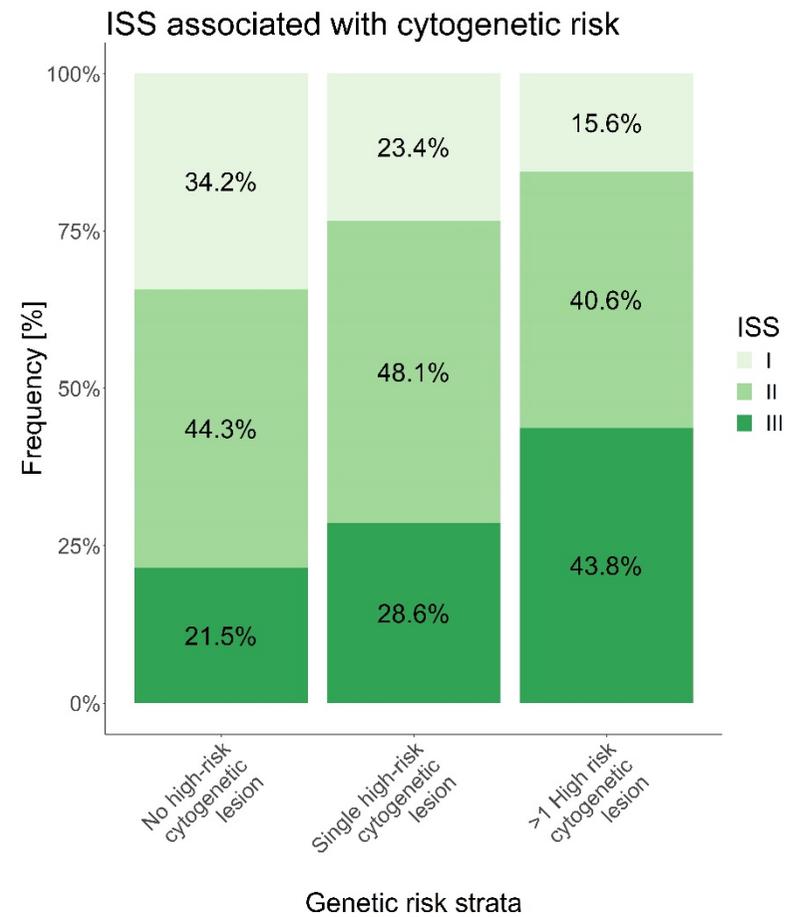
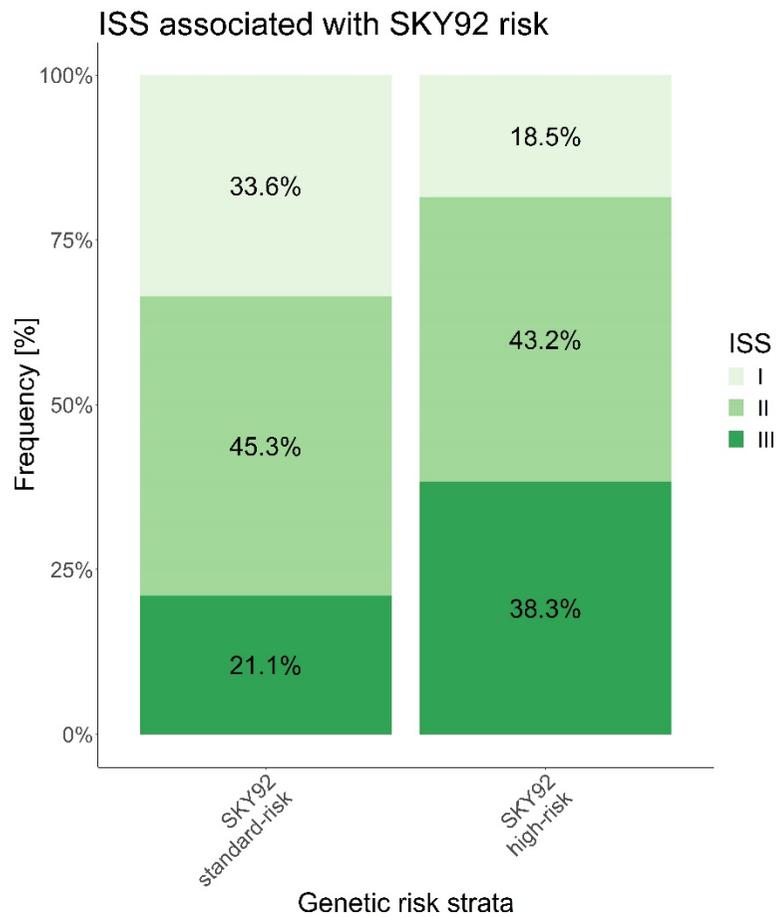


Figure 77: Proportion of patients with each ISS status for groups with no, single or double-hit genetics and patients compared to patients with absence or presence of SKY92 high-risk rating.

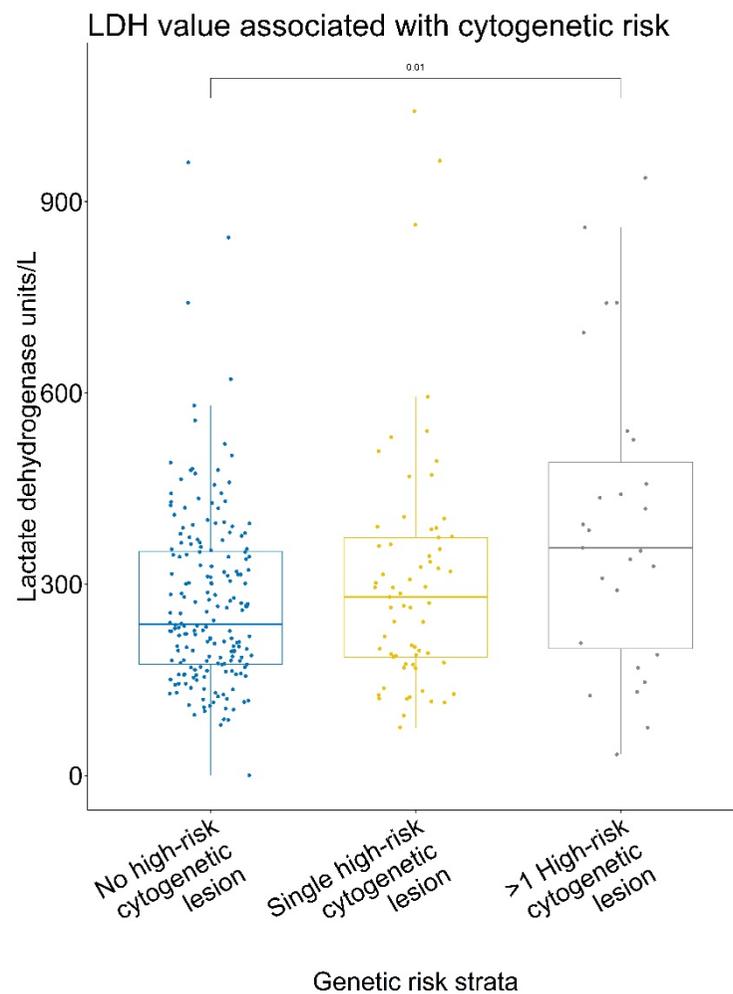
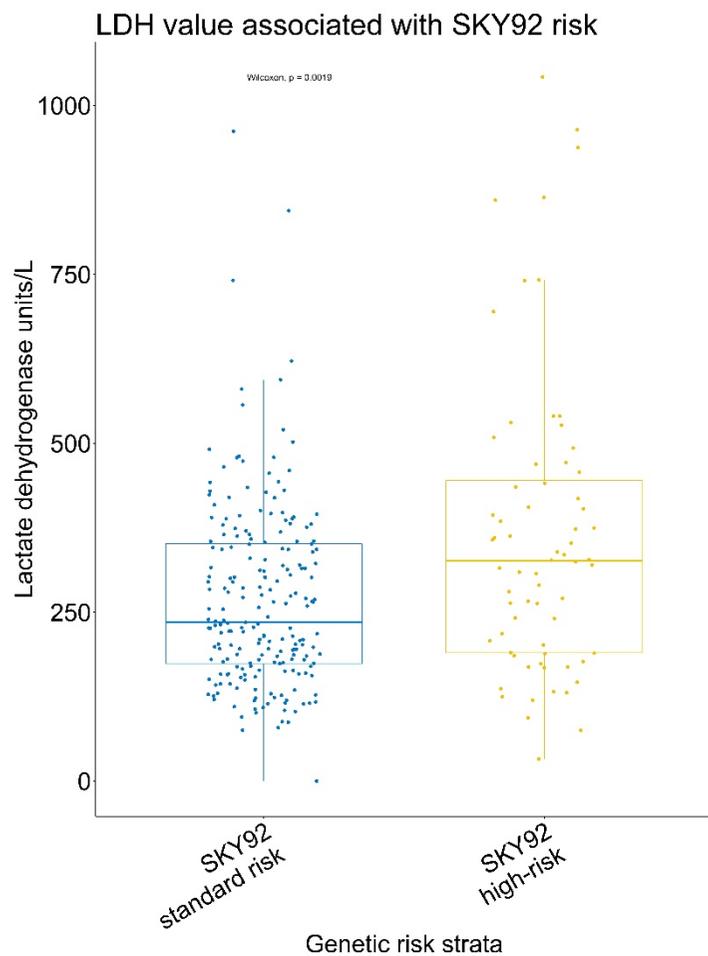


Figure 78: LDH values at presentation for patients with no, single or double-hit genetics and patients or with absence or presence of SKY92 high-risk rating

6.3.6 SKY92 GEP identifies patients with poor outcomes who were treated with IMiD-based induction

We previously analysed the continuous EMC92 signature in the context of other signatures that are commonly used in MM. For the validation of the diagnostic test we tested the association with SKY92 high-risk dichotomised signature and survival in more detail. By univariate analysis, the presence of a SKY92 high-risk rating was associated with shorter PFS (median 16.0 vs. 33.8 months; $P=4.1 \times 10^{-11}$; HR 2.6, 95% CI: 2.0-3.5) and OS (median 36.7 months vs. not reached; $P=2.5 \times 10^{-13}$; HR 3.9, 95% CI: 2.7-5.7) in these largely IMiD-treated patients (Figure 79 ,Table 66). We found similar results for CTD or CRD induction; SKY92 was independently associated with shorter survival periods, regardless of which IMiD treatment was used (Figure 80;Table 68, Table 69).

By multivariable Cox-regression analysis with both clinical and genetic high-risk markers, SKY92 high-risk was associated with a short OS (HR 2.7, 95% CI: 1.8-4.2, $P=4.4 \times 10^{-6}$). Shorter overall survival was associated with adverse translocations (HR 1.8, 95% $P=0.007$; CI: 1.2-2.9) and del(17p) (HR 2.5, 95% CI: 1.5-4.1, $P=0.0007$). Perhaps due to its correlation SKY92 high risk shown in the previous section, ISS staging lost its independent association with poor survival. Similarly, shorter PFS was also associated with SKY92 high-risk ratings (HR 2.1, 95% CI: 1.5-3.0, $P=4.8 \times 10^{-6}$) and adverse translocations (HR 1.9, 95% CI: 1.4-2.6, $P=0.0002$) (Table 66).

For comparison, we also tested the UAMS GEP70 dichotomised high-risk signature with other cytogenetic and clinical high-risk abnormalities in a multivariate analysis (Table 67). This multivariable analysis demonstrated the negative association with survival of UAMS GEP70 high-risk (HR=2.54; 95% CI: 1.56-4.13; $P=0.00018$) in addition to del(17p) (HR=2.22; 95% CI: 1.32-3.72, $P=0.0025$) and adverse translocation (HR=2.11; 95% CI: 1.35-3.28; $P=0.00095$). However, only the presence of adverse translocations (HR=2.01; 95% CI: 1.45-2.79; $P=0.00003$) and age but not UAMS GEP70 were associated with shorter PFS in this data set.

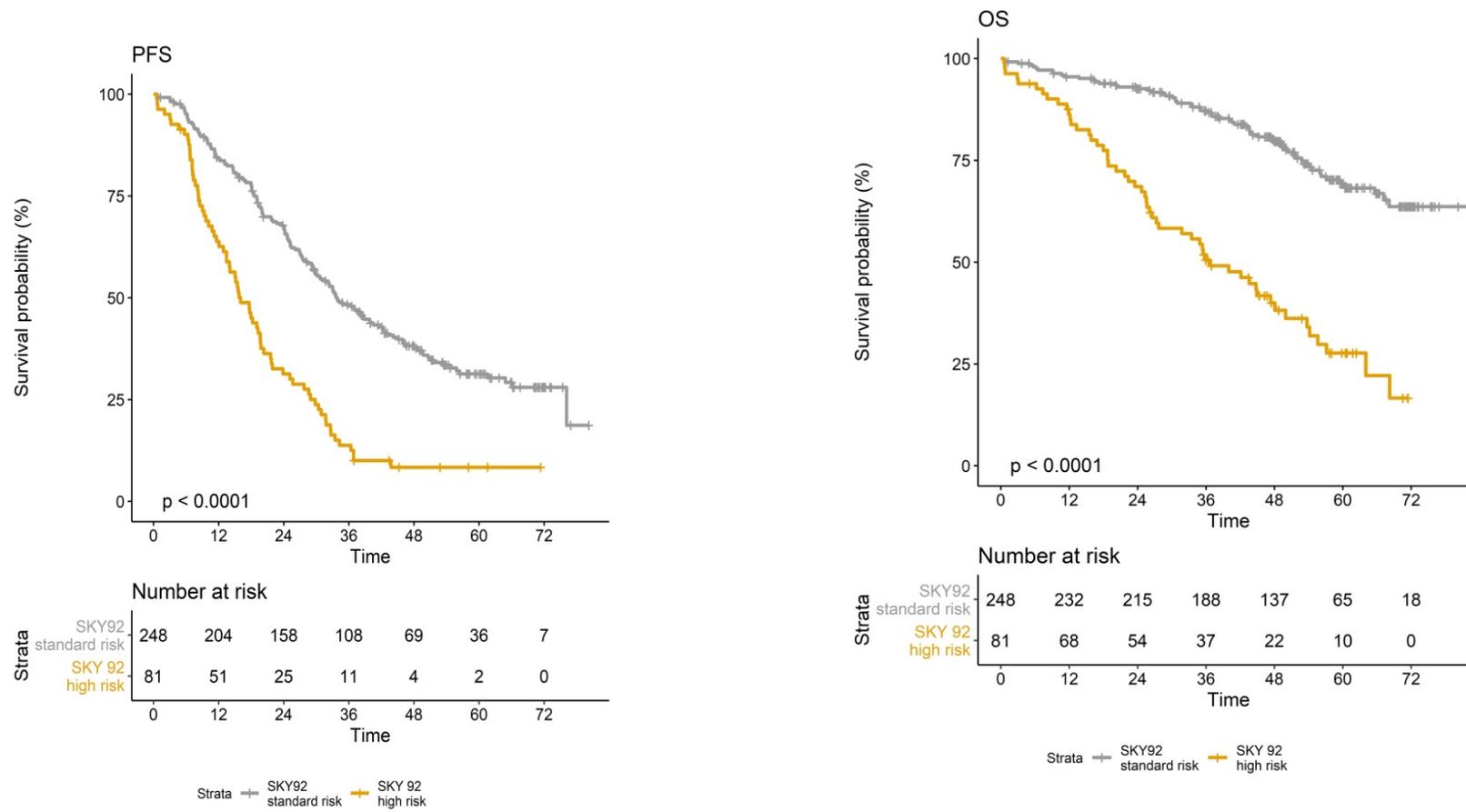


Figure 79: Kaplan-Meier plot of the analysed representative NCRI Myeloma XI trial patients (n=329) in context of SKY92 risk-profiling results for PFS and OS from induction randomisation

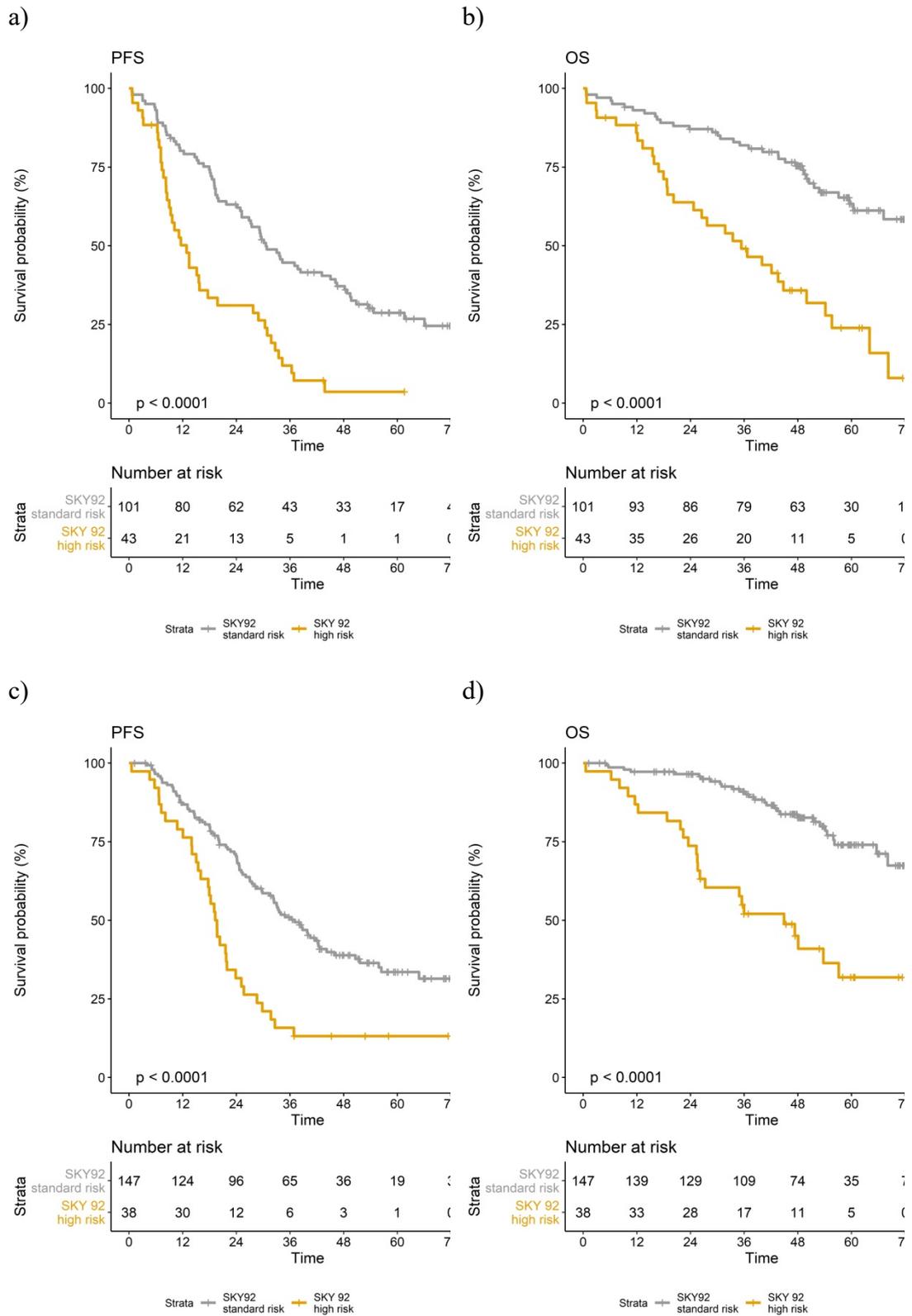


Figure 80: Kaplan-Meier plots for groups defined by presence or absence of SKY92 GEP risk status in patients randomised (ITT) to induction with CTD (a, b) or CRD (c, d)

	Univariate analysis	
Progression free survival		
	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	2·6 (1·96-3·45)	4·08x10 ⁻¹¹
Hyperdiploid	0·74 (0·57-0·95)	0·0198
Adverse translocation	2·04 (1·53-2·72)	1·12x10 ⁻⁰⁶
Del(1p) [CDKN2C]	1·47 (1·2-1·8)	0·0514
Del(17p) [TP53]	1·63 (1·09-2·42)	0·016
Gain(1q)	1·44 (1·11-1·88)	0·00634
Age	1·04 (1·02-1·06)	0·00012
Induction randomisation	0·77 (0·59-0·99)	0·0417
ISS	1·33 (1·12-1·58)	0·0012
Overall survival		
	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	3·94 (2·73-5·69)	2·54x10 ⁻¹³
Hyperdiploid	0·6 (0·42-0·87)	0·00717
Adverse translocation	2·5 (1·72-3·64)	1·67x10 ⁻⁰⁶
Del(1p) [CDKN2C]	2·38 (1·49-3·79)	0·000271
Del(17p) [TP53]	3·02 (1·87-4·87)	5·76x10 ⁻⁰⁶
Gain(1q)	2·39 (1·66-3·44)	2·98x10 ⁻⁰⁶
Age	0·62 (0·43-0·9)	0·0113
Induction randomisation	0·62 (0·43-0·9)	0·0113
ISS	1·38 (1·08-1·76)	0·0101

	Multivariate analysis	
Progression free survival		
	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	2·14 (1·54-2·96)	0·00000475
Hyperdiploid	0·93 (0·7-1·24)	0·634
Adverse translocation	1·89 (1·36- 2·62)	0·00015
Del(1p) [CDKN2C]	1·01 (0·65-1·56)	0·979
Del(17p) [TP53]	1·32 (0·87-2·0)	0·198
Gain(1q)	0·88 (0·65-1·2)	0·425
Age	1·04 (1·02-1·06)	0·000144
Induction randomisation	1·2 (0·92-1·55)	0·176
ISS	1·13 (0·95-1·36)	0·176
Overall survival		
	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	2·72 (1·78-4·16)	0·00000396
Hyperdiploid	0·91 (0·6-1·37)	0·647
Adverse translocation	1·85 (1·19-2·88)	0·0061
Del(1p) [CDKN2C]	1·29 (0·76-2·2)	0·343
Del(17p) [TP53]	2·48 (1·48-4·17)	0·000602
Gain(1q)	1·3 (0·85-1·97)	0·222
Age	1·02 (0·99-1·05)	0·2
Induction randomisation	1·31 (0·9-1·91)	0·153
ISS	1·09 (0·84-1·43)	0·512

Table 66: Univariate and multivariate Cox proportional hazard survival analyses of genetic, gene expression and clinical risk markers for PFS and OS for 329 representative Myeloma XI NDMM patients from induction randomisation. N=328, events for PFS=232, events for OS = 117

Progression free survival

	Univariate analysis			
	N	events	HR	Wald P
UAMS70 High-risk	329	232	1.86 (1.34-2.59)	0.000234
Del(1p) [CDKN2C]	329	232	1.47 (1-2.18)	0.0514
Del(17p) [TP53]	329	232	1.63 (1.09-2.42)	0.016
Gain(1q)	329	232	1.44 (1.11-1.88)	0.00634
Adverse translocation	329	232	2.04 (1.53-2.72)	1.12E-06
Hyperdiploid	329	232	0.74 (0.57-0.95)	0.0198
ISS	328	232	1.33 (1.12-1.58)	0.0012
Age	329	232	1.04 (1.02-1.06)	0.000115
Induction randomisation	329	232	1.31 (1.01-1.69)	0.0417

	Multivariate analysis	
	HR(95% CI)	Wald P
UAMS70 High-risk	1.37 (0.91 - 2.04)	0.129
Del(1p) [CDKN2C]	1.04 (0.66 - 1.64)	0.867
Del(17p) [TP53]	1.35 (0.89 - 2.05)	0.163
Gain(1q)	1.02 (0.75 - 1.38)	0.891
Adverse translocation	2.01 (1.45 - 2.79)	0.00003
Hyperdiploid	0.93 (0.7 - 1.23)	0.605
ISS	1.17 (0.97 - 1.4)	0.106
Age	1.04 (1.02 - 1.06)	0.000143
Induction randomisation	1.21 (0.93 - 1.57)	0.152

Table 67: Cox-based univariate and multivariable analysis from trial entry for PFS and OS with UAMS70 gene expression risk signature. N=328, events for PFS=232, events for OS = 117

	Univariate analysis			
	N	events	HR	Wald P
UAMS70 High-risk	329	117	3.79 (2.55-5.65)	5.51E-11
Del(1p) [CDKN2C]	329	117	2.38 (1.49-3.79)	0.000271
Del(17p) [TP53]	329	117	3.02 (1.87-4.87)	5.76E-06
Gain(1q)	329	117	2.39 (1.66-3.44)	2.98E-06
Adverse translocation	329	117	2.5 (1.72-3.64)	1.67E-06
Hyperdiploid	329	117	0.6 (0.42-0.87)	0.00717
ISS	328	117	1.38 (1.08-1.76)	0.0101
Age	329	117	1.03 (1-1.05)	0.033
Induction randomisation	329	117	1.6 (1.11-2.31)	0.0113

	Multivariate analysis	
	HR(95% CI)	Wald P
UAMS70 High-risk	2.54 (1.56 - 4.13)	0.000175
Del(1p) [CDKN2C]	1.19 (0.68 - 2.08)	0.545
Del(17p) [TP53]	2.22 (1.32 - 3.72)	0.00253
Gain(1q)	1.39 (0.91 - 2.12)	0.125
Adverse translocation	2.11 (1.35 - 3.28)	0.000951
Hyperdiploid	0.95 (0.62 - 1.44)	0.806
ISS	1.1 (0.84 - 1.43)	0.505
Age	1.02 (1 - 1.05)	0.0972
Induction randomisation	1.39 (0.96 - 2.02)	0.079

Table 68. Cox-based univariate and multivariate analysis for induction randomisation arms cyclophosphamide, thalidomide and dexamethasone (CTD)

CTD induction		Univariate analysis		Progression-free survival			Multivariate analysis	
	N	HR (95% CI)	Wald <i>P</i>		HR (95% CI)	Wald <i>P</i>		
SKY92 high-risk	144	2.67 (1.78-4)	1.81E-06	SKY92 high-risk	2.32 (1.41-3.84)	0.001		
Hyperdiploid	144	0.62 (0.42-0.91)	0.0142	Hyperdiploid	0.81 (0.54-1.22)	0.317		
Adverse translocation	144	2.39 (1.59-3.58)	2.49E-05	Adverse translocation	1.94 (1.21-3.12)	0.00577		
Del(1p) [CDKN2C]	144	1.74 (0.97-3.12)	0.0637	Del(1p) [CDKN2C]	0.69 (0.32-1.46)	0.329		
Del(17p) [TP53]	144	1.83 (1.06-3.17)	0.0308	Del(17p) [TP53]	1.68 (0.86-3.29)	0.132		
Gain(1q)	144	1.55 (1.06-2.27)	0.0227	Gain(1q)	0.89 (0.55-1.44)	0.632		
ISS	144	1.37 (1.08-1.75)	0.00987	ISS	1.1 (0.85-1.43)	0.477		
Age	144	1.02 (1-1.05)	0.0612	Age	1.03 (1.01-1.06)	0.0197		

Overall survival				Overall survival		
	N	HR (95% CI)	Wald <i>P</i>		HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	144	3.51 (2.14-5.76)	6.71E-07	SKY92 high-risk	2.64 (1.42-4.91)	0.00213
Hyperdiploid	144	0.81 (0.49-1.33)	0.404	Hyperdiploid	1.07 (0.63-1.83)	0.792
Adverse translocation	144	2.1 (1.27-3.48)	0.00374	Adverse translocation	1.52 (0.82-2.81)	0.182
Del(1p) [CDKN2C]	144	2.69 (1.4-5.17)	0.00303	Del(1p) [CDKN2C]	0.97 (0.43-2.16)	0.936
Del(17p) [TP53]	144	2.85 (1.51-5.38)	0.00125	Del(17p) [TP53]	2.55 (1.18-5.5)	0.0174
Gain(1q)	144	2.49 (1.52-4.09)	0.000319	Gain(1q)	1.27 (0.68-2.37)	0.445
ISS	144	1.45 (1.04-2.02)	0.0273	ISS	1.1 (0.76-1.61)	0.607
Age	144	1.02 (0.99-1.06)	0.178	Age	1.02 (0.98-1.06)	0.323

Table 69: Cox-based univariate and multivariate analysis for induction randomisation arms cyclophosphamide, lenalidomide and dexamethasone (CRD)

		Univariate analysis	
CRD induction			
Progression-free survival			
	N	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	185	2.45 (1.63-3.68)	1.67E-05
Hyperdiploid	185	0.87 (0.61-1.25)	0.454
Adverse translocation	185	1.69 (1.1-2.58)	0.0156
Del(1p) [CDKN2C]	185	1.38 (0.81-2.33)	0.237
Del(17p) [TP53]	185	1.44 (0.81-2.57)	0.215
Gain(1q)	185	1.3 (0.89-1.89)	0.174
ISS	184	1.32 (1.03-1.69)	0.0256
Age	185	1.05 (1.02-1.08)	0.00069

		Multivariate analysis	
CRD induction			
Progression-free survival			
		HR (95% CI)	Wald <i>P</i>
SKY92 high-risk		2.01 (1.3-3.13)	0.00187
Hyperdiploid		1.1 (0.72-1.67)	0.668
Adverse translocation	2	(1.22-3.28)	0.00623
Del(1p) [CDKN2C]		1.24 (0.7-2.18)	0.46
Del(17p) [TP53]		1.28 (0.72-2.3)	0.403
Gain(1q)		0.86 (0.57-1.31)	0.489
ISS		1.15 (0.89-1.5)	0.284
Age		1.04 (1.01-1.07)	0.0038

Overall survival			
	N	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	185	4.22 (2.43-7.32)	2.96E-07
Hyperdiploid	185	0.46 (0.26-0.8)	0.00643
Adverse translocation	185	2.9 (1.64-5.11)	0.000234
Del(1p) [CDKN2C]	185	2.29 (1.17-4.48)	0.015
Del(17p) [TP53]	185	3.04 (1.47-6.28)	0.00262
Gain(1q)	185	2.12 (1.23-3.65)	0.00678
ISS	184	1.36 (0.94-1.97)	0.0994
Age	185	1.03 (0.99-1.07)	0.154

Overall survival		
	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	3.19 (1.76-5.8)	0.000139
Hyperdiploid	0.84 (0.41-1.69)	0.619
Adverse translocation	2.69 (1.32-5.46)	0.00626
Del(1p) [CDKN2C]	1.83 (0.87-3.87)	0.113
Del(17p) [TP53]	2.51 (1.17-5.37)	0.0176
Gain(1q)	1.38 (0.76-2.52)	0.293
ISS	1.11 (0.75-1.65)	0.601
Age	1.01 (0.97-1.05)	0.624

Of the 161 patient tumours in which no cytogenetic high-risk marker was detected, the majority belonged to hyperdiploid or t(11;14) pathogenetic sub-groups. Of these, 20 (12%) of the tumours were identified as SKY92 high-risk. These SKY92 high-risk patients were found to be independently associated with shorter PFS (HR=3.18; 95% CI: 1.86-5.46, $P=2.6 \times 10^{-6}$; median 15.8 vs. 41.7 months) and OS (HR=2.42; 95% CI: 1.04-5.67, $P=0.04$; estimated four-year OS 55% vs. 86%) (Table 70, Figure 81). This represents a subset of patients who would be classified as standard risk by other methods and therefore would have no escalation in induction treatment upfront at diagnosis. It is possible that the utility of this risk prediction signature would be for patients with no known risk markers rather than those with established risk markers as a way to confirm low risk.

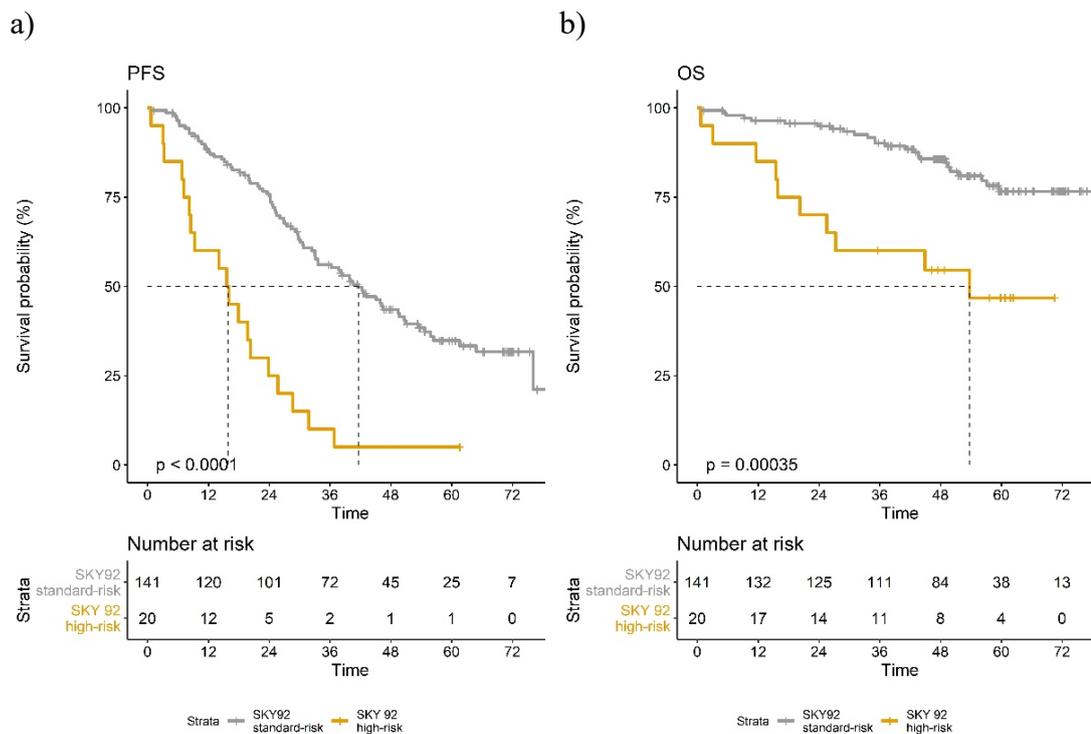


Figure 81: Kaplan-Meier plots of subset of 161 patients with no established genetic high-risk markers in context of presence or absence of SKY92 high-risk status. a) PFS, b) OS

Table 70: Cox-based univariate and multivariate survival analysis of prognostic association of molecular or clinical variables for 161 patients without established high-risk markers

Univariate analysis				
Progression-free survival				
	N	events	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	161	104	3.91 (2.34-6.54)	1.85E-07
Del(1p) [CDKN2C]	161	104	3.48 (1.73-7)	0.000464
Hyperdiploidy	161	104	1.1 (0.73-1.65)	0.66
Age	161	104	1.05 (1.02-1.08)	0.00157
Induction CTD vs. CRD	161	104	1.02 (0.69-1.5)	0.933
ISS	161	104	1.23 (0.96-1.58)	0.0991

Multivariate analysis		
Progression-free survival		
	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	3.18 (1.86-5.46)	2.57E-05
Del(1p) [CDKN2C]	2.26 (1.07-4.78)	0.0335
Hyperdiploidy	1.14 (0.75-1.73)	0.529
Age	1.04 (1.01-1.06)	0.0162
Induction CTD vs. CRD	1.05 (0.69-1.58)	0.834
ISS	1.09 (0.85-1.41)	0.496

Overall survival				
	N	events	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	161	35	3.51 (1.68-7.31)	0.000809
Del(1p) [CDKN2C]	161	35	3.36 (1.3-8.68)	0.0124
Hyperdiploidy	161	35	0.95 (0.48-1.89)	0.884
Age	161	35	1.02 (0.97-1.06)	0.448
Induction CTD vs. CRD	161	35	1.59 (0.82-3.1)	0.172
ISS	161	35	1.5 (0.97-2.32)	0.0656

Overall survival		
	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	2.42 (1.04-5.67)	0.0412
Del(1p) [CDKN2C]	2.33 (0.77-7.06)	0.134
Hyperdiploidy	1.03 (0.5-2.13)	0.927
Age	1 (0.95-1.05)	0.951
Induction CTD vs. CRD	1.78 (0.87-3.62)	0.113
ISS	1.37 (0.87-2.15)	0.177

6.3.7 SKY 92 GEP identifies high-risk patients after autologous stem cell transplantation and maintenance randomisation

Next, we examined a group of patients who had been landmarked from the time of HDM-ASCT. The analysis confirms that the risk associated with SKY92 high-risk is not abrogated by transplantation (Table 72). Of additional note is finding that the proportion of patients that reaches HDM-ASCT is reduced in the SKY92 high-risk cohort. These patients were originally planned to receive this intervention at induction randomisation according to the decision of the patients' physicians (Table 71). This suggests that patients who undergo ASCT have already self-selected themselves into a slightly lower risk group. Studies that retrospectively evaluate the effect of transplantation in myeloma should take close account of the risk groups that enter into transplantation to account for this possible confounding factor.

Similarly, in a landmarked analysis of maintenance randomisation (lenalidomide vs. observation), groups with SKY92 high-risk continued to show a negative association with survival (HR 3.0, 95% CI: 1.4 – 6.1, $P=0.0031$). Additionally, gain(1q) (HR 2.1, 95% CI: 1.0 – 4.4, $P=0.047$) and del(17p) (HR 3.6, 95% CI: 1.6 – 7.8, $P=0.0015$) remained associated with shorter OS rates. In terms of progression free survival and those with SKY92 high-risk (HR 2.6, 95% CI: 1.6 – 4.3, $P=9.5 \times 10^{-5}$) and adverse translocations (HR 2.1, 95% CI: 1.3 – 3.4, $P=0.0035$) remained associated with shorter PFS.

For patients with SKY92 standard-risk tumours, application of lenalidomide maintenance significantly extended PFS (HR=0.29; 95% CI: 0.16-0.51, $P=1.5 \times 10^{-5}$) but not OS when compared with observation alone, whereas those with SKY92 high-risk disease did not appear to derive benefit from lenalidomide maintenance therapy (Table 73; Figure 82). These two analyses suggest that the SKY92 high-risk profile at induction is not abrogated by high-dose melphalan therapy or by lenalidomide maintenance.

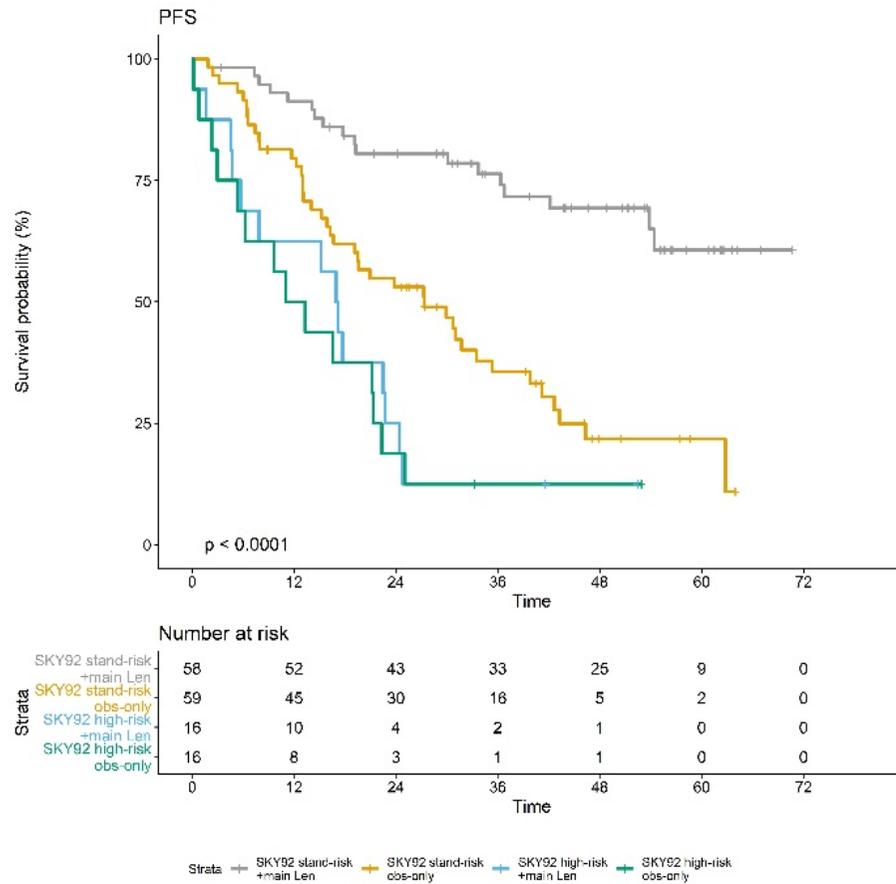
Table 71: Baseline table with details of treatment arm randomisation in patients classified as SKY92 standard risk vs. high risk

	SKY92 standard-risk (N=248)	SKY92 high-risk (N=81)	<i>P</i>
Treatment characteristics			
Intensive pathway (%)	248 (100.0)	81 (100.0)	n/a
Induction randomisation treatment			
CRD (%)	147 (59.3)	38 (46.9)	0.069
CTD (%)	101 (40.7)	43 (53.1)	0.069
Response dependent consolidation randomisation (%)	58 (26.6)	12 (23.5)	
VCD consolidation (%)	29 (50.0)	5 (41.7)	0.835
No consolidation (%)	29 (50.0)	7 (58.3)	0.835
VCD without randomisation for refractory disease (%)	8 (3.2)	6 (7.4)	
Underwent consolidation bone marrow transplant (%)	171 (72.8)	47 (60.3)	0.052
Underwent maintenance randomisation	121 (48.8)	35 (43.2)	0.456
Maintenance randomisation (%)			0.841
No maintenance	61 (50.4)	17 (48.6)	
Lenalidomide maintenance	59 (48.8)	18 (51.4)	
Lenalidomide & Vorinostat maintenance	1 (0.8)	0 (0.0)	

Table 72: Cox-based univariate and multivariate analysis landmarked from timepoint of HDM-ASCT for PFS and OS

			Univariate analysis		Multivariate analysis		
Progression-free survival					Progression-free survival		
	N	n events	HR (95% CI)	Wald <i>P</i>		HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	205	123	2.95 (2-4.37)	5.99E-08	SKY92 high-risk	2.53 (1.61 - 3.97)	5.22E-05
Adverse translocation	205	123	2.19 (1.48-3.24)	8.82E-05	Adverse translocation	2.25 (1.44 - 3.5)	0.000341
Del(1p) [CDKN2C]	205	123	1.72 (1.02-2.92)	0.0434	Del(1p) [CDKN2C]	1.07 (0.59 - 1.95)	0.826
Del(17p) [TP53]	205	123	2.12 (1.27-3.55)	0.00412	Del(17p) [TP53]	1.63 (0.95 - 2.8)	0.0765
Gain(1q)	205	123	1.62 (1.13-2.32)	0.00916	Gain(1q)	0.97 (0.63 - 1.5)	0.902
Hyperdiploid	205	123	0.84 (0.59-1.19)	0.326	Hyperdiploid	1.12 (0.76 - 1.66)	0.567
Age	205	123	1.03 (1.01-1.06)	0.00781	Age	1.04 (1.01 - 1.06)	0.00676
ISS	204	123	1.2 (0.94-1.52)	0.136	ISS	0.97 (0.76 - 1.25)	0.825
Double-hit	205	123	3.17 (2.1-4.8)	4.47E-08			
Overall survival					Overall survival		
	N	n events	HR (95% CI)	Wald <i>P</i>		HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	205	56	3.93 (2.31-6.69)	4.76E-07	SKY92 high-risk	2.92 (1.57 - 5.42)	0.000675
Adverse translocation	205	56	2.26 (1.31-3.9)	0.00357	Adverse translocation	2.01 (1.05 - 3.82)	0.0346
Del(1p) [CDKN2C]	205	56	3.5 (1.83-6.71)	0.000161	Del(1p) [CDKN2C]	1.55 (0.73 - 3.31)	0.254
Del(17p) [TP53]	205	56	4.76 (2.58-8.77)	5.69E-07	Del(17p) [TP53]	3.94 (2 - 7.76)	7.13E-05
Gain(1q)	205	56	2.91 (1.71-4.94)	8.16E-05	Gain(1q)	1.67 (0.9 - 3.12)	0.105
Hyperdiploid	205	56	0.77 (0.46-1.3)	0.334	Hyperdiploid	1.28 (0.69 - 2.37)	0.438
Age	205	56	1.02 (0.99-1.06)	0.256	Age	1 (0.96 - 1.04)	0.989
ISS	204	56	1.19 (0.83-1.7)	0.337	ISS	0.91 (0.62 - 1.34)	0.637
Double-hit	205	56	5.03 (2.93-8.64)	4.98E-09			

a)



b)

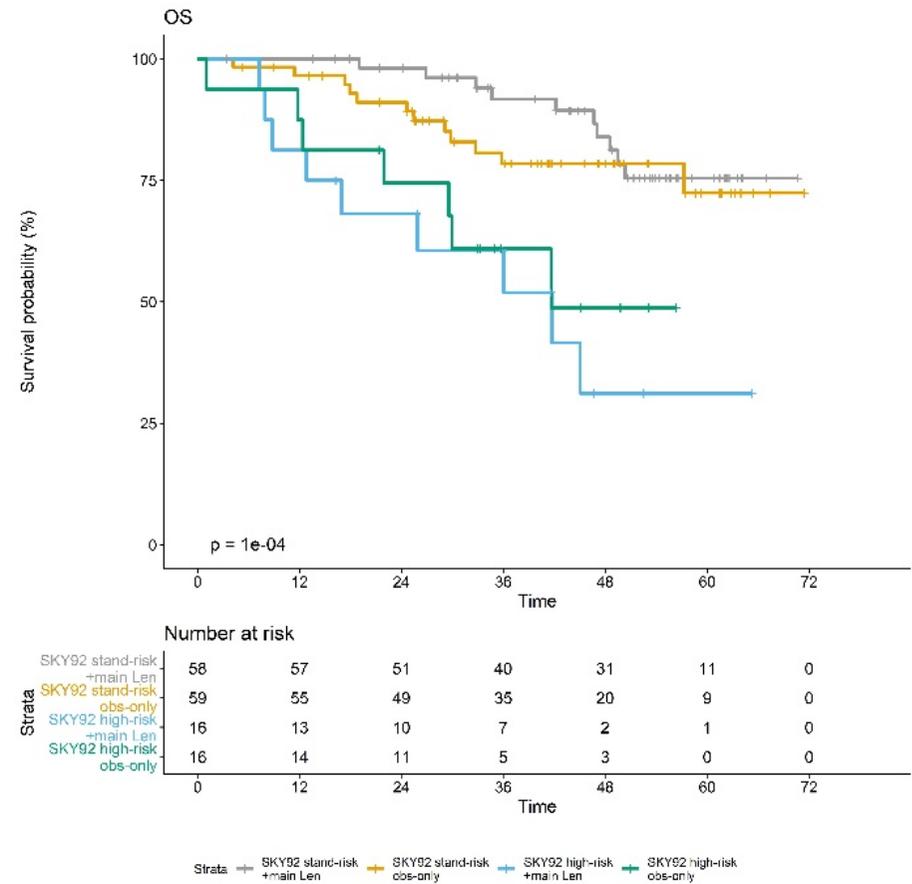


Figure 82: Kaplan-Meier plot of the analysed representative NCRI Myeloma XI trial patients (n=329) in context of SKY92 risk profiling results for a) PFS, b) OS from maintenance randomisation

		Univariate analysis		
Progression-free survival				
	N	n events	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	149	87	3.34 (2.1-5.31)	3.55E-07
Adverse translocation	149	87	2.09 (1.3-3.36)	0.00234
Del(1p) [CDKN2C]	149	87	1.77 (0.88-3.55)	0.108
Del(17p) [TP53]	149	87	2.05 (1.13-3.71)	0.0176
Gain(1q)	149	87	1.73 (1.12-2.66)	0.0128
Hyperdiploid	149	87	0.84 (0.55-1.28)	0.427
Age	149	87	1.03 (1-1.07)	0.0258
ISS	148	87	1.17 (0.89-1.54)	0.257
Maintenance randomisation len vs. observation	149	87	0.42 (0.27-0.65)	0.000116

		Multivariate analysis	
Progression-free survival			
	HR (95% CI)	Wald <i>P</i>	
SKY92 high-risk	2.61 (1.54-4.41)	0.000343	
Adverse translocation	2.01 (1.15-3.51)	0.0142	
Del(1p) [CDKN2C]	1.35 (0.6-3.05)	0.475	
Del(17p) [TP53]	1.94 (1.02-3.72)	0.0444	
Gain(1q)	1.31 (0.77-2.21)	0.32	
Hyperdiploid	1.09 (0.67-1.76)	0.739	
Age	1.03 (1-1.06)	0.0811	
ISS	0.92 (0.69-1.22)	0.563	
Maintenance Randomisation len vs. observation	0.34 (0.21-0.54)	6.74E-06	

Table 73: Cox-based univariate and multivariate analysis landmarked from timepoint of maintenance randomisation to lenalidomide or observation for PFS and OS

		Overall survival		
	N	n events	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	149	38	3.92 (2.04-7.54)	4.23E-05
Adverse translocation	149	38	2.18 (1.11-4.26)	0.0231
Del(1p) [CDKN2C]	149	38	3.2 (1.39-7.36)	0.00607
Del(17p) [TP53]	149	38	4.29 (2.08-8.87)	8.33E-05
Gain(1q)	149	38	3.18 (1.67-6.07)	0.00045
Hyperdiploid	149	38	0.85 (0.45-1.62)	0.63
Age	149	38	1.03 (0.98-1.07)	0.245
ISS	148	38	1.09 (0.72-1.66)	0.677
Maintenance randomisation len vs. observation	149	38	0.88 (0.47-1.67)	0.707

		Overall survival	
	HR (95% CI)	Wald <i>P</i>	
SKY92 high-risk	3.13 (1.46-6.71)	0.00324	
Adverse translocation	1.84 (0.81-4.16)	0.144	
Del(1p) [CDKN2C]	1.26 (0.48-3.35)	0.637	
Del(17p) [TP53]	4.4 (1.94-9.99)	0.000401	
Gain(1q)	2.4 (1.11-5.17)	0.0257	
Hyperdiploid	1.34 (0.61-2.94)	0.466	
Age	1 (0.95-1.05)	0.968	
ISS	0.82 (0.53-1.28)	0.381	
Maintenance randomisation len vs. observation	0.56 (0.28-1.14)	0.108	

6.3.8 SKY 92 GEP high-risk profile is independent of ‘double hit’ in myeloma

We previously demonstrated association of double-hit tumours (≥ 1 established cytogenetic high-risk lesion) with unfavourable outcome in the first results chapter of this thesis. Next we investigated whether SKY92 retained its prognostic association in this setting.

We found that SKY92 and genetic double-hit were both independently prognostic by multivariable analysis, with HRs of 2.9 (95% CI: 1.9-4.2, $P=2.6 \times 10^{-7}$) and 2.3 (95% CI: 1.5-3.6, $P=0.0002$) for OS, and HRs of 2.0 (95% CI: 1.5–2.8, $P=6.8 \times 10^{-6}$) and 1.6 (95% CI: 1.2-2.3, $P=0.005$) for PFS, respectively (Table 74). The association with adverse outcomes was maintained post induction/pre-HDM-ASCT (Table 76) and when assessed from maintenance randomisation (Table 75).

Overall, an estimated 67.8% (95% CI 53.3-77.7%) of patients with double-hit and 68.7% (95% CI 56.7-77.4%) of those with SKY92 high-risk signature tumours progressed or died within the first 24 months from initial randomisation. Based on the specifically adverse outcome for these groups and their independent association with outcome, we split the overall patient group into four risk strata: double-hit AND SKY92 (9.7% of patients), double-hit OR SKY92 (23.4% of patients), one isolated genetic risk factor (24.0% of patients) and no risk factor (42.9% of patients). Hazard ratios for these risk strata for OS were 11.0 (95% CI: 6.3-19.1, $P < 2.2 \times 10^{-16}$), 3.8 (95% CI: 2.3-6.3, $P=2 \times 10^{-7}$) and 1.9 (95% CI: 1.1-3.3, $P=0.03$) in relation to those without risk factor; HRs for PFS were 4.5 (95% CI: 3.0-6.9, $P=2.3 \times 10^{-12}$), 2.3 (95% CI: 1.7-3.3, $P=4.4 \times 10^{-7}$) and 1.3 (95% CI: 0.9-1.9, $P=0.118$), respectively from the time of induction randomisation (Table 77; Figure 83). Similar results were seen for landmarked analyses post-induction/prior to HDM-ASCT (Table 77; Figure 84).

In terms of maintenance randomisation, patients with tumours with no and, in particular, those with a single high-risk marker who were randomised to lenalidomide maintenance had significantly improved PFS compared with those who were randomised to observation only (HR 0.26; 95% CI: 0.12-0.58; $P=0.001$ and HR 0.11; 95% CI: 0.03-0.41; $P=0.0001$, respectively). In contrast, those with either SKY92 high-risk or double-hit (HR PFS 0.67; 95% CI: 0.32-1.37, $P=0.27$) or combined

SKY92 high-risk and double-hit (HR PFS 0.67; 95% CI: 0.12-1.72, $P=0.24$) did not derive consistent benefit from lenalidomide maintenance (Figure 85).

Patients with combined double-hit and SKY92 high-risk status had detrimental outcomes: all patients (100%) progressed within 48 months from initial randomisation and predicted OS at 48 months was 12.5%.

In order to validate our findings, we performed a meta-analysis that included an additional 116 genetically and GEP profiled patients from the transplant treatment arm of the MRC Myeloma IX trial, of whom eight (6.9%) carried combined double-hit and EMC92 high-risk tumours; all patients progressed within 36 months and died within 48 months. Meta-analysis using a random-effect model confirmed the detrimental outcome for this group across both trials, with a HR of 3.5 (95% CI: 2.5-4.9; $P=6.9 \times 10^{-13}$) for PFS and HR of 6.0 (95% CI: 4.1-8.9; $P=4.8 \times 10^{-20}$) for OS.

Table 74: Cox-based univariate and multivariate analysis of PFS and OS from timepoint of induction randomisation

From induction randomisation

			Univariate analysis	
Progression-free survival				
	N	events	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	329	232	2.6 (1.96-3.45)	4.08E-11
Double-hit	329	232	2.36 (1.73-3.22)	5.43E-08
Hyperdiploid	329	232	0.74 (0.57-0.95)	0.0198
ISS	328	232	1.33 (1.12-1.58)	0.0012
Age	329	232	1.04 (1.02-1.06)	0.000115
Induction CTD vs CRD	329	232	1.31 (1.01-1.69)	0.0417
Overall survival				
			Univariate analysis	
	N	events	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	329	117	3.94 (2.73-5.69)	2.54E-13
Double-hit	329	117	3.72 (2.54-5.47)	1.98E-11
Hyperdiploid	329	117	0.6 (0.42-0.87)	0.00717
ISS	328	117	1.38 (1.08-1.76)	0.0101
Age	329	117	1.03 (1-1.05)	0.033
Induction CTD vs CRD	329	117	1.6 (1.11-2.31)	0.0113

			Multivariate analysis	
Progression-free survival				
	HR (95% CI)	Wald <i>P</i>		
SKY92 high-risk	2.02 (1.49 - 2.75)	6.76E-06		
Double-hit	1.64 (1.16 - 2.31)	0.00499		
Hyperdiploid	0.85 (0.65 - 1.12)	0.248		
ISS	1.15 (0.96 - 1.38)	0.12		
Age	1.03 (1.01 - 1.05)	0.000866		
Induction CTD vs CRD	1.18 (0.91 - 1.53)	0.223		
Overall survival				
			Multivariate analysis	
	HR (95% CI)	Wald <i>P</i>		
SKY92 high-risk	2.85 (1.91 - 4.24)	2.63E-07		
Double-hit	2.3 (1.49 - 3.57)	0.000193		
Hyperdiploid	0.83 (0.56 - 1.24)	0.373		
ISS	1.15 (0.88 - 1.49)	0.315		
Age	1.02 (0.99 - 1.04)	0.263		
Induction CTD vs CRD	1.27 (0.87 - 1.85)	0.218		

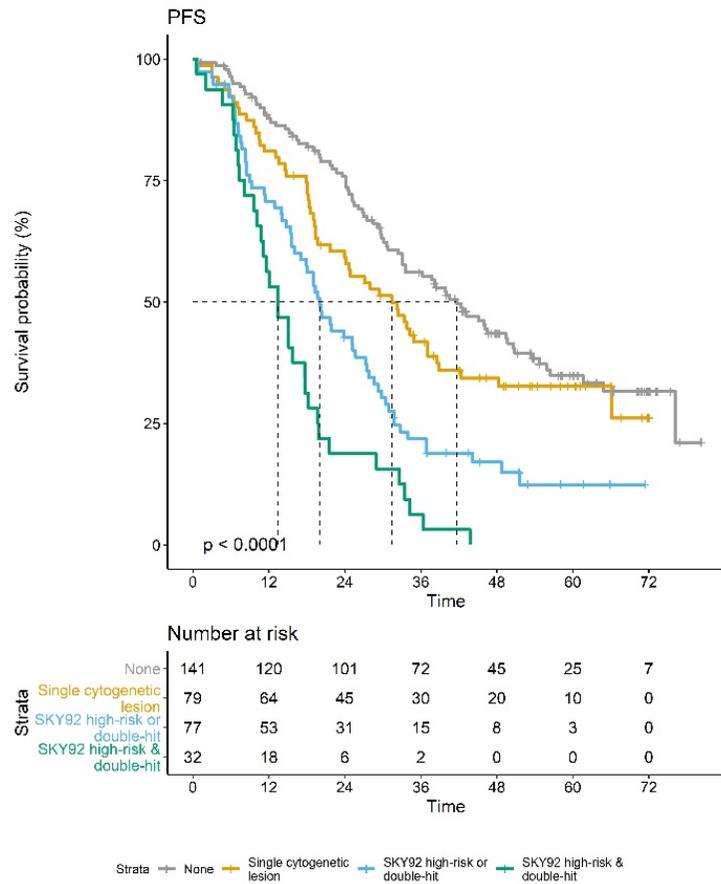
Table 75: Cox-based univariate and multivariate analysis of PFS and OS from timepoint of maintenance randomisation

Landmarked from maintenance randomisation							
Progression Free Survival					Progression Free Survival		
			Univariate Analysis			Multivariate Analysis	
	N	events	HR (95% CI)	Wald <i>P</i>		HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	149	87	3.34 (2.1-5.31)	3.55E-07	SKY92 high-risk	2.71 (1.64 - 4.48)	9.64E-05
Double-hit	149	87	3.15 (1.93-5.13)	4.28E-06	Double-hit	2.78 (1.61 - 4.8)	0.000246
Hyperdiploid	149	87	0.84 (0.55-1.28)	0.427	Hyperdiploid	1.02 (0.65 - 1.62)	0.918
ISS	148	87	1.17 (0.89-1.54)	0.257	ISS	0.92 (0.7 - 1.22)	0.571
Age	149	87	1.03 (1-1.07)	0.0258	Age	1.03 (0.99 - 1.06)	0.107
Maintenance Len vs. Obs	149	87	0.42 (0.27-0.65)	0.000116	Maintenance Len vs. Obs	0.39 (0.25 - 0.6)	2.73E-05
Overall Survival					Overall Survival		
			Univariate Analysis			Multivariate Analysis	
	N	events	HR (95% CI)	Wald <i>P</i>		HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	149	38	3.92 (2.04-7.54)	4.23E-05	SKY92 high-risk	3.31 (1.64 - 6.69)	0.000866
Double-hit	149	38	4.74 (2.46-9.14)	3.27E-06	Double-hit	4.58 (2.15 - 9.79)	8.36E-05
Hyperdiploid	149	38	0.85 (0.45-1.62)	0.63	Hyperdiploid	1.25 (0.61 - 2.56)	0.544
ISS	148	38	1.09 (0.72-1.66)	0.677	ISS	0.79 (0.52 - 1.23)	0.298
Age	149	38	1.03 (0.98-1.07)	0.245	Age	1.01 (0.96 - 1.05)	0.776
Maintenance Len vs. Obs	149	38	0.88 (0.47-1.67)	0.707	Maintenance Len vs. Obs	0.77 (0.4 - 1.5)	0.449

Table 76: Cox-based multivariable analysis landmarked from time point of transplantation for PFS and OS

Progression-free survival		
	Multivariate	
	HR(95% CI)	Wald P
SKY92 high-risk	2.38 (1.56 - 3.63)	5.94E-05
Double-hit	2.5 (1.6 - 3.91)	5.61E-05
Hyperdiploid	1.01 (0.7 - 1.46)	0.962
Age	0.97 (0.76 - 1.25)	0.835
ISS	1.03 (1.01 - 1.06)	0.0144
Overall survival		
	Multivariate	
	HR(95% CI)	Wald P
SKY92 high-risk	3.05 (1.73 - 5.41)	0.000126
Double-hit	4.19 (2.28 - 7.67)	3.59E-06
Hyperdiploid	1.19 (0.67 - 2.09)	0.554
Age	0.88 (0.6 - 1.28)	0.509
ISS	1.01 (0.97 - 1.05)	0.74

a)



b)

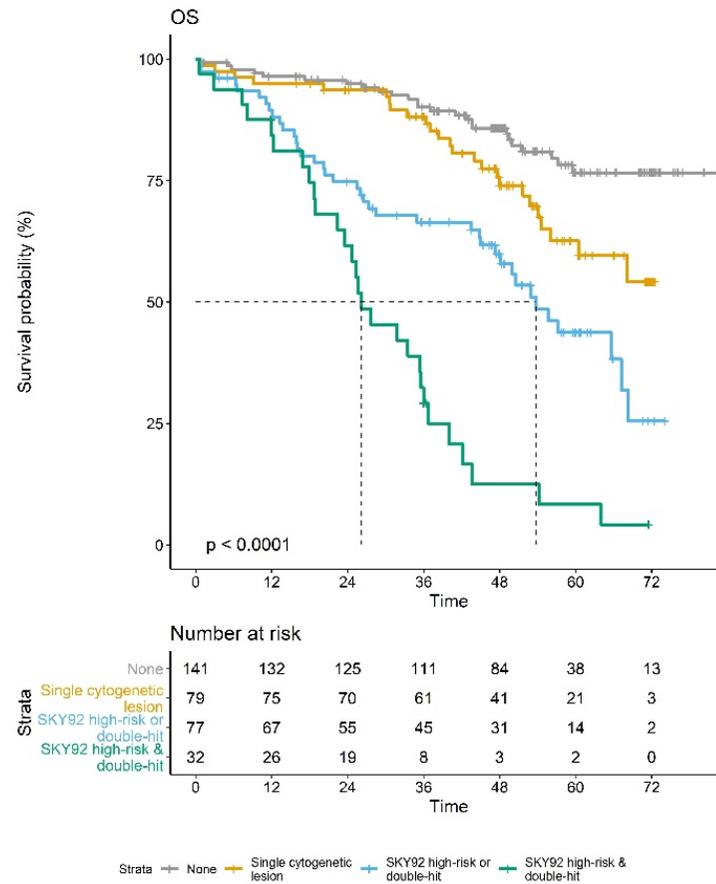
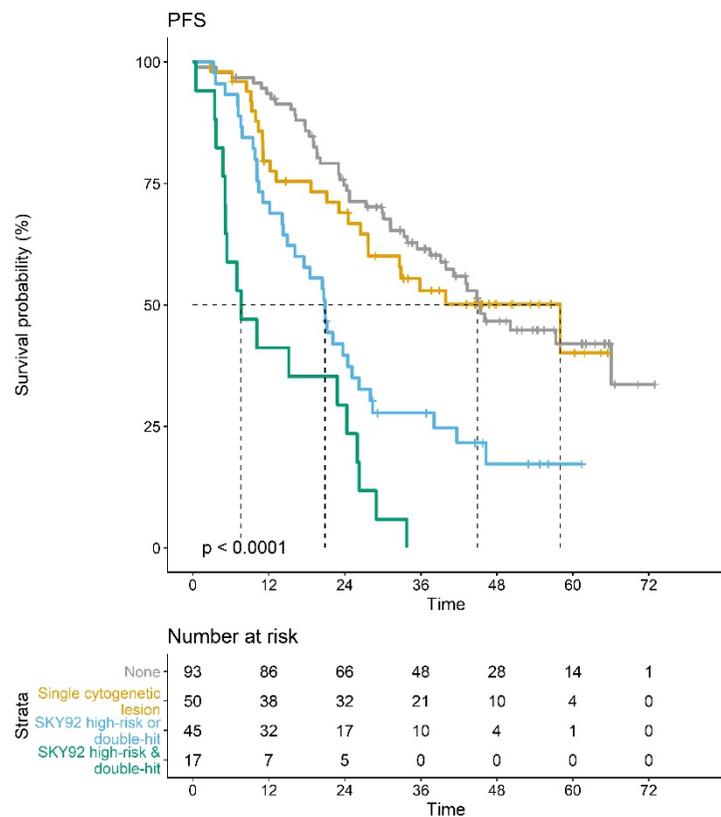


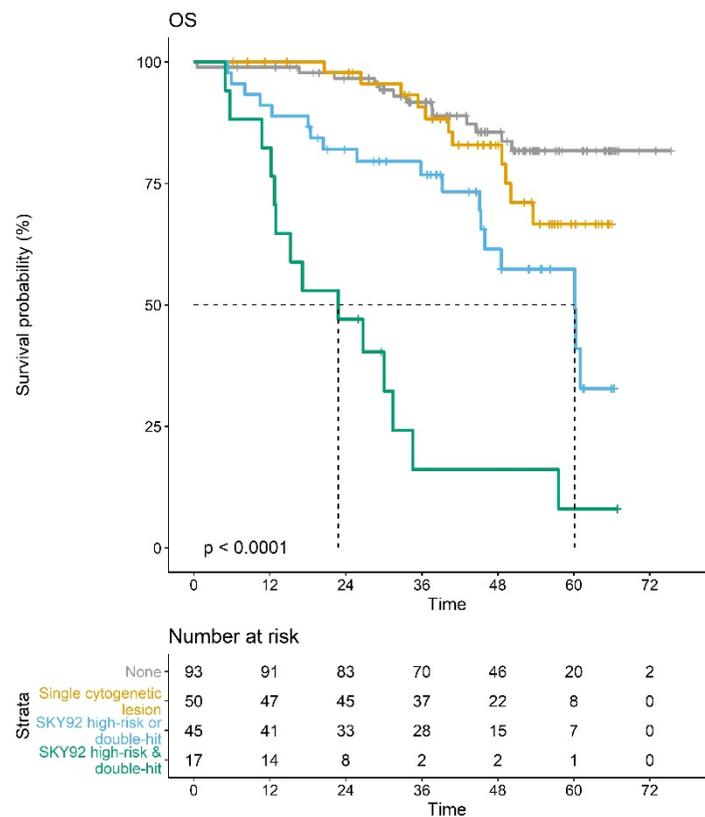
Figure 83: Kaplan-Meier plots of molecular risk groups defined by absence of any high-risk marker, presence of a single genetic marker, presence of either double-hit or SKY92 high-risk or combined double-hit and SKY92 high-risk for a) PFS b) OS from induction randomisation

a)



Strata — None — Single cytogenetic lesion — SKY92 high-risk or double-hit — SKY92 high-risk & double-hit

b)



Strata — None — Single cytogenetic lesion — SKY92 high-risk or double-hit — SKY92 high-risk & double-hit

Figure 84: Kaplan-Meier plots of molecular risk groups defined by absence of any high-risk marker, presence of a single genetic marker, presence of either double-hit or SKY92 high-risk or combined double-hit and SKY92 high-risk for a) PFS and b) OS landmarked from HDM-ASCT consolidation

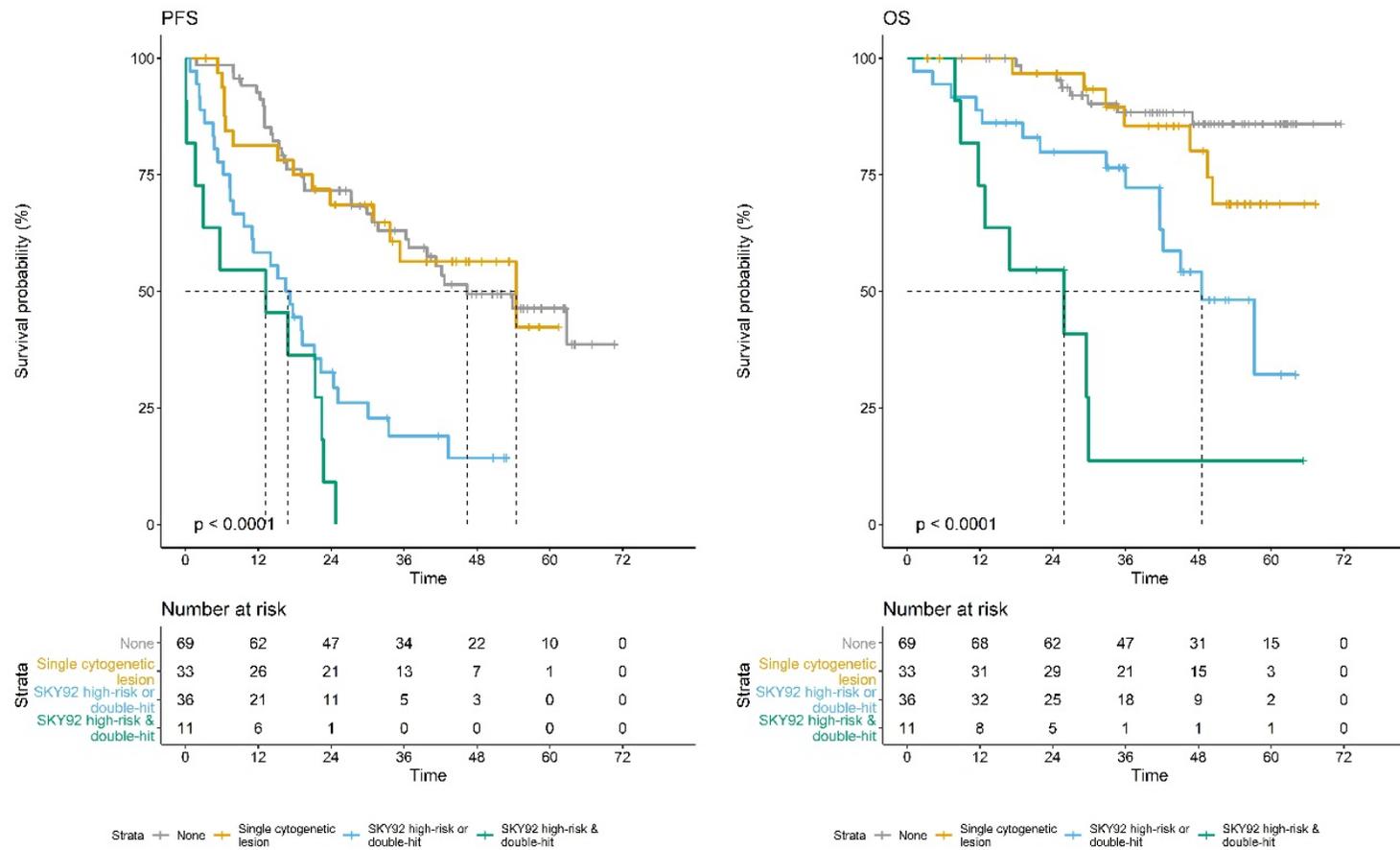


Figure 85: Kaplan-Meier plots of molecular risk groups defined by absence of any high-risk marker, presence of a single genetic marker, presence of either double-hit or SKY92 high-risk or combined double-hit and SKY92 high-risk for a) PFS and b) OS from maintenance randomisation

Table 77: Cox-based multivariable analysis of PFS and OS from time point of a) induction randomisation and b) transplant consolidation of cytogenetic and SKY92 risk scoring strata

a) **Multivariate analysis**

Progression-free survival

	HR (95% CI)	Wald <i>P</i>
No high-risk	1	n/a
1 cytogenetic high-risk lesion	1.32 (0.93 - 1.86)	0.118
Double-hit OR SKY92 high-risk	2.34 (1.68 - 3.26)	4.44E-07
Double-hit AND SKY92 high-risk	4.5 (2.96 - 6.86)	2.25E-12

Overall survival

	HR (95% CI)	Wald <i>P</i>
No high-risk	1	n/a
1 cytogenetic high-risk lesion	1.88 (1.08 - 3.28)	0.0255
Double-hit OR SKY92 high-risk	3.8 (2.3 - 6.29)	2.00E-07
Double-hit AND SKY92 high-risk	10.98 (6.32 - 19.09)	2.01E-17

b) **Multivariate analysis**

Progression-free survival

	HR (95% CI)	Wald <i>P</i>
No high-risk	1	n/a
1 cytogenetic high-risk lesion	1.12 (0.68 - 1.83)	0.653
Double-hit OR SKY92 high-risk	2.61 (1.68 - 4.08)	2.31E-05
Double-hit AND SKY92 high-risk	6.2 (3.49 - 11.02)	4.59E-10

Overall survival

	HR (95% CI)	Wald <i>P</i>
No high-risk	1	n/a
1 cytogenetic high-risk lesion	1.65 (0.74 - 3.69)	0.221
Double-hit OR SKY92 high-risk	3.59 (1.76 - 7.34)	0.000454
Double-hit AND SKY92 high-risk	14.47 (6.69 - 31.26)	1.08E-11

6.3.9 Capture of clinical high-risk with integrated molecular high-risk profiling

The clinical risk markers ISS score and LDH level have been described as independently prognostic and are hence used in combination with t(4;14) and del(17p) in the revised ISS (R-ISS) risk stratifier (69). We noted significantly higher β 2-microglobulin ($P=0.002$) and LDH ($P<0.001$) levels in SKY92 high-risk patients compared with standard risk. Consequently, SKY92 high-risk was significantly associated with ISS stage III (38% vs. 21% in SKY92 standard-risk; $P=0.003$).

ISS status was associated with outcome by univariate analysis, but in the multivariable model that included GEP and genetic risk factors neither ISS nor LDH retained

significant prognostic association for PFS or OS (Table 66;

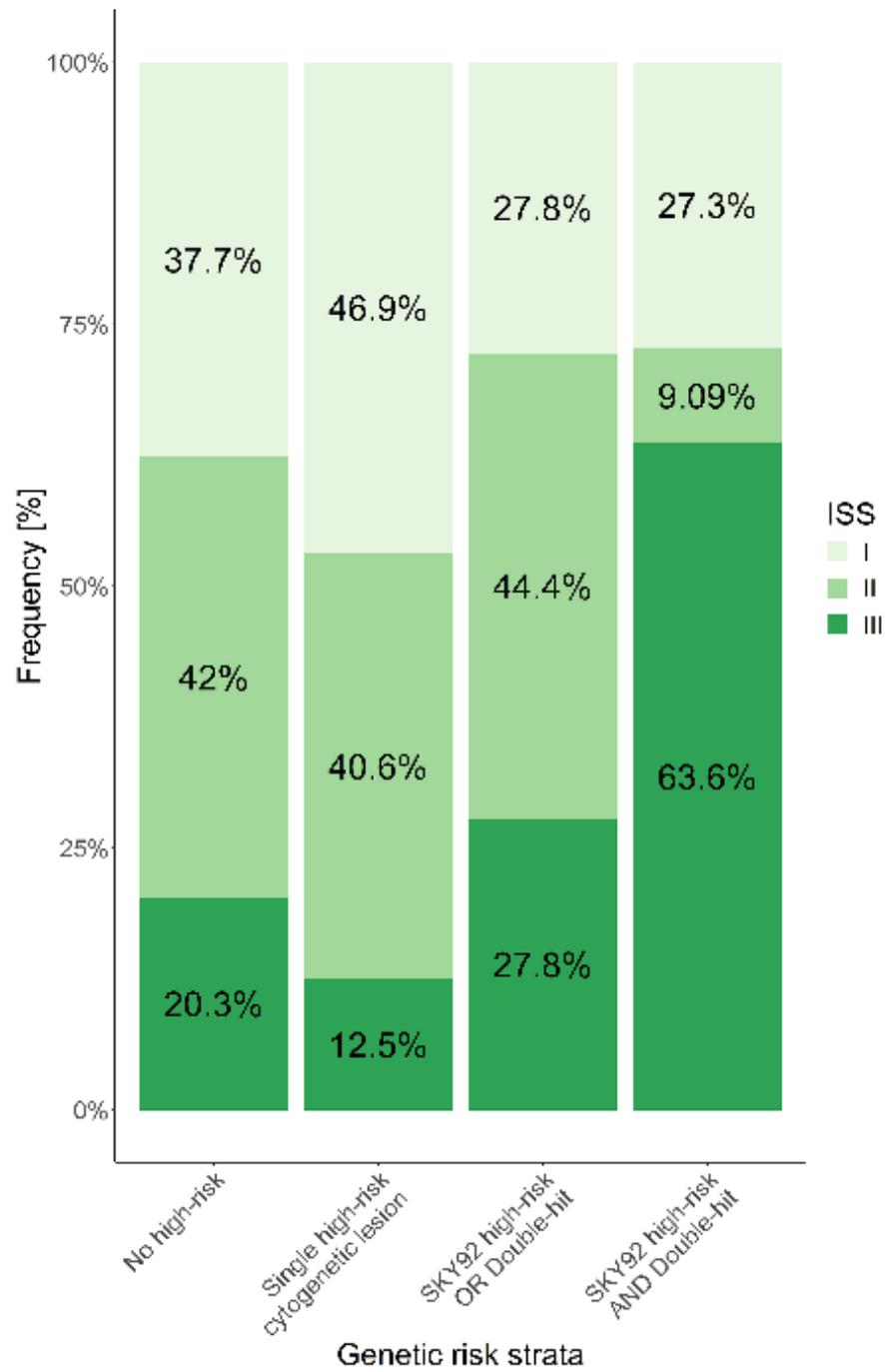


Figure 87: ISS at diagnosis as per defined risk strata.

Table 78: Cox-based multivariable analysis of PFS and OS from induction randomisation including LDH level and other clinical and molecular risk markers

). Patients with ISS stage III were found to be high-risk by genetic or SKY92 profiling in 71% of cases. The proportion of patients with ISS III increased as per the risk strata that are defined above: 21% and 23% in the groups with no or one genetic risk factor, respectively, 29% in patients with SKY92 or double-hit and 44% in those with combined double-hit/SKY92 tumours (Figure 87). Baseline serum LDH concentration also increased along genetic risk strata (mean 280 IU/l vs. 394 IU/l; $P=0.002$ for no high-risk lesion vs. SKY92 high-risk AND double-hit) and there were more patients with reduced platelet count of $< 150 \times 10^9/l$ in the ultra-high risk group (44% vs. 5.7% for no high-risk lesion; $P<0.001$) (Figure 86).

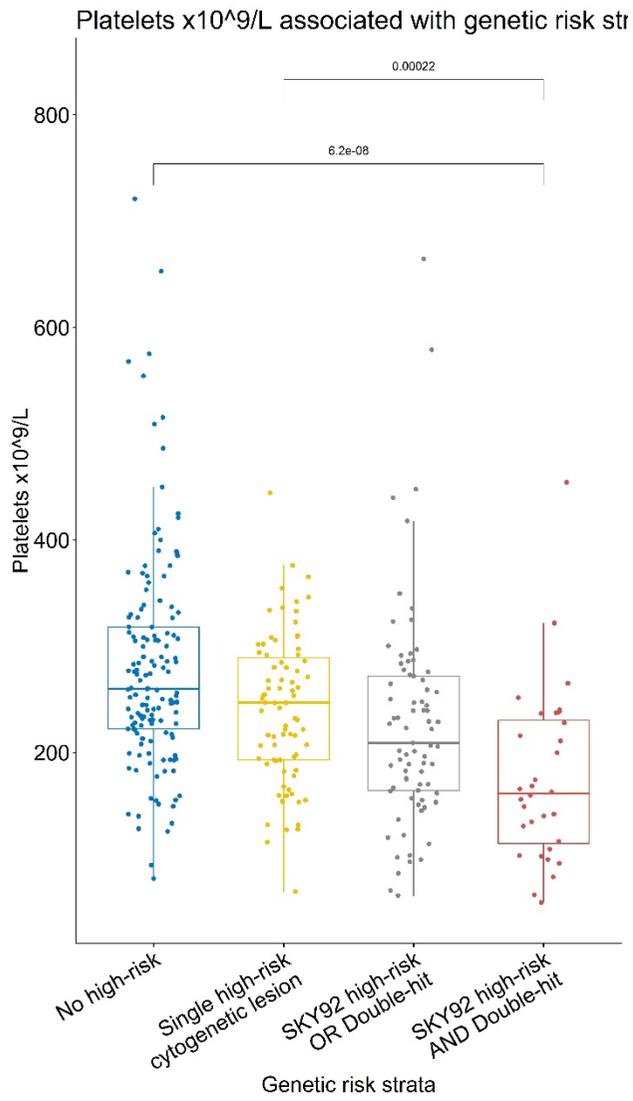
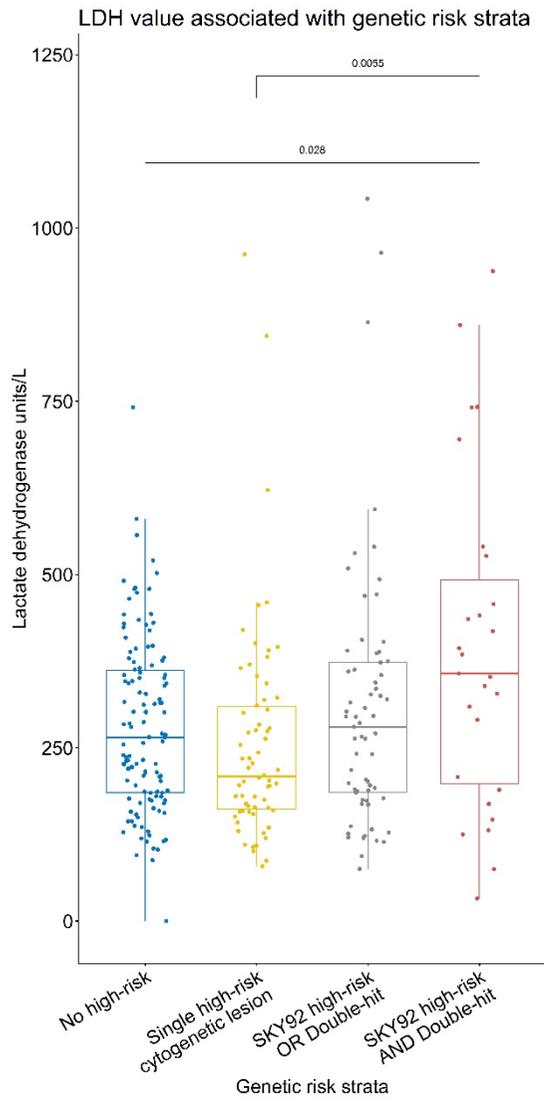


Figure 86: LDH level and platelet count at diagnosis as per defined risk strata.

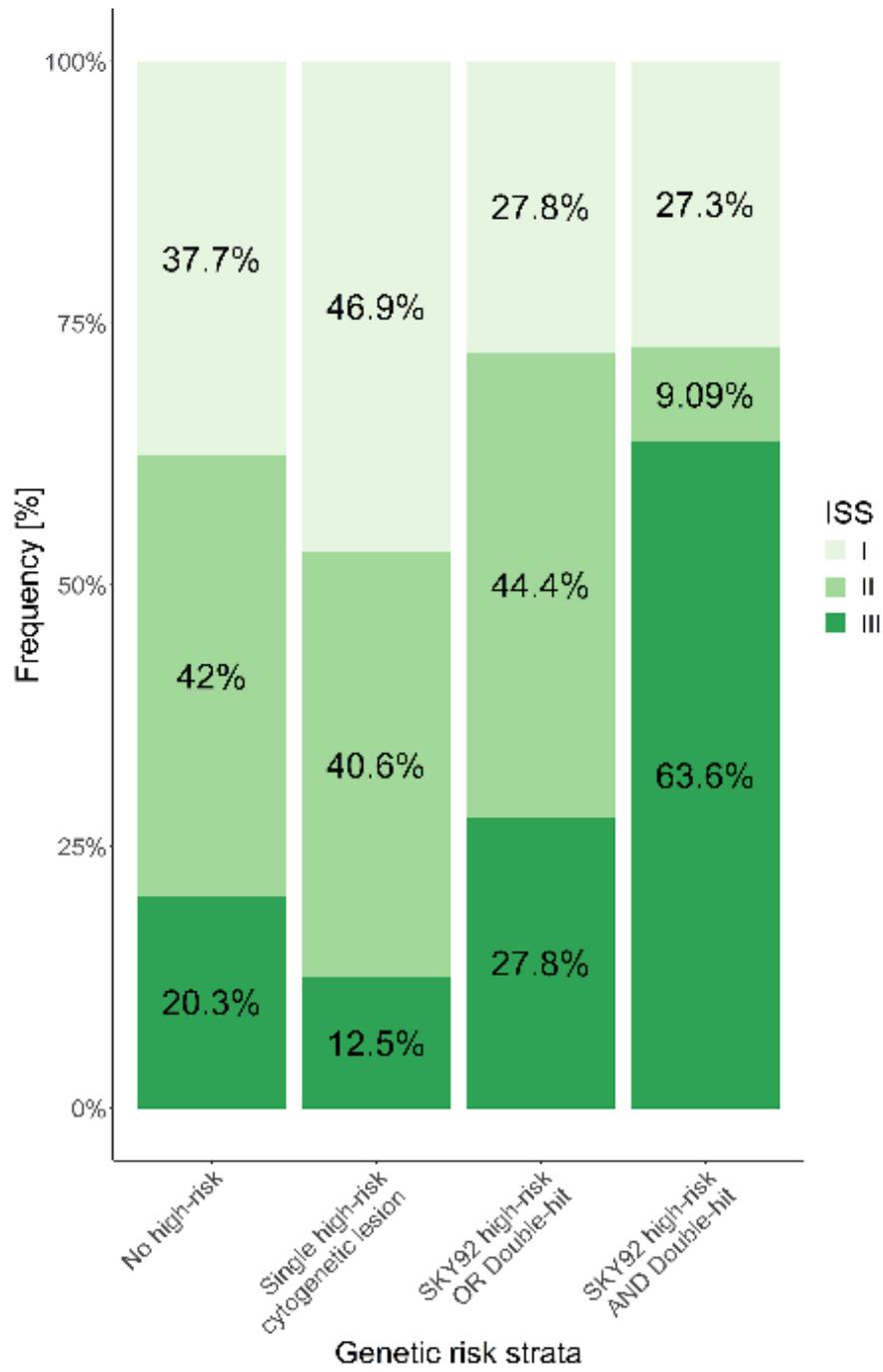


Figure 87: ISS at diagnosis as per defined risk strata.

Table 78: Cox-based multivariable analysis of PFS and OS from induction randomisation including LDH level and other clinical and molecular risk markers

Multivariate analysis

Progression-free survival

	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	1.9 (1.34 - 2.69)	0.000282
Double-hit	1.84 (1.28 - 2.65)	0.000953
ISS	1.09 (0.9 - 1.33)	0.373
Age	1.03 (1.01 - 1.05)	0.00221
Induction CTD vs CRD	1.13 (0.85 - 1.5)	0.404
Lactate dehydrogenase units/l	1 (1 - 1)	0.805

Overall survival

	HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	3.01 (1.93 - 4.7)	1.30E-06
Double-hit	2.24 (1.41 - 3.57)	0.000631
ISS	1.12 (0.84 - 1.49)	0.449
Age	1.02 (0.99 - 1.05)	0.156
Induction CTD vs CRD	1.25 (0.83 - 1.9)	0.284
Lactate dehydrogenase units/l	1 (1 - 1)	0.283

6.4 Discussion

Our results demonstrate the independent prognostic association of the SKY92 signature compared with several other signatures. This includes the only other gene expression signature that is commercially available for diagnostic prognostication in multiple myeloma, the UAMS70 risk signature.

We found 6.1% of patients who had a high-risk gene expression signature in the absence of any other high risk chromosomal abnormality. In clinical practice, identification of this small subset of patients has to be balanced against the high cost of gene expression based risk calling and the fact that these patients are likely to declare themselves as high risk over time. Adoption of this method of risk stratification into routine clinical practice is only plausible if there is a demonstrable difference in overall survival through early identification of this subgroup of patients.

The EMC92/SKY92 signature was developed based on the HOVON-65/GMMG-HD4 dataset of proteasome inhibitor-treated patients (272). Our results demonstrate the prospective prognostic validity of use of SKY92 profiling in the wider context as a means of identifying patients at diagnosis who have high-risk MM in a largely IMiD-based study. The signature therefore is not specifically associated with resistance to specific treatment of myeloma, but to aggressive disease itself. In the era of constantly changing treatment, its validity in our study in addition to the HOVON-65/GMMG-HD4 trials may predict its validity in triple therapy-based regimens and perhaps also in novel antibody-based regimens. Further trials will elucidate this. The MUK9 study of treatment in high-risk patients that combines IMiD (lenalidomide), proteasome inhibitors (bortezomib) and anti-CD38 antibody treatment (daratumumab) upfront in the newly diagnosed setting should help to answer this question further. The results from the Myeloma XI trial will help to form a comparator with patients in the MUK9 trial (NCT03188172) as established within its protocol.

Structural aberrations that include CNAs are increasingly recognised as key drivers of prognosis across multiple cancers (400, 401). Our results also show the independent association of SKY92 and established high-risk chromosomal aberrations with

outcome. Our results highlight the molecular diversity of MM that has been elucidated by combined GEP and chromosomal high-risk profiling

Challenges of the identification of deletion of 17p/*TP53* by GEP have been reported previously (379). Within our study, del(*TP53*) has been consistently and independently associated with poor prognosis congruent with the finding that many cases of del(17p) are not found to be high risk by gene expression signatures such as SKY92. Improvement of such signatures in the future therefore could focus on improving the ability of such signatures to recognise CNA associated changes in gene expression more closely.

Conversely, gain(1q) was not consistently associated with shorter survival by multivariable analysis when GEP signatures were added to the analysis. This is consistent with significant overlap of SKY92 with gain(1q21) within our study suggesting GEP is able to pick up this abnormality with higher accuracy. However overlap of this abnormality with SKY92 is still incomplete (100). We did however find that gain(1q) was still independently associated with a poorer prognosis in the context of maintenance IMiD therapy. This finding raises the possibility that gain(1q) may interact with IMiD-based therapy. Interaction studies of maintenance with gain(1q) however showed no statistical significance.

Likewise, a substantial group of patients with high-risk tumours are not identified through use of DNA chromosomal aberration profiling alone, which demonstrates the additional value of GEP-based risk scoring.

The finding that more than 70% of patients with ISS III status show molecular high-risk markers suggests that integrated molecular diagnostics can increase specificity over clinical proxy markers. This finding is particularly relevant in the context of individual patient treatment stratification. Additionally, a high degree of correlation that is shown between LDH levels and increasing numbers of high-risk markers also demonstrates this. ISS scores and serum LDH levels are widely used as surrogate markers for tumour burden and/or proliferation; however, both can be sensitive to biological (*e.g.* β -2-microglobulin in renal failure) and/or technical (*e.g.* LDH levels in haemolysis) confounders. We demonstrate that, in the context of combined SKY92

and chromosomal profiling, ISS ratings and LDH levels are not independently predictive. This is perhaps to be expected, since GEP risk scores reflect, at least partly, proliferation, and therefore complement the information on genomic instability that is assessed by chromosomal profiling. Our analysis was, however, limited to younger, fitter, transplant-eligible patients compared with average MM patients, and clinical risk markers such as ISS may have greater and independent relevance in older or frailer patients (402).

We demonstrate, for the first time, that the SKY92 gene-expression signature is independently prognostic in the setting of transplantation. We show that transplantation does not abrogate the association with short survival with SKY92 high risk in a transplant-landmarked analysis. We also demonstrate that few patients are able to progress even to receive a HDM-ASCT in the setting of SKY-92, which suggests that perhaps early death and resistance to treatment may prevent these patients from progression to receive this form of therapy. However, we demonstrate that patients with SKY92 high-risk signatures are associated with a poor WHO performance status, which may in turn offer a challenge to the delivery of this kind of treatment to these high-risk patients.

Additionally, we demonstrate that single timepoint combined GEP and chromosomal high-risk profiling at diagnosis, without longitudinal re-sampling or post-hoc response assessment, can predict clinical outcome with significant precision. The strength of complementary prognostic transcriptional and chromosomal/aneuploidy profiling is in line with recent reports across a number of cancers (400, 401).

Importantly, randomisation of maintenance arms within this study has enabled us to identify differential responses to maintenance treatment in the context of the SKY92 risk signature, which may help clinicians to choose maintenance regimens in this setting. Our results clearly show that patients with double-hit or GEP high-risk status are unlikely to benefit from current treatment approaches, which include single-agent lenalidomide maintenance therapy. In such patients, intensified ongoing therapy with combination agents may be beneficial but should be investigated (147). This will be prospectively assessed in clinical studies such as the prospective, risk-stratified UK MUK9 trial.

Our results further demonstrate that patients with double-hit or GEP high-risk status do not seem to benefit sufficiently from current treatment approaches, including lenalidomide maintenance therapy. In particular, patients with combined double-hit and GEP risk features are in urgent need of improved therapeutic strategies.

The specific genes that are identified as high risk in the different signatures have little overlap. This reflects the nature of the high number of co-variables within gene expression arrays (403-405). We show a high degree of overlap of the SKY92 signature with other prognostic signatures that are produced by other laboratories despite little overlap in the probes or genes that are used within these signatures. This finding suggests that signatures identify similar pathways but that the large number of variables does not enable the easy identification of specifically targetable genes by this method. This is a common issue with high-dimensional data due to issues with assessment of co-linear variables.

Despite this, most GEP signatures seem to capture core characteristics of high-risk disease. This assumption is suggested by the correlation that is observed between most signatures, especially in identified high-risk patients (399). However, there may be other differences in expression signatures, such as differences in the methods used for signature construction, the patient populations, and the therapies that are used, which vary greatly between different research groups (399). Unsurprisingly, the signature with least correlation to all other signatures, which were based on patient samples, was the cell line-based gene expression signature. This is likely to reflect the real differences between cell lines and patient-based research.

There are significant challenges to delivery of gene expression signatures to the clinic. The first of these is to do with technical reasons. Although the Micro Array Quality Control (MAQC) consortium has demonstrated through comprehensive analysis that the technology is reliable for clinical and regulatory purposes (406, 407), the evaluation was performed on high-quality RNA that was extracted under optimal conditions. RNA degrades quickly and old samples may have issues regarding quality. Within the Myeloma XI trial, only a subset of data from patients could be analysed through this method due to sample quality requirements. In addition, the MUK9 trial,

which is prospectively validating this signature to assign risk for adaptation of therapy, had several issues with sample quality. For example, to gain adequate samples, many patients had to undergo repeat bone-marrow biopsies. In clinical practice, these issues are even more challenging, and it is likely that many patients would not be able to have undergo such analysis due to pre-analytical issues. Dying cells may also alter their transcription profile altering the risk signature results.

Gene expression arrays also often fail quality parameters downstream of RNA quality issues. Running expression arrays require a high degree of expertise to run reliably and consistently. This is demonstrated by batch specific differences in arrays that frequently need to be accounted for through software such as COMBAT to compensate for such differences.

Thousands of such signatures have now been developed for cancers across the world. However, given that high rates of false-positive results occur, very few have been validated or been introduced to routine diagnostics for tumour profiling (408). Gene expression profiling is a resource-intensive investigation with significant issues with regard to reproducibility and sample quality issues. It is not routine to profile MM patients through use of this method in the vast majority of centres across the world. Validation of such signatures through comparison as well as investigation of whether they add value to current methods of prognostication is important to discover whether they can ever be used routinely in the management of MM or whether they should remain a research tool only.

7 Discussion and conclusions

Despite significant progress in the development of treatment in the last two decades, the outlook for MM patients is still relatively poor as the disease remains incurable and only 33% of patients survive for up to 10 years after diagnosis (7). Part of the reasons for this are genomic instability and clonal evolution in response to treatment pressures, and acquisition of genetic mutations all of which result in relapse and progression of the disease. As a result, there is interest in the investigation of genomic aberrations and mutations that drive disease progression, to gain an improved understanding of disease biology and to develop targeted therapies. As novel treatments are developed, the prognosis that is associated with known genetic lesions is changing. The broad aims of this project were the identification of risk factors and investigation of genetic lesions to define risk groups that could be applied in a practical fashion to the treatment of patients.

7.1 Findings in this study

The findings of the work that is described in each chapter have been discussed within the thesis, but we provide a summary of the findings in this section. This project started with a broad overview of high-risk cytogenetic data in both the Myeloma XI and Myeloma IX trials. We found continued poor prognosis that was conferred by the known high-risk genetic markers $t(4;14)$, $t(14;16)$, $t(14;20)$, $del(17p)$ and $gain(1q)$. This finding came despite use of the latest therapies in the Myeloma XI trial. We found an especially poor prognosis in patients with double- and triple-hit myeloma, which was consistent with previous findings from Myeloma IX. We went on to validate the combined cytogenetic risk and ISS signatures that were developed through application of the results of the Myeloma IX trial. In addition, the analysis from this trial demonstrated that high risk was not abrogated in patients with hyperdiploidy and defined molecular subgroups of hyperdiploid myeloma, which appear to be correlated with specific copy number changes and cyclin-D expression.

Next, we addressed several areas of debate that involve important clinical applications in myeloma with regard to high-risk cytogenetics. Due to the ongoing debate with

regard to the association of survival periods with del(17p) and clonality, we evaluated this through numerous methods to find the optimal cut-off for prognostic association with regard to del(17p). We used several statistical methods of evaluation to investigate this lesion robustly in the context of data from our trial. We found that subclonal lesions (<60%) were still associated with shortened survival periods in all stages of treatment within the trial, including induction randomisation as well as landmarked analyses post-transplantation and maintenance randomisation.

Furthermore, we found no evidence of improvement in survival rates when thalidomide treatment was compared with lenalidomide at induction in the context of this lesion. We also found no evidence of improvement in survival rates when lenalidomide maintenance was compared with no maintenance in the context of this lesion. Our results demonstrated some improvement in survival in the limited number of patients who received a short, response-dependent course of bortezomib-based intensification after randomisation. However, despite improvement, the higher risk that was associated with this lesion was not abrogated. This is in line with other study findings (62).

Our analysis of genetic and clinical association in the context of these lesions led to interesting findings of correlation between del(13q) and del(17p) and the possibility of co-evolution of these lesions, with the primary lesion being del(13q). Findings from our study were limited as only bulk DNA methods such as MLPA could be utilised and there was a limited selection of samples that were suitable for iFISH that were available at the time. Future work to validate these associations should utilise single-cell sequencing methods to validate these findings. Additionally, samples from relapse tumours may also help to validate these findings.

The positive key clinical associations of poor performance status and del(17p) suggest that frailty, which is considered to be an independent patient-associated prognostic factor, has associations with high-risk genetics. These inter-relationships have clinical implications in the form of delivery of therapy for these patients, as discussed in the chapter. The association with LDH levels and del(17p) also demonstrates the inter-relatedness of patients with biochemical markers of risk (as LDH is part of the R-ISS prognostic score) and high-risk cytogenetics. This is a feature we demonstrated again

within the work that was performed on gain(1q) and which is described in the relevant chapter of this thesis.

Lastly, we integrated copy number and exome sequencing results to examine mono- and bi-allelic *TP53* aberrations and found that mono-allelic aberrations continued to have a prognostic impact. The presence of bi-allelic aberrations was associated with a particularly poor prognosis. The elucidation of the mechanisms by which mono-allelic aberrations are associated with poor prognosis would need further experimental and longitudinal tumour analysis as detailed in the *TP53* chapter of this thesis. Evaluation of the *TP53* mutational status of patients should move urgently into the clinic and should be reported routinely in clinical trials, to improve therapies for this specifically adverse lesion. Of note, it was the recognition of the abysmal prognosis conferred by aberrations in *TP53* which led to the rapid development of therapies for patients with CLL with this lesion such as BTK inhibitors.

We went on to evaluate another key high-risk cytogenetic marker, gain(1q), in the work that was described in the next chapter of this thesis. We analysed two separate studies that used different methods of analysis of 1q copy number status the distribution of these abnormalities in newly diagnosed patients. We went on to find an association with survival of both gain(1q) and amp(1q), which is currently under intense debate.

We examined both gain(1q) and amp(1q), which are likely to be part of the same spectrum of disease, in terms of clinical and genetic associations. We showed that there was a progressive increase in both LDH levels and ISS ranking with increasing 1q copy number. This finding also has key implications with regard to the current prognostic indices that are used in clinical practice.

Although we found occurrence of gain(1q) and amp(1q) in all primary cytogenetic abnormalities, there was a lower incidence of both these abnormalities within the hyperdiploid and t(11;14) subgroups of MM compared with other subgroups. Conversely there was a higher incidence in the t(4;14), t(14;16) and t(14;20) subgroups of MM. We have described a subgroup of HRD which is positively associated with gain(1q) and del(13q) in the first results chapter of this thesis. This subgroup was

found to be associated with high cyclin D2 levels. The t(4;14), t(14;16) and t(14;20) lesions all appear to demonstrate correlation with gain(1q). The high incidence of patients with amp(1q) within these MM subtypes suggests a dependency of disease progression on this lesion, which has presumably developed from gain(1q). As with the HRD-gain(1q) subtype of MM, all these lesions also show associations with del(13q) and cyclin D2 over-expression. Together, these findings suggest that the cyclin-D2-dependent subtypes of MM may depend on increasing 1q copy number and or other copy number abnormalities to progress to the symptomatic stage, but that the cyclin-D1 subtypes do not. However, despite these associations, a causal relationship cannot be established with regard to this inference. Discovery of such a relationship will again rely on performance of single-cell studies and longitudinal sampling to investigate this further.

The final chapter of this thesis focused on high-risk gene expression signatures that were commercially available in the clinical setting. We validated the SKY92 signature in terms of its prognostic-independent association with short survival periods. We also explored the inter-relationships between the cytogenetic high-risk variables that were explored in the work described in the previous chapters and the gene expression signature. We found an independent prognostic association of del(17p) and high-risk translocations within this analysis. Furthermore, we demonstrated that the combination of the presence of high-risk chromosomal aberrations and high-risk genetics identified an especially high-risk association, which was stronger than double-hit MM. These results can be readily applied in the clinical setting but, as mentioned in the next section, will be compared with the high-risk MUK9 trial, which intensifies treatment that is dependent on risk.

I summarise my findings from the thesis with a hierarchy of risk associated lesions in reference to newly diagnosed MM.

Table 79: Table summarising hierarchy of risk markers in newly diagnosed MM.

Ultra-Low risk	No high risk translocation, Gain(1q), Del(17p), SKY92 standard risk, Normal LDH, ISS I
Low risk	No high-risk translocation, Normal LDH, ISS 1
Standard Risk	1 High risk lesion (excluding del(17p))m+ ISS I or ISS II
High Risk	≥2 high risk lesions (excluding del(17p)) Monoallelic TP53 alteration SKY92 high risk
Ultra-High-Risk	≥2 high risk cytogenetic lesions including of del(17p) ≥2 high risk cytogenetic lesions and SKY 92 high risk Bi-allelic TP53 alteration

7.2 Critical appraisal

The work that is described in each chapter has been discussed in the context of known work within the field in the discussion sections of the results chapters. Here we discuss the main disadvantages and advantages of the work as a whole.

A key advantage of these data is that they are derived from two large, well-conducted, randomised controlled trials with broad inclusivity. Often clinical trials are restricted to young and/or fit patients, which limits the applicability of analyses to the general population. As shown through the eligibility requirements for both trials, both the Myeloma IX and XI trials included patients of all ages who were not restricted from entering due to performance status. Additionally, both trials allowed entry of patients with poor renal function.

The trial is well annotated with robust data. The genetics datasets that are available from the largest pool of data in the world; results from many thousands of patients have been analysed. The size of this dataset improves the power to detect associations and enables subgroup analysis that would not be possible with smaller datasets. Integration of clinical data, subclonal copy number changes and gene expression provided a holistic assessment of clinical and molecular associations of specific lesions.

Despite their advantages, both trials have limitations. The inclusion of bortezomib, which became the standard-of-care for patients with renal dysfunction in myeloma, and lenalidomide dosing are limited in renal dysfunction, which potentially limits treatment for these patients within the trial. Applicability of our findings in the setting of patients with renal dysfunction may therefore be more limited.

Additionally, triple therapy with the inclusion of a proteasome inhibitor and an IMiD became the standard-of-care during the course of patient recruitment for the Myeloma XI trial. Although another treatment arm was added (CCRD), the results of the analyses of this report had not reached sufficient follow-up for the clinical trials unit to release endpoints from this arm. This situation therefore limits the applicability of our study to some extent. Some of our findings also need validation therefore once the CCRD arm survival results are released or with other trials which involve triple-therapy induction. However, as velcade became standard of care as second line treatment during the trial, response to proteasome inhibitors has, at least in part, been taken into account within evaluation of overall survival of this analysis.

Both therapies and diagnostics within myeloma are moving rapidly. The latest antibody-based therapies such as daratumumab therapies show remarkable efficacy in the newly diagnosed setting. The definition of high-risk disease may change with the adoption of new treatments. This is another limitation of our evaluation. The addition of daratumumab has been shown to be beneficial for cytogenetic high-risk patients both in the front-line transplant-eligible and transplant-ineligible, newly diagnosed setting. This is shown with VTD (227), VRD (228), VMP (229) and RD (230). However, all these studies still demonstrate that patients with high-risk cytogenetics show poorer responses than standard-risk patients. Additionally, high risk is defined

as the occurrence of del(17p) and t(4;14) in most of these studies, and there is limited or no analysis of the effect of individual cytogenetic abnormalities. As new, clearly efficacious treatments are adopted into the front-line setting, it is important to request analyses of individual, defined, high-risk lesions to improve treatments further.

The tumour samples that were investigated in this study were all taken from bone-marrow biopsies from the iliac crest. Recently, studies have shown that myeloma involves complex spatial heterogeneity (409). The data that were used within this study could not be employed to study such heterogeneity and this may be part of the reason why prognostic models are not able to predict completely which patients will do badly with upfront therapy. New technologies such as those that measure circulating tumour DNA may help to investigate this tumour heterogeneity in the future. The heterogeneity may also be partially represented by biochemical markers such as LDH levels and ISS ratings, which are independent of tumour site.

Limitations within the study were also due to the methods that were used for evaluation. As I was not able to access all sequencing data within these studies, concurrent evaluation of mutations with copy number was more limited. However, such studies, as with MLPA, all rely on bulk tumour sequencing. The use of the Myeloma IX trial data has therefore enabled exploration of single-level events and the correlation of single-cell with bulk-tumour sequencing methods to be demonstrated, especially within the gain(1q) results chapter. It highlights the importance of the study of single-cell-based methods for the evaluation of tumour heterogeneity and single-cell sequencing will enable the evaluation of several interactions of copy number and mutational analysis at the single-cell level at the same time.

Although the large numbers of patients who were included in the evaluation of chromosomal aberrations in this study allowed for a comprehensive assessment of the association of lesions with clinical outcomes, the more limited availability of gene expression arrays and next generation sequencing data restricted the ability to evaluate these in as great a depth, for instance within subgroups. As more sequenced samples undergo additional testing, these analyses will become possible with time.

Survival analysis for Myeloma XI that is described in the first chapter had a short follow-up. Myeloma IX analysis, on the other hand, had a much longer follow-up period. As the project progressed, longer follow-up data were shared but assessment of the above findings with further follow up will be important to discover whether any findings are altered. This was also a major shortcoming of the original whole exome sequencing study that was based on the Myeloma XI trial, in which re-evaluation of several genetic associations with mutations with longer follow up available to us suggested that *TP53* was the only mutation which had a persistent association with shortened survival.

7.3 Future directions

There are several elements of this work that will be taken forward within this research group. Some will continue in the context of Myeloma XI, with further investigation of newly diagnosed samples that undergo whole genome and RNA sequencing analysis. This work is underway by other members of the group. Whole genome sequencing will provide improved genomic coverage, coverage of non-coding transcript analysis as well as more reliable copy number calling. RNA sequencing will enable the identification of differentially modulated transcripts, splice variant analysis, novel transcripts and single nucleotide variants.

In addition, this work has demonstrated that there is essentially missing information with regard to genetic aberrations and gene expression with regard to prognosis. The study of epigenetic changes is important for the investigation of these alterations as there is evidence from previous studies of the importance of epigenetic changes in MM; they may be the cause of some differential gene expression that is evaluated by gene expression risk signatures. Differential methylation has been demonstrated in MM (410). Several epigenetic modifiers are also known to be mutated in MM (37). Additionally, in the clinical setting, histone deacetylase inhibitors demonstrate activity in relapsed/refractory MM (217-220). More than 500 newly diagnosed tumour samples have undergone methylation array analysis and evaluation of this work with its correlation to genomic aberrations, gene expression and treatment resistance is currently underway (411). This along with other research teams' work may aid in

addressing some of the knowledge gaps in terms of how epigenetic changes also influence gene expression. If changes are found that correlate with gene expression, these methods may be easier to evaluate in the diagnostic setting as they have fewer issues with sample quality than RNA-based evaluation.

In addition to this, more than 500 patient samples from the Myeloma XI trial have undergone high resolution SNP array analysis. Integration of minimally amplified regions in 1q and the gene expression arrays may allow further evaluation of this region and other regions of interest.

Limited work has been conducted on the genetics of relapsed myeloma compared with newly diagnosed myeloma. It is clear that both the mutational burden and the numbers of chromosomal aberrations increase with disease progression as the presence of pathogenic mutations, bi-allelic events and copy number gains increase, as has been detailed in the previous chapters (91, 114, 131, 412).

In this project, we have explored tumour heterogeneity in the newly diagnosed setting prior to the application of treatment selection pressure. The Myeloma XI trial also evaluated patient tumour samples at the time of relapse. These samples have been evaluated both by whole exome sequencing (413) and by copy number analysis and gene expression (data not published). Validation of some of the associations that were found in this study is being actively researched within the group.

In terms of clinical information, survival data with regard to carfilzomib-based triple therapy (CCRD) will mature shortly and will enable comparison in the context of genetic lesions with lenalidomide and thalidomide-based induction. I have curated data regarding genetic lesions and initial analysis of progression-free survival has been completed. This work has more applications in the modern treatment algorithms, as triplet therapy has now become the standard-of-care for remission induction in several centres across the world. Together with longer follow up and additional clinical data, these results will also enable the development of accurate prognostic models.

Other trials are also underway that are aimed at addressing the treatments within the high-risk newly diagnosed MM setting (414-416). Direct comparison of the work that

was described in the gene expression signature chapter of this thesis will occur; there will be comparison of 446 Myeloma XI patients with gene expression array, copy number and translocation results from the MUK9 trial (NCT03188172) (416), which treated patients based on high-risk ratings that were assigned either by gene expression or genomic aberration studies (>1 of gain(1q21), del(17p), del(1p32), t(4;14), t(14;16) or t(14;20)). This project has resulted in the curation of these patient tumour samples within the Myeloma XI trial and the results will be compared directly with those of MUK9 by trial design to evaluate whether these patients have improved overall survival with more intensive, prolonged treatment for MM. A disadvantage of the approach that was applied in the Myeloma XI trial was the use of a historic high-risk MM arm rather than the randomisation arms that have been used in other high-risk MM studies. Although there may be some validity in comparing PFS in this context, the more valid outcome of OS cannot be compared appropriately as treatments overall within MM have advanced over this time period and will impact on overall survival rates.

Another high risk trial is the SWOG 1211 trial which uses a randomised trial design to assign patients that have been defined as high risk to elotuzumab-VRd vs VRd (415). The definition of high-risk MM in this trial includes the GEP70 gene expression model, t(14;16), t(14;20) and/or deletion (17p), primary plasma cell leukaemia or LDH levels that are >2x normal. Both the above trials have not used *TP53* mutations within their risk assignments. Although the SWOG 1211 trial does not utilise gain(1q) in the model, our analysis did not find that gain(1q) retained its independent prognostic association with poor survival in the context of the GEP70 signature, possibly as a result of significant overlap in these. Disappointingly, preliminary results do not demonstrate a PFS benefit of this approach.

The GMMG-CONCEPT trial for high-risk MM that is treated with isatuximab in combination with carfilzomib, lenalidomide and dexamethasone is based on the presence of del(17p) or t(4;14) or t(14;16) or amp(1q21)(>3 copies), and ISS II or III. Unfortunately, this trial also does not have a randomisation arm, so comparison of treatments is difficult. Comparison of even the three trials will not be possible as they all chose different definitions of high-risk disease. This highlights the issues of not resolving debate with regards to the definition of high risk disease across the world.

In terms of therapy related to actionable genetic alterations, a basket study has recently been developed in the context of relapsed MM. It uses therapies against genetic alterations such as *IDH2* mutations, *RAF/RAS*, *FGFR3* mutations, t(11;14), and *CDK2* alterations in combination with ixazomib, pomalidomide and dexamethasone treatment vs the addition of daratumumab in those without actionable mutations (417). This trial has small numbers of patients in each arm, so the interpretation of results is challenging. Nevertheless, it may lead to larger trials of such targeted drugs versus the current standard of care in a randomised controlled fashion with sufficient power to answer questions on whether directly targetable drugs improve overall survival in such patients.

There are no trials that are currently designed to test de-escalation of therapy based on low-risk MM upfront. This is probably due to industry pressure, as most trials are funded at least in part by industry for which de-escalation of therapy would result in loss of revenue. Careful examination of comprehensively profiled data will help to provide support for such a trial in the future in order to improve healthcare resource allocation as well as to improve quality of life of patients who may not require intensive therapy. It is clear from the work that is described in this thesis and in other studies that there are several unknown features of high-risk disease that form challenges for clinicians who must make upfront decisions on how to treat patients. It is therefore possible that de-escalation may be a possible subject in trials that de-escalate therapy through assessment of minimal residual disease (MRD) as well as through genetic assessment to address whether de-escalation of therapy is beneficial or non-inferior to the other randomised arm of therapy. Future trials are due to open in the UK, including the Myeloma XV trial which will treat patients differently according to genetic risk as well as MRD status. Several other trials have also started that will address MRD-based assessment for consolidation therapy or to consider stopping maintenance therapy.

In addition, evolution of disease from the asymptomatic to symptomatic stages may produce too many difficulties to treat the disease effectively by the time current timepoints for treatment are reached. Studies on high-risk asymptomatic or smouldering MM, randomised on genetics, may help to inform the scientific

community regarding whether high risk can be abrogated, for example prior to the development of bi-allelic *TP53* aberrations. It is important to show conclusive evidence of an overall survival benefit in this setting given the effects of potential drug toxicity, the impact on quality of life and the cost of treatment in this setting.

In summary, although we now know a great deal more about the genetic basis of myeloma, limited progress has been made in application of this knowledge to the clinic. The knowledge that is gained from this thesis as well as other studies must be applied in the clinic through systematic clinical trials to evaluate individual and combined genetic risk features. Commercial clinical trials should be required to show the effect of a standardised list of cytogenetic and clinical risk-marker results so that they can be evaluated in the context of high-risk MM. Through these efforts as well as targeted treatment of myeloma molecular dependencies, we will achieve improvements in treatment and abrogation of high-risk features of this disease.

8 Appendix

8.1 Publications that have arisen from work associated with this study

Shah V, Johnson DC, Sherborne AL, Ellis S, Aldridge FM, Howard-Reeves J, Begum F, Price A, Kendall J, Chiecchio L, Savola S, Jenner MW, Drayson MT, Owen RG, Gregory WM, Morgan GJ, Davies FE, Houlston RS, Cook G, Cairns DA, Jackson G, Kaiser MF. Subclonal *TP53* copy number is associated with prognosis in multiple myeloma. *Blood*. 2018 Dec 6;132(23):2465-2469.

Shah V, Sherborne AL, Johnson DC, Ellis S, Price A, Chowdhury F, Kendall J, Jenner MW, Drayson MT, Owen RG, Gregory WM, Morgan GJ, Davies FE, Cook G, Cairns DA, Houlston RS, Jackson G, Kaiser MF. Predicting ultrahigh risk multiple myeloma by molecular profiling: an analysis of newly diagnosed transplant eligible myeloma XI trial patients. *Leukemia*. 2020 Mar 11.

Shah V, Sherborne AL, Walker BA, Johnson DC, Boyle EM, Ellis S, Begum DB, Proszek PZ, Jones JR, Pawlyn C, Savola S, Jenner MW, Drayson MT, Owen RG, Houlston RS, Cairns DA, Gregory WM, Cook G, Davies FE, Jackson GH, Morgan GJ, Kaiser MF. Prediction of outcome in newly diagnosed myeloma: a meta-analysis of the molecular profiles of 1905 trial patients. *Leukemia*. 2018 Jan;32(1):102-110.

Cook G, Royle KL, Pawlyn C, Hockaday A, **Shah V**, Kaiser MF, Brown SR, Gregory WM, Child JA, Davies FE, Morgan GJ, Cairns DA, Jackson GH. A clinical prediction model for outcome and therapy delivery in transplant-ineligible patients with myeloma (UK Myeloma Research Alliance Risk Profile): a development and validation study. *Lancet Haematol*. 2019 Mar;6(3): e154-e166.

Pawlyn C, Cairns D, Kaiser M, Striha A, Jones J, **Shah V**, Jenner M, Drayson M, Owen R, Gregory W, Cook G, Morgan G, Jackson G, Davies F. The relative importance of factors predicting outcome for myeloma patients at different ages: results from 3894 patients in the Myeloma XI trial. *Leukemia*. 2020 Feb;34(2):604-612.

8.2 Abstract publications/presentations

Shah V, Johnson DC, Sherborne AL, Ellis S, Price A, Kendall J, Chiecchio L, Striha A, Hockaday A, Pawlyn C, Jenner M, Drayson MT, Owen RG, Gregory WM, Morgan GJ, Davies FE, Houlston RS, Cook G, Cairns DA, Jackson GH, Kaiser MF.

Characterisation of Long-Term Responders to First-Line Myeloma Therapy - Results from the UK Myeloma IX and XI Trials. *Blood* 2018; 132 (Supplement 1)

Shah V, Sherborne AL, Ellis S, Johnson DC, Begum F, Kendall J, Walker BA, Jones JR, Pawlyn C, Savola S, Jenner MW, Drayson MT, Owen RG, Cairns DA, Gregory WM, Houlston RS, Davies, FE Cook G, Morgan GJ, Jackson G, Kaiser MF. Molecular Characterisation of *TP53* Aberrations in 1,777 Myeloma Trial Patients. *Blood* 2017; 130 (Supplement 1): 4331.

Jenner M, Sherborne AL, Hall A, **Shah V**, Walker K, Ellis S, Sharp K, Price A, Croft J, Jackson G, Flanagan L, Drayson MT, De Tute R, Owen RG, Pratt R, Cook G, Brown SR, Kaiser MF. Molecular Treatment Stratification for Newly Diagnosed High-Risk Myeloma, Including Plasma Cell Leukemia - Feasibility Results of the Ukmra Optimum: MUK9 Trial (NCT03188172). *Blood* 2019; 134 (Supplement_1): 3162. Presented at American Society of Haematology, USA, 2018

Sherborne, AL., **Shah, V.**, Ellis, S., Begum, F., Kendall, J., Johnson, DC., Owen, RG., Drayson, MT., Flanagan, L., Sherratt, D., Gregory, WM., Jackson, G., Pratt, G., Cook, G., Hinsley, S., Brown, S., Jenner, MW., & Kaiser, MF. Improving Outcomes for Patients with High-Risk Myeloma Via Prospective Trial Evidence: The Myeloma UK Nine Optimum Trial. *Blood* 2017,130(Suppl 1), 1767.

Sherborne AL, **Shah V**, Hall A, Walker K, Ellis S, Begum F, Johnson DC, Croft J, Kendall J, Pawlyn C, Lilit A, Savola S, Flanagan L, Thakurta A, Garg M, Cook G, Boyd K, Brown S, Kaiser MF. Molecular Profiling of Relapsed and Refractory Myeloma Identifies Frequent Bi-Allelic Driver Events — Interim Results of the 'Myeloma UK Seven' Biomarker Trial. *Blood* 2017; 130 (Supplement 1): 1851.

8.3 Publications that contain data curated for this thesis by the author

Lenalidomide maintenance versus observation for patients with newly diagnosed multiple myeloma (Myeloma XI): a multicentre, open-label, randomised, phase 3 trial. Jackson GH, Davies FE, Pawlyn C, Cairns DA, Striha A, Collett C, Hockaday A, Jones JR, Kishore B, Garg M, Williams CD, Karunanithi K, Lindsay J, Jenner MW, Cook G, Russell NH, Kaiser MF, Drayson MT, Owen RG, Gregory WM, Morgan GJ; UK NCRI Haemato-oncology Clinical Studies Group. *Lancet Oncol.* 2019 Jan;20(1):57-73.

Response-adapted intensification with cyclophosphamide, bortezomib, and dexamethasone versus no intensification in patients with newly diagnosed multiple myeloma (Myeloma XI): a multicentre, open-label, randomised, phase 3 trial.

Jackson GH, Davies FE, Pawlyn C, Cairns DA, Striha A, Collett C, Waterhouse A, Jones JR, Kishore B, Garg M, Williams CD, Karunanithi K, Lindsay J, Wilson JN, Jenner MW, Cook G, Kaiser MF, Drayson MT, Owen RG, Russell NH, Gregory WM, Morgan GJ; UK NCRI Haematological Oncology Clinical Studies Group. *Lancet Haematol*. 2019 Dec;6(12):e616-e629.

Lenalidomide before and after ASCT for transplant-eligible patients of all ages in the randomized, phase III, Myeloma XI trial. Jackson GH, Davies FE, Pawlyn C, Cairns DA, Striha A, Collett C, Waterhouse A, Jones JR, Kishore B, Garg M, Williams CD, Karunanithi K, Lindsay J, Allotey D, Shafeek S, Jenner MW, Cook G, Russell NH, Kaiser MF, Drayson MT, Owen RG, Gregory WM, Morgan GJ; UK NCRI Haematological Oncology Clinical Studies Group.

Optimising the value of immunomodulatory drugs during induction and maintenance in transplant ineligible patients with newly diagnosed multiple myeloma: results from Myeloma XI, a multicentre, open-label, randomised, Phase III trial. Jackson GH, Pawlyn C, Cairns DA, Striha A, Collett C, Waterhouse A, Jones JR, Wilson J, Taylor C, Kishore B, Garg M, Williams CD, Karunanithi K, Lindsay J, Jenner MW, Cook G, Russell NH, Drayson MT, Kaiser MF, Owen RG, Gregory WM, Davies FE, Morgan GJ; UK NCRI Haemato-oncology Clinical Studies Group. *Br J Haematol*. 2020 Jul 12.

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ORIGINAL ARTICLE

Prediction of outcome in newly diagnosed myeloma: a meta-analysis of the molecular profiles of 1905 trial patients

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Robust establishment of survival in multiple myeloma (MM) and its relationship to recurrent genetic aberrations is required as outcomes are variable despite apparent similar staging. We assayed copy number alterations (CNA) and translocations in 1036 patients from the NCRI Myeloma XI trial and linked these to overall survival (OS) and progression-free survival. Through a meta-analysis of these data with data from MRC Myeloma IX trial, totalling 1905 newly diagnosed MM patients (NDMM), we confirm the association of t(4;14), t(14;16), t(14;20), del(17p) and gain(1q21) with poor prognosis with hazard ratios (HRs) for OS of 1.60 ($P=4.77 \times 10^{-7}$), 1.74 ($P=0.0005$), 1.90 ($P=0.0089$), 2.10 ($P=8.86 \times 10^{-14}$) and 1.68 ($P=2.18 \times 10^{-14}$), respectively. Patients with 'double-hit' defined by co-occurrence of at least two adverse lesions have an especially poor prognosis with HRs for OS of 2.67 ($P=8.13 \times 10^{-27}$) for all patients and 3.19 ($P=1.23 \times 10^{-18}$) for intensively treated patients. Using comprehensive CNA and translocation profiling in Myeloma XI we also demonstrate a strong association between t(4;14) and *BIRC2/BIRC3* deletion ($P=8.7 \times 10^{-15}$), including homozygous deletion. Finally, we define distinct sub-groups of hyperdiploid MM, with either gain(1q21) and *CCND2* overexpression ($P < 0.0001$) or gain(11q25) and *CCND1* overexpression ($P < 0.0001$). Profiling multiple genetic lesions can identify MM patients likely to relapse early allowing stratification of treatment.

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INTRODUCTION

While survival for multiple myeloma (MM) has improved over the last decade with the introduction of immunomodulatory drugs and proteasome inhibitors most MM patients will still relapse.¹ Upfront identification of patients who are likely to relapse early offers the prospect of intervening pre-emptively to maintain remission. Furthermore, identifying tumor sub-groups with targetable molecular dependencies has the potential to inform on biologically driven therapy.

Myeloma cells are typified by recurrent chromosomal aberrations, a number of which have been variously associated with poor prognosis, notably t(4;14), t(14;16), t(14;20), deletion 17p and gain of 1q.² We and others have recently reported that the co-occurrence of multiple genetic lesions may have greater significance for predicting patient outcome than any single abnormality.^{3,4} Since many of the molecular abnormalities in MM are only present at relatively low frequency, robustly establishing the impact of molecular sub-classes on prognosis is contingent on the analysis of large patient series that have been uniformly treated.

Here we report a meta-analysis of the relationship between genetic profile and prognosis in newly diagnosed MM (NDMM) using data from two UK multi-center phase III clinical trials, totalling 1905 patients. This dataset includes previously generated

data on the MRC Myeloma IX trial and an expanded analysis of the NCRI Myeloma XI trial. In addition, we analysed molecular copy number profiling in 1036 Myeloma XI patients to identify sub-groups with molecular additions that could be therapeutically targetable.^{5,6}

MATERIALS AND METHODS

Myeloma XI trial patients

1036 patients with NDMM enrolled in the UK NCRI Myeloma XI phase III trial were molecularly profiled. Trial characteristics are described in Supplementary Methods. At the time of analysis, the trial endpoints have not been published. Median follow-up was 36.0 months. The study was undertaken with written informed consent from patients and ethical approval was obtained from the Oxfordshire Research Ethics Committee (MREC 17/09/09, ISRCTN49407852).

Myeloma IX trial patients

Detailed characteristics and main outcomes of MRC Myeloma IX have been reported previously and summarised in Supplementary Methods.⁷ The study was undertaken with written informed consent from patients and ethical approval was obtained from the MRC Leukaemia Data Monitoring and Ethics committee (MREC 02/08/95, ISRCTN68454111). For the present analysis we included data from 869 of the 1960 NDMM patients with available clinical and comprehensive cytogenetic data.³ Median follow-up

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for this group was 72 months.^{3,8} Accompanying gene expression and mapping array data have been previously published (GSE15695).^{6,9,10}

Samples

For both trials myeloma cells from bone marrow aspirate samples were obtained at diagnosis and purified (>95%) using immune-magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). RNA and DNA were extracted using RNA/DNA mini kit or Allprep kits (QIAGEN) according to manufacturers' instructions.

Copy number and translocation detection

Technical details about fluorescence *in situ* profiling of Myeloma IX have been published previously.¹¹ Myeloma XI cases were centrally analysed using MLPA and qRT-PCR. The SALSA MLPA P425-B1 MM probemix (MRC Holland, Amsterdam, The Netherlands) was used as previously described.^{12,13} The newly developed probemix X073-A1 was used to profile 1007 of the 1036 cases in an identical fashion (MLPA Probe Mix:

Supplementary Table 1). Copy number at each locus was determined as described previously.^{12,13}

Multiplexed qRT-PCR was used to determine *IGH* translocation status using a translocation and cyclin D (TC)-classification based algorithm (Supplementary Methods), as previously described.¹⁰

Statistical methods

All statistical analyses were undertaken using R version 3.3 and the 'survival', 'rms', 'metafor', 'survC1', 'JAGS' and 'BayesMed' packages.¹⁴

Progression-free survival (PFS) was defined as the time from the date of randomization to progression, according to IMWG criteria, or death from any cause. Overall survival (OS) was defined as the time from the date of randomization to death from any cause. Kaplan–Meier survival curves were generated and the homogeneity between groups was evaluated with the log-rank test. Cox regression analysis was used to estimate hazard ratios (HRs) and respective 95% confidence intervals (CI) and adjustment for variables was performed by multivariable analysis. Fixed effects meta-analysis was performed using individual patient data. Correlations between structural aberrations were analysed using Bayesian inference. A Bayes

Table 1. Clinical characteristics and frequency of genetic aberrations in myeloma IX and myeloma XI trial patients

	Myeloma IX trial		Myeloma XI trial		P-value
	(Total n = 869)	Missing information	(Total n = 1036)	Missing information	
<i>Clinical characteristics</i>					
Female	339 (39.0%)		398 (38.4%)		0.81
Male	530 (61.0%)		638 (61.6%)		0.81
Intensive treatment pathway	511 (58.8%)		598 (57.5%)		0.64
Non-intensive treatment pathway	358 (41.2%)		438 (42.3%)		0.64
ISS I	130 (20.7%)	240	225 (23.1%)	61	0.27
ISS II	253 (40.2%)	240	429 (44.0%)	61	0.15
ISS III	246 (39.1%)	240	321 (32.9%)	61	0.01
Median age (years)	65 (range 34–89)		67 (range 34–88)		1.0
<i>Primary lesions (translocations, HRD)</i>					
t(4;14)	104 (11.9%)		137 (13.2%)		0.45
t(4;14) FGFR3-negative	–		26 (2.5%)		
t(6;14)	8 (0.9%)	1	7 (0.7%)		0.61
t(11;14)	129 (14.8%)		175 (16.9%)		0.23
t(14;16)	27 (3.1%)		38 (3.7%)		0.53
t(14;20)	13 (1.5%)		13 (1.3%)		0.69
HRD	499 (58.9%)	22	488 (47.1%)		3×10^{-7}
<i>Copy number abnormalities</i>					
Del(1p32)	87 (10.7%)	60	107 (10.3%)		0.82
Gain(1q) or Amp(1q)	340 (39.1%)		357 (34.5%)		0.04
Gain(1q)	–		277(26.7%)		
Amp(1q)	–		80 (7.7%)		
Gain(6p) or Amp(6p)	–		122 (12.1%)	29	
Gain(6q) or Amp(6q)	–		69 (6.9%)	29	
Del(6q)	–		157 (15.6%)	29	
Del(8p)	–		164 (16.3%)	29	
Gain(8q)	–		43 (4.3%)	29	
Gain(11q25)	–		418 (41.5%)	29	
Del(12p)	–		78 (7.5%)		
Del(13q)	389 (45.1%)	6	425 (41.0%)		0.07
Del(14q)	–		144 (13.9%)		
Del(16q)	153 (17.6%)	46	175 (16.9%)		0.36
Del(17p)	78 (8.9%)		96 (9.3%)		0.87
Del(22q)	100 (13.1%)	103	103 (10.2%)	29	0.04
<i>Focal copy number abnormalities/mutations</i>					
<i>CDKN2C</i> homozygous del	–		19 (1.8%)		
<i>BIRC2/BIRC3</i> homozygous del	–		22 (2.2%)	29	
<i>MYC</i> amplification	–		28 (2.8%)	29	
<i>CCND1</i> focal gain	–		46 (4.6%)	29	
<i>BRAF</i> V600E mutation	–		36 (3.6%)	29	

Abbreviations: HRD, hyperdiploid; ISS, International Staging System.

Table 2. Relationship between genetic abnormalities and patient survival

	Myeloma IX n = 869		Myeloma XI n = 1036		Combined n = 1905		Heterogeneity
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	P-value
(a) Progression-free survival							
t(4;14)	1.88 (1.52–2.23)	5.31 × 10 ⁻⁹	1.51 (1.22–1.88)	0.0001	1.69 (1.45–1.96)	9.30 × 10 ⁻¹²	0.16
t(14;16)	1.50 (1.01–2.22)	0.0425	1.51 (1.05–2.17)	0.0256	1.50 (1.15–1.96)	0.0026	0.98
t(14;20)	1.13 (0.64–1.99)	0.6852	1.54 (0.80–2.97)	0.1987	1.29 (0.84–1.98)	0.2509	0.48
Adverse translocations	1.77 (1.47–2.13)	1.88 × 10 ⁻⁹	1.58 (1.31–1.91)	2.05 × 10 ⁻⁶	1.67 (1.46–1.91)	2.69 × 10 ⁻¹⁴	0.41
Del(17p)	1.54 (1.21–1.95)	0.0003	1.61 (1.26–2.06)	0.0002	1.57 (1.33–1.87)	2.07 × 10 ⁻⁷	0.79
Gain(1q)	1.53 (1.33–1.77)	6.70 × 10 ⁻⁹	1.53 (1.31–1.80)	1.34 × 10 ⁻⁷	1.53 (1.38–1.71)	4.61 × 10 ⁻¹⁵	1.00
Del(1p32)	0.99 (0.78–1.25)	0.9202	1.30 (1.02–1.66)	0.0331	1.13 (0.95–1.34)	0.1571	0.11
ISS II	1.40 (1.12–1.76)	0.0036	1.54 (1.23–1.92)	0.0002	1.47 (1.25–1.72)	2.50 × 10 ⁻⁶	0.58
ISS III	1.64 (1.30–2.06)	2.34 × 10 ⁻⁵	2.46 (1.96–3.09)	6.88 × 10 ⁻¹⁶	2.02 (1.71–2.37)	1.73 × 10 ⁻¹⁷	0.01
1 Adverse lesion	1.41 (1.21–1.65)	1.73 × 10 ⁻⁵	1.46 (1.23–1.74)	1.44 × 10 ⁻⁵	1.44 (1.28–1.61)	1.07 × 10 ⁻⁹	0.76
'Double hit' > 1 adverse lesion	2.24 (1.83–2.76)	1.11 × 10 ⁻¹⁴	2.22 (1.78–2.77)	1.05 × 10 ⁻¹²	2.23 (1.92–2.59)	7.92 × 10 ⁻²⁶	0.94
Intermediate risk-ISS	1.50 (1.25–1.79)	1.48 × 10 ⁻⁵	1.95 (1.63–2.33)	1.56 × 10 ⁻¹³	1.71 (1.51–1.95)	9.48 × 10 ⁻¹⁷	0.04
'Double hit'-ISS	2.76 (2.13–3.57)	1.54 × 10 ⁻¹⁴	2.93 (2.29–3.09)	2 × 10 ⁻¹⁶	2.85 (2.38–3.40)	8.32 × 10 ⁻³¹	0.74
	Myeloma IX n = 869		Myeloma XI n = 1036		Combined n = 1905		Heterogeneity
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	P-value
(b) OS							
t(4;14)	1.72 (1.36–2.17)	5.12 × 10 ⁻⁶	1.42 (1.06–1.91)	0.0188	1.60 (1.33–1.92)	4.77 × 10 ⁻⁷	0.33
t(14;16)	1.52 (0.98–2.35)	0.0607	2.00 (1.28–3.11)	0.0021	1.74 (1.27–2.37)	0.0005	0.39
t(14;20)	1.64 (0.88–3.07)	0.1213	2.35 (1.11–4.97)	0.0259	1.90 (1.17–3.07)	0.0089	0.47
Adverse translocations	1.74 (1.42–2.14)	1.15 × 10 ⁻⁷	1.71 (1.33–2.20)	3.23 × 10 ⁻⁵	1.73 (1.47–2.03)	1.63 × 10 ⁻¹¹	0.90
Del(17p)	1.92 (1.49–2.48)	6.07 × 10 ⁻⁷	2.40 (1.77–3.24)	1.61 × 10 ⁻⁸	2.10 (1.73–2.56)	8.86 × 10 ⁻¹⁴	0.27
Gain(1q)	1.61 (1.37–1.91)	1.81 × 10 ⁻⁸	1.80 (1.44–2.24)	1.76 × 10 ⁻⁷	1.68 (1.47–1.92)	2.18 × 10 ⁻¹⁴	0.44
Del(1p32)	1.23 (0.94–1.61)	0.1170	1.83 (1.35–2.48)	0.0001	1.46 (1.20–1.78)	0.0002	0.06
ISS II	1.98 (1.47–2.68)	8.11 × 10 ⁻⁶	1.90 (1.30–2.77)	0.0009	1.95 (1.54–2.47)	2.76 × 10 ⁻⁸	0.86
ISS III	2.62 (1.94–3.53)	2.69 × 10 ⁻¹⁰	3.85 (2.66–5.56)	7.41 × 10 ⁻¹⁴	3.05 (2.42–3.85)	4.38 × 10 ⁻²¹	0.11
1 Adverse lesion	1.42 (1.18–1.71)	0.0002	1.81 (1.41–2.32)	3.57 × 10 ⁻⁶	1.55 (1.33–1.79)	9.97 × 10 ⁻⁹	0.13
'Double hit' > 1 Adverse lesion	2.54 (2.02–3.18)	7.77 × 10 ⁻¹⁶	2.91 (2.17–3.89)	1.11 × 10 ⁻¹²	2.67 (2.23–3.19)	8.13 × 10 ⁻²⁷	0.47
Intermediate risk-ISS	1.96 (1.57–2.45)	4.26 × 10 ⁻⁹	2.59 (1.96–3.41)	1.62 × 10 ⁻¹¹	2.19 (1.84–2.61)	1.3 × 10 ⁻¹⁸	0.13
'Double hit'-ISS	3.93 (2.93–5.27)	2 × 10 ⁻¹⁶	4.37 (3.13–6.12)	2 × 10 ⁻¹⁶	4.12 (3.30–5.14)	2.85 × 10 ⁻³⁶	0.64

Abbreviations: CI, confidence interval; HR, hazard ratio; ISS, International Staging System; OS, overall survival.

factor (BF₀₁) of BF₀₁ < 0.01 was considered significant. The association between categorical variables was examined using the Fisher exact test. The association between myeloma subtype and gene expression was assessed using the Mann–Whitney test. A two-sided P-value < 0.05 was considered significant.

RESULTS

Descriptive patient characteristics and structural aberrations

The clinical characteristics of the 1036 newly profiled Myeloma XI trial patients and the 869 Myeloma Trial IX patients are detailed in Table 1. Overall there were no significant differences between trial patients in terms of gender, age and proportion that had been in receipt of intensive/non-intensive therapy. Although the frequencies of the primary IGH translocations, del(17p), del(1p32), del(13q) and del(16q) in tumours were similar in Myeloma IX and XI trial patients, a higher proportion of Myeloma IX patients had hyperdiploidy (HRD), gain(1q) and del(22q) (Table 1). Amongst Myeloma XI trial patients, homozygous deletion of *CDKN2C* (1p32), *BIRC2/BIRC3* (11q22) and amplification of *CKS1B* (1q21) and *MYC* (8q24) were the commonest focal homozygous copy number changes, which were seen at similar frequencies to those previously reported (Table 1).¹⁵

Relationship between cytogenetic aberrations and survival

In both trial series, the archetypically high-risk lesions del(17p), gain(1q) and t(4;14) were each significantly associated with shorter PFS and OS (Table 2). In the combined analysis, respective HR for OS were 2.1 for del(17p) (P = 8.86 × 10⁻¹⁴), 1.68 for gain(1q) (P = 2.18 × 10⁻¹⁴) and 1.60 for t(4;14) (P = 4.77 × 10⁻⁷; Table 2; Supplementary Figures 1 and 2). In addition, the t(14;16) and t(14;20) translocations involving *MAF* and *MAFB* were also associated with shorter OS with respective HRs of 1.74 (P = 0.0005) and HR 1.90 (P = 0.0089). Respective inference C-statistic estimates for adequacy of risk prediction are shown in Supplementary Tables 5 and 6.

Deletion of 1p32 (*CDKN2C*) was significantly associated with shorter OS (HR 1.46; P = 0.0002; Table 2). This association was confined to patients in receipt of intensive treatment (in the combined analysis: HR 1.89; P = 1.23 × 10⁻⁵ vs HR 1.05; P = 0.72 for non-intensive treatment). The association of del(1p32) with OS was independent from gain(1q21) by multivariable analysis (P < 0.05) in the intensive treatment groups of both trials.

To examine the relationship between 1q21 status and outcome in more detail we sub-classified Myeloma XI patients (n = 1036) by diploid vs gain vs amplification status. 1q21 gain was confirmed as a high-risk lesion and was associated with significantly shorter PFS (HR 1.56; P = 3.53 × 10⁻⁷) and OS (HR 1.67; P = 3.30 × 10⁻⁵) than

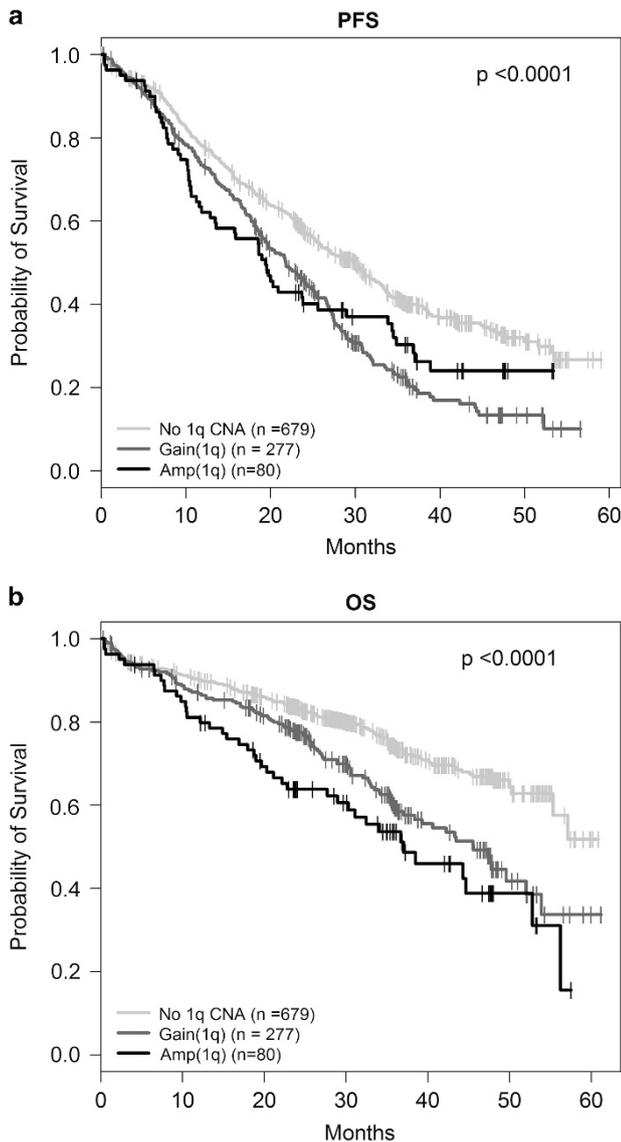


Figure 1. Chromosome 1q21 copy number status and outcome in Myeloma XI. Kaplan–Meier curves and log-rank *P*-values for (a) PFS (b) OS for normal vs gain vs amplification of 1q21.

normal 1q copy number status. Amp(1q) was also associated with shorter PFS (HR 1.44; $P=0.01$) and OS (HR 2.28; $P=2.32 \times 10^{-6}$) compared to normal 1q, but there was no significant difference to gain(1q) (PFS: HR 0.91; $P=0.54$; OS: HR 1.36; $P=0.09$ for OS). Median PFS was 19.4 vs 21.8 vs 30.1 months ($P < 0.0001$) and 24-months OS 63.8 vs 77.5 vs 83.5% ($P < 0.0001$) for amp(1q), gain(1q) and normal 1q, respectively (Figure 1; Supplementary Table 7).

'Double-hit' as a high-risk classifier

We next examined the impact of a 'double-hit' based on the co-occurrence of at least any two of the following: (1) Adverse translocations t(4;14), t(14;16), t(14;20); (2) gain(1q); (3) del(17p).³ For Myeloma XI the three risk groups, defined by 'double-hit', 1 or no adverse lesions, were associated with median PFS of 17.0, 24.2 and 31.1 months (log-rank $P=5.7 \times 10^{-13}$), with corresponding median 24-months OS of 66.1, 76.6 and 86.4% ($P=4.4 \times 10^{-13}$). These findings were consistent with Myeloma IX (Table 2). In the

combined analysis of all 1905 patients the HR for 'double-hit' was 2.23 for PFS ($P=7.92 \times 10^{-26}$) and 2.67 for OS ($P=8.13 \times 10^{-27}$; Table 2; Supplementary Figures 1 and 2). Similarly to Myeloma IX, the 'triple-hit' of an adverse translocation, Gain(1q) and del(17p) was associated with a very short median OS of 19 months with a HR of 6.23 ($P=1.31 \times 10^{-7}$) vs no adverse lesion (Supplementary Figure 5).

In both Myeloma IX and XI trials the impact of a 'double-hit' on patient outcome was independent of International Staging System (ISS; Supplementary Table 3). Moreover, integration of ISS and genetic risk defined 'double-hit'-ISS ultra high risk (ISS II or III and 'double-hit'; 12.0%), intermediate risk (ISS I and 'double-hit'; ISS II and 1 adverse lesion; ISS III and no or 1 adverse lesion; 44.1%) and favourable risk groups (ISS I and no or 1 adverse lesion; ISS II and no adverse lesion; 43.9%). 'double hit'-ISS ultra high risk was associated with HR 2.85 ($P=8.32 \times 10^{-31}$) for PFS and HR 4.12 ($P=2.85 \times 10^{-36}$) for OS in the meta-analysis (Table 2).

Genetic markers and survival in intensively treated patients

Since young and fit patients are most likely to be considered for intensified combination therapy, we subsequently focused on the relationship between molecular profile and survival of this subgroup of Myeloma XI ($n=598$) and Myeloma IX ($n=511$) patients.

In these 1109 intensively treated patients, del(17p), gain(1q) and t(4;14) were consistently associated with shorter PFS and OS; combined HRs of 2.65 (3.04×10^{-12}), 1.77 (1.65×10^{-8}) and 1.87 (7.62×10^{-7}), respectively (Table 3; Supplementary Figures 3 and 4). In this group, t(14;16) was associated with shorter PFS (HR 1.80; $P=0.0021$) and OS (HR 1.82; $P=0.013$). The t(14;20) was not associated with adverse PFS or OS, but the lesion was only present in eight Myeloma IX and five Myeloma XI cases (Table 3; Supplementary Figures 3 and 4).

In Myeloma XI, the groups with a 'double-hit', 1 adverse or no adverse lesion were associated with median PFS of 19.7, 30.9 and 44.8 months (log-rank $P=2.5 \times 10^{-13}$) and 24-months OS of 72.3, 86.2 and 92.2% ($P=1.6 \times 10^{-10}$; Supplementary Figure 3). By meta-analysis, intensively treated patients with a 'double-hit' had a HR for PFS of 2.61 ($P=1.07 \times 10^{-20}$) and HR for OS of 3.19 ($P=1.23 \times 10^{-18}$; Table 3). Survival time increased for all risk groups of intensively treated patients in Myeloma XI compared to Myeloma IX (median PFS: 14.4, 21.9 and 30.8 months; 24 month OS: 63.9, 75.4 and 86.0%, respectively). Median PFS was 5.3 months longer for 'double-hit' in Myeloma XI vs IX, but 14 months longer for the group without any risk lesion.

On the basis of clinical and genetic information (Supplementary Table 4) the 'double-hit'-ISS ultra high-risk group comprising 12.5% of patients were associated with a HR of 3.11 ($P=1.59 \times 10^{-20}$) for PFS and HR 4.79 ($P=5.10 \times 10^{-23}$) for OS.

Associations of copy number changes with translocations and targetable lesions

We next focused on genetic sub-groups of MM that could be specifically targetable using copy number and translocation data on the 1036 Myeloma XI patients. Figure 2 provides an overview of correlations between CNA and translocations (Supplementary Table 2). Of particular note was a relationship between NFκB-pathway CNA and translocation groups.

Potentially targetable NFκB-pathway gene deletions are common in myeloma

Deletions of NFκB-pathway modulating genes *TNFAIP3*, *BIRC2/BIRC3*, *TRAF3* or *CYLD* were identified in 16.6, 4.8, 13.9 and 16.9% of Myeloma XI cases, respectively. Nearly half of all tumors (43.2%) harbored an NFκB-pathway gene abnormality. Overall, a deletion of more than one NFκB-pathway gene was detectable in 9.7% of

Table 3. Relationship between genetic abnormalities and patient survival for intensively treated patients

	Myeloma IX n = 511		Myeloma XI n = 598		Combined n = 1109		Heterogeneity
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	P-value
(a) Progression-free survival							
t(4;14)	1.96 (1.49–2.59)	1.80 × 10 ⁻⁶	2.03 (1.56–2.64)	2.18 × 10 ⁻⁷	2.00 (1.65–2.42)	1.85 × 10 ⁻¹²	0.88
t(14;16)	1.60 (0.96–2.69)	0.0729	2.03 (1.19–3.47)	0.0099	1.80 (1.23–2.60)	0.0021	0.54
t(14;20)	0.96 (0.46–2.03)	0.9192	0.64 (0.09–4.54)	0.6524	0.91 (0.45–1.84)	0.7987	0.70
Adverse translocations	1.81 (1.42–2.31)	1.79 × 10 ⁻⁶	2.09 (1.62–2.68)	8.88 × 10 ⁻⁹	1.94 (1.63–2.31)	1.07 × 10 ⁻¹³	0.42
Del(17p)	1.81 (1.30–2.51)	0.0004	1.81 (1.29–2.52)	0.0005	1.81 (1.43–2.28)	7.25 × 10 ⁻⁷	1.00
Gain(1q)	1.48 (1.22–1.80)	7.44 × 10 ⁻⁵	1.65 (1.31–2.07)	2.03 × 10 ⁻⁵	1.55 (1.34–1.80)	7.59 × 10 ⁻⁹	0.49
Del(1p32)	1.05 (0.76–1.47)	0.7556	1.48 (1.04–2.09)	0.0286	1.23 (0.97–1.57)	0.0833	0.17
ISS II	1.34 (1.01–1.77)	0.0409	1.48 (1.11–1.99)	0.0085	1.40 (1.15–1.72)	0.0009	0.61
ISS III	1.43 (1.07–1.91)	0.0168	2.20 (1.61–3.01)	7.88 × 10 ⁻⁷	1.74 (1.40–2.16)	3.11 × 10 ⁻⁷	0.04
1 Adverse lesion	1.50 (1.21–1.85)	0.0002	1.49 (1.15–1.93)	0.0024	1.50 (1.27–1.76)	1.36 × 10 ⁻⁶	0.99
'Double hit' > 1 adverse lesion	2.31 (1.75–3.05)	3.67 × 10 ⁻¹⁴	3.00 (2.24–4.02)	2.17 × 10 ⁻¹³	2.61 (2.13–3.20)	1.07 × 10 ⁻²⁰	0.21
Intermediate risk-ISS	1.47 (1.16–1.86)	0.0015	1.87 (1.45–2.41)	1.45 × 10 ⁻⁶	1.64 (1.38–1.95)	2.10 × 10 ⁻⁸	0.17
'Double hit'-ISS	2.78 (1.96–3.95)	9.85 × 10 ⁻⁹	3.42 (2.47–4.75)	1.92 × 10 ⁻¹³	3.11 (2.45–3.95)	1.59 × 10 ⁻²⁰	0.40
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	Myeloma IX n = 511		Myeloma XI n = 598		Combined n = 1109		Heterogeneity
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	P-value
(b) OS							
t(4;14)	1.74 (1.26–2.41)	0.0008	2.09 (1.41–3.07)	0.0002	1.87 (1.46–2.40)	7.62 × 10 ⁻⁷	0.49
t(14;16)	1.51 (0.79–2.84)	0.2059	2.31 (1.13–4.73)	0.0218	1.82 (1.13–2.92)	0.01359	0.38
t(14;20)	1.44 (0.59–3.49)	0.4181	1.91 (0.26–13.70)	0.5219	1.51 (0.67–3.39)	0.3169	0.80
Adverse translocations	1.74 (1.30–2.33)	0.0002	2.30 (1.60–3.31)	6.99 × 10 ⁻⁶	1.94 (1.55–2.43)	9.91 × 10 ⁻⁹	0.24
Del(17p)	2.31 (1.61–3.31)	5.87 × 10 ⁻⁶	3.19 (2.10–4.85)	5.74 × 10 ⁻⁸	2.65 (2.01–3.48)	3.04 × 10 ⁻¹²	0.25
Gain(1q)	1.79 (1.40–2.27)	2.33 × 10 ⁻⁶	1.72 (1.22–2.43)	0.0020	1.77 (1.45–2.15)	1.65 × 10 ⁻⁸	0.86
Del(1p32)	1.84 (1.28–2.64)	0.0010	1.99 (1.25–3.18)	0.0037	1.89 (1.42–2.52)	1.23 × 10 ⁻⁵	0.78
ISS II	1.96 (1.32–2.90)	0.0008	1.88 (1.14–3.11)	0.0140	1.92 (1.41–2.63)	3.27 × 10 ⁻⁵	0.90
ISS III	2.56 (1.72–3.81)	3.59 × 10 ⁻⁶	3.22 (1.94–5.35)	6.56 × 10 ⁻⁶	2.79 (2.04–3.81)	1.30 × 10 ⁻¹⁰	0.49
1 Adverse lesion	1.73 (1.32–2.26)	6.91 × 10 ⁻⁵	1.62 (1.08–2.44)	0.0207	1.69 (1.35–2.12)	4.30 × 10 ⁻⁶	0.79
'Double hit' > 1 Adverse lesion	2.84 (2.05–3.94)	3.70 × 10 ⁻¹⁰	3.88 (2.55–5.92)	2.98 × 10 ⁻¹⁰	3.19 (2.47–4.14)	1.23 × 10 ⁻¹⁸	0.25
Intermediate risk-ISS	2.36 (1.71–3.25)	1.44 × 10 ⁻⁷	2.26 (1.49–3.43)	0.0001	2.32 (1.80–3.00)	7.15 × 10 ⁻¹¹	0.87
'Double hit'-ISS	4.51 (2.97–6.83)	1.26 × 10 ⁻¹²	5.18 (3.24–8.27)	5.78 × 10 ⁻¹²	4.79 (3.51–6.54)	5.10 × 10 ⁻²³	0.66

Abbreviations: CI, confidence interval; HR, hazard ratio; ISS, International Staging System; OS, overall survival.

tumors and in 42.4% of these cases involved deletions of both *TNFAIP3* and *CYLD*.

t(4;14) myeloma is associated with *BIRC2/BIRC3* deletions

We identified *BIRC* NFκB-pathway deletions to be enriched in t(4;14) MM (Figure 2). Specifically, 29/135 (21.5%) t(4;14) vs 20/852 (2.3%) non-t(4;14) ($P = 8.7 \times 10^{-15}$) tumors were *BIRC2/BIRC3* deleted. Intriguingly, homozygous *BIRC2/BIRC3* deletions were present in 15/135 (11%) of t(4;14) as compared to 7/872 non-t(4;14) tumors (0.8%; $BF_{01} = 4.3 \times 10^{-12}$; $P = 1.0 \times 10^{-8}$; Supplementary Table 2). Almost all t(4;14) tumors (28/29) with any *BIRC2/BIRC3* deletion expressed *FGFR3*, more than expected ($P = 0.015$). Deletions of *TRAF3* (14q32) seen in t(4;14) were mutually exclusive of *BIRC* deletions ($P = 0.016$) and more than expected *FGFR3*-negative: 20/29 (69%; $P = 0.0001$). In contrast, deletions of *CYLD* were generally significantly under-represented in the t(4;14) group (6/137; 4.4%) as compared with non-t(4;14) (169/899; 18.8%; $BF = 0.007$; $P = 4.0 \times 10^{-6}$).

High-risk and hypodiploidy-associated lesions

The t(4;14) subgroup was significantly associated with hypodiploidy lesions (HYL) del(12p) ($BF = 1.1 \times 10^{-4}$), del(13q) ($BF = 1.1 \times 10^{-25}$) and del(22q) ($BF = 1.1 \times 10^{-7}$; Figure 2).¹⁶ Deletion of 17p was also associated with deletions of 12p ($BF = 0.0004$),

13q ($BF = 3.7 \times 10^{-6}$) and 22q ($BF = 0.0076$), but there was no correlation between del(17p) and t(4;14). MM with t(4;14) was associated with gain(1q) ($BF = 3.0 \times 10^{-8}$), but not with del(1p32), which was only significantly correlated with del(8q) ($BF = 0.0009$) and del(16q23) ($BF = 0.0003$). In contrast, t(11;14) and HRD cases, the latter defined by extra copies of any two of chromosomes 5, 9 or 15, were negatively associated with gain(1q) ($BF = 1.6 \times 10^{-3}$ and $BF = 0.06$, respectively). Collectively, HRD cases were negatively associated with del(13q) ($BF = 1.9 \times 10^{-21}$) and del(22q) ($BF = 0.0002$).

Molecular sub-classification of hyperdiploid myeloma

We noted heterogeneity within the HRD subgroup in terms of co-occurrence of lesions. Although HRD as a whole group was strongly correlated with gain(11q25) ($BF = 1.2 \times 10^{-66}$), a subgroup lacking gain(11q25) was characterised by gain(1q) (Figure 3; Supplementary Figure 6).

Of the 488 HRD cases in Myeloma XI, 68% had gain(11q25) and 29% gain(1q). Both lesions co-occurred in 15% of HRD cases, less than expected ($BF = 0.0004$). Accordingly, most HRD patients could be classified as having gain(1q)-HRD, gain(11q25)-HRD or gain(1q) + gain(11q25)-HRD (Figure 2a). Gain(1q)-HRD was associated with overexpression of *CCND2* and silenced *CCND1* ($P < 0.0001$). In contrast, gain(11q25) was associated with *CCND1* expression and

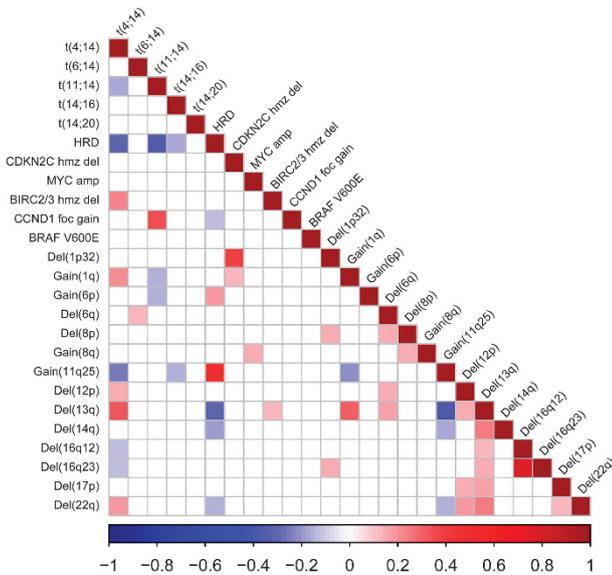


Figure 2. Associations between copy number aberrations and translocations in Myeloma XI. A Bayesian approach was used to identify all potential associations between genetic lesions. Significant interactions (BF < 0.01) are colour-coded, red representing positive and blue negative associations. Correlation factors and Bayes Factors are provided in Supplementary Table 2. amp, amplification; foc gain, focal gain; hmsz del, homozygous deletion.

silenced *CCND2* ($P < 0.0001$). We validated this correlation between gain(1q)-HRD and *CCND2* and gain(11q25)-HRD and *CCND1* expression in the Myeloma IX dataset (Supplementary Figure 7). The TC classification-defined D1 and D2 sub-groups of HRD MM on the basis of *CCND1* and *CCND2* overexpression¹⁷ and our findings suggest similarity between gain(11q25)-HRD and the D1, gain(1q)-HRD and the D2 and gain(1q)+gain(11q)-HRD and the D1+D2 TC classification subgroup.

Further differences between the HRD subtypes were noted: 13q was deleted in 41.1% (58/141) of gain(1q)-HRD (BF = 6.0×10^{-6} ; $P < 0.0001$), but only in 15.4% (50/325) of gain(11q25)-HRD (BF = 5.5×10^{-11} ; $P < 0.0001$). We validated this finding in the Myeloma IX dataset, where del(13q) was also positively associated with gain(1q)-HRD ($P = 0.024$) and negatively associated with gain(11q25) ($P = 0.041$).

Prognostic impact of molecular sub-groups in HRD

Gain of 1q, del(1p32) and del(17p) was associated with shorter OS (HR 1.81, $P = 0.001$; HR 2.44, $P = 0.0004$; HR 1.89, $P = 0.022$; respectively) in the 488 HRD cases. Gain(1q) and del(1p32) but not del(17p) was also associated with shorter PFS (HR 1.56, $P = 0.0003$; HR 1.66, $P = 0.005$; HR 1.30, $P = 0.23$ respectively; (Supplementary Table 8, Supplementary Figure 8)). Gain of 11q25, del(13q) and del(22q) were not associated with shorter OS or PFS. At least one of the lesions gain(1q), del(1p32) or del(17p) were present in 39.3% (192/488) of HRD cases, defining a risk population with significantly shorter PFS ($P = 4.9 \times 10^{-6}$) and OS ($P = 2.7 \times 10^{-6}$; Supplementary Figure 9) compared to HRD MM lacking any of these lesions. Interestingly, the 28.5% of all patients (296/1036) that had HRD without any demonstrable adverse lesion, had the longest survival of all sub-groups, indeed longer than those with t(11;14) MM (Figure 4).

DISCUSSION

Our analysis confirms the association with outcome in MM for the archetypal high-risk lesions del(17p), gain(1q) and adverse

translocations and emphasises the importance of ‘double-hit’ as a risk biomarker. Importantly, we demonstrate that this information can be combined with the ISS to further refine risk prediction.^{3,18–21} To our knowledge, this study represents the largest analysis investigating the additive effect of multiple genetic lesions on outcome in NDMM. Importantly, our analysis has been based on trials that recruited between 2003 and 2016, a timeframe during which treatment for MM has undergone significant change.²² The consistent adverse impact of high-risk genetics on survival in Myeloma IX and XI is striking and highlights the need for intensified efforts to target the biology of high-risk disease. Although survival time increased for all risk groups in Myeloma XI vs IX, absolute improvement was smallest for the ‘double-hit’ high-risk group. Median PFS for ‘double-hit’ in Myeloma XI patients receiving intensive treatment was 19.7 months, meaning that about half of patients relapsed 12 months following autologous transplant.

Comprehensive assessment of the inter-relationship of CNAs and translocations in the Myeloma XI trial led to characterisation of genetic sub-groups with putative therapeutic relevance. We found that half of Myeloma XI tumors carried a deletion of NFκB-pathway genes, and 10% of tumors had two co-occurring deletions.^{23–25} Intriguingly, our data suggests NFκB-inducing kinase (NIK)-specific addiction of the t(4;14) group: *BIRC2/BIRC3* deletions, including homozygous deletions, were enriched in t(4;14) tumours. The t(4;14) MM without *BIRC2/BIRC3* deletions were frequently *TRAF3* deleted. *BIRC2*, *BIRC3* and *TRAF3* proteins all interact directly with NIK, suppressing NFκB-pathway activity. (25) MM cell lines with deletions of *BIRC2/BIRC3* or *TRAF3*, predominantly t(4;14), have high NIK levels and activated NFκB-pathway signalling, as demonstrated by Keats *et al*. Recently, specific NIK inhibitors have been developed which might be used to target high-risk t(4;14) MM.^{26–28} Virtually all *BIRC2/3* deletions were found in *FGFR3*-positive tumors. They were mutually exclusive of *TRAF3* deletions, which were present in *FGFR3*-negative tumors, a pattern which may indicate convergent evolution. Deletions of *FGFR3*, which often occur as loss of der14 that includes *TRAF3*, may constitute ‘collateral damage’ of NIK addiction in t(4;14).^{15,24,25,29,30}

Although t(4;14) and del(17p) were not correlated with each other, both groups were strongly associated with hypodiploidy-associated lesions del(12p), del(13q) and del(22q).^{16,17,31,32} This suggests the consequences of t(4;14) and del(17p) may share molecular mechanisms. Gain of chromosome 1q21 was strongly associated with t(4;14), but not with del(17p). Gain(1q21) was confirmed as a high-risk lesion that is independent of del(1p32).^{33–35}

HRD MM constitutes the largest genetic sub-group of patients, with substantial heterogeneity.¹⁷ We describe two sub-groups of HRD with either gain(11q25) and *CCND1* biology or gain(1q21) and *CCND2* overexpression. These groups are similar to the D1 and D2 sub-groups of the TC classification, which pioneered biologic classification of HRD MM. Application of the TC classification in routine diagnostics has unfortunately been restricted due to access limitations to array-based gene expression profiling.¹⁷ Pragmatic classification of HRD based on gain(11q25) and gain(1q) may facilitate sub-grouping in clinical practice and open opportunities for improving therapy for these patients. Recently, activity of bcl-2 inhibitors has been reported in *CCND1*-driven t(11;14) MM, and *CCND1*-driven gain(11q25)-HRD may constitute another target group.³⁶ We also found a high frequency of del(13q) in gain(1q)-HRD, in contrast to gain(11q25)-HRD. Interestingly, del(13q) and gain(1q) also frequently co-occur in t(4;14), suggesting similarities in the genetic sequelae of these pathogenetic groups.³⁷ An inter-relationship between del(13q) and gain(1q)-HRD was suggested based on GEP in the TC classification by Bergsagel *et al*, but has been demonstrated here for the first time on a DNA level.^{17,38} Moreover, HRD MM without any of risk lesions

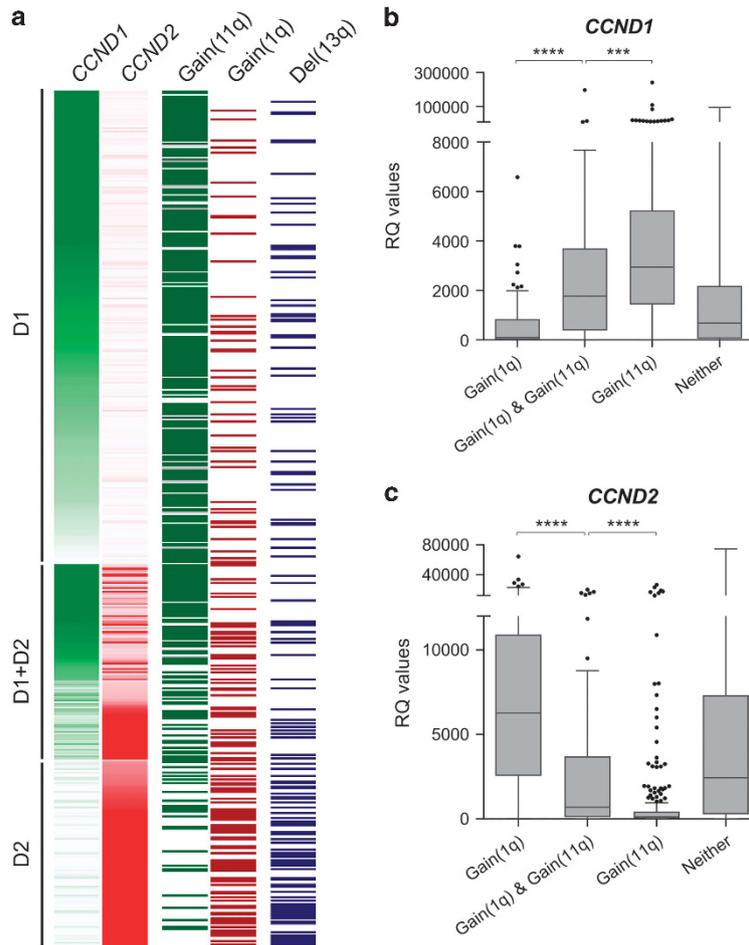


Figure 3. HRD genetic sub-groups in Myeloma XI. (a) Each row represents one of in total 1007 cases. Expression intensity is coded in green for *CCND1* and red for *CCND2* expression. Gain of 11q25 is shown in dark green, gain of 1q in dark red and deletion 13q in dark blue; white = no abnormality detected. B+C. *CCND1* (b) and *CCND2* (c) qRT-PCR expression levels (relative quantitative RQ values, *GAPDH* normalised) for HRD cases with gain(1q), gain(1q)+gain(11q25), gain(11q25) or neither. Gene expression levels were significantly different for all possible group-wise comparisons (two-sided Mann-Whitney *U* test; *****P* < 0.0001; ****P* < 0.001).

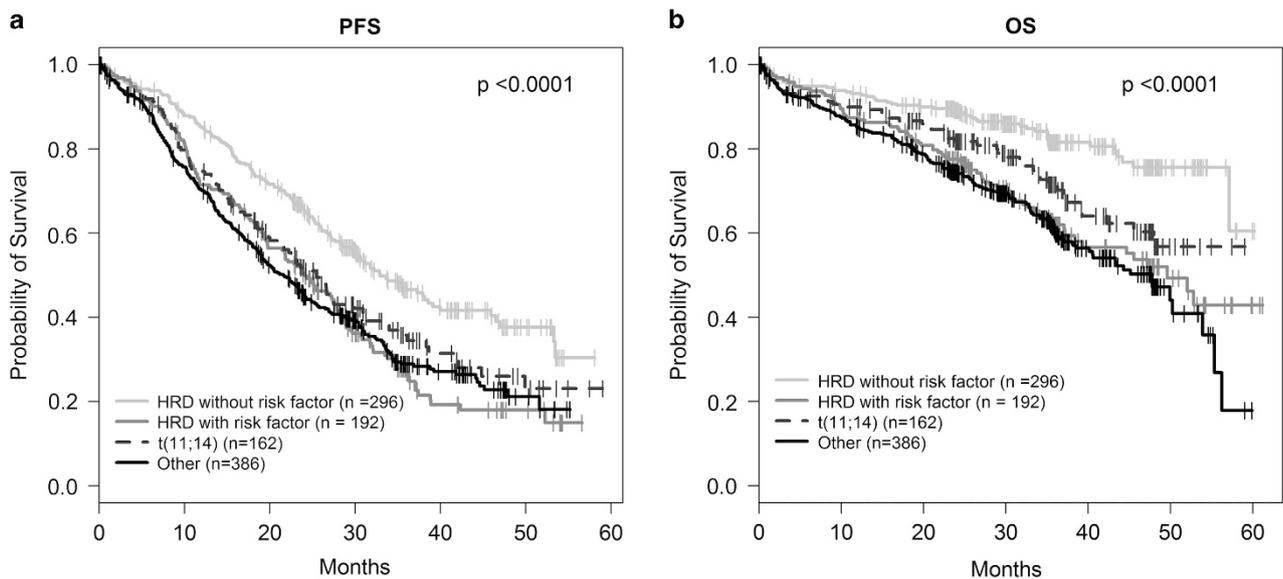


Figure 4. Survival in HRD MM with and without risk factors in Myeloma XI. Kaplan-Meier curves and log-rank *P*-values for (a) PFS (b) OS.

gain(1q)-HRD, del(17p) and del(1p32) had longer remissions and survival than any other sub-group and may be sufficiently treated with single-novel agent/immunomodulatory drug-based approaches, potentially reducing additional side effects and costs of novel agent combinations.^{39–42}

In summary, we demonstrate the utility of profiling multiple molecular genetic lesions to identify patients most likely to benefit from molecularly targeted therapies. The molecular tools used for profiling Myeloma XI are readily applicable within diagnostic settings and should therefore help implementing stratified treatment approaches as part of routine patient care.

CONFLICT OF INTEREST

Honoraria from Novartis, Pfizer, Takeda, Janssen by EMB; research support from Celgene by JRJ; consultancy and honoraria from Celgene, Novartis, Takeda by CP; SS was employed by MRC Holland; Honoraria and consultancy from Janssen, Novartis, Amgen, Takeda, Celgene by MWJ; Research funding: Janssen, Celgene; employment, equity ownership, royalties from Abingdon Health/Serascience by MTD; honoraria, consultancy from Celgene, Takeda, Janssen by RGO; Research funding from Celgene by DAC; Research Funding from Celgene by WMG; consultancy and research funding from Janssen, Celgene, Takeda Oncology, Sanofi, Amgen and BMS by GC; honoraria from Takeda-Millennium, Onyx/Amgen, Celgene and Janssen by FED; GHJ: honoraria and consultancy from Celgene, Takeda, MSD, Janssen, Roche and Amgen; research funding and travel support from Celgene and Takeda; honoraria, consultancy, research funding from Celgene, Takeda-Millennium, BMS by GJM; honoraria from Amgen, Celgene, Janssen: Consultancy for Takeda, Amgen, Chugai Pharma, BMS, Janssen; Research Funding from Celgene by MFK. The remaining authors declare no conflict of interest.

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LYMPHOID NEOPLASIA

Subclonal *TP53* copy number is associated with prognosis in multiple myeloma

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KEY POINTS

- *TP53* deletion of minor tumor subclones is independently prognostic in newly diagnosed multiple myeloma.
- Assessment of subclonal *TP53* deletions by MLPA is readily applicable in standard diagnostics, enabling stratified patient management.

Multiple myeloma (MM) is a genetically heterogeneous cancer of bone marrow plasma cells with variable outcome. To assess the prognostic relevance of clonal heterogeneity of *TP53* copy number, we profiled tumors from 1777 newly diagnosed Myeloma XI trial patients with multiplex ligation-dependent probe amplification (MLPA). Subclonal *TP53* deletions were independently associated with shorter overall survival, with a hazard ratio of 1.8 (95% confidence interval, 1.2-2.8; $P = .01$). Clonal, but not subclonal, *TP53* deletions were associated with clinical markers of advanced disease, specifically lower platelet counts ($P < .001$) and increased lactate dehydrogenase ($P < .001$), as well as a higher frequency of features indicative of genomic instability, *del*(13q) ($P = .002$) or *del*(1p) ($P = .006$). Biallelic *TP53* loss-of-function by mutation and deletion was rare (2.4%) and associated with advanced disease. We present a framework for identifying subclonal *TP53* deletions by MLPA, to improve patient stratification in MM and tailor therapy, enabling management strategies. (*Blood*. 2018;132(23):2465-2469)

Introduction

Despite recent improvements in survival, patient outcomes remain variable in multiple myeloma (MM). It is increasingly recognized that tumor heterogeneity is a determinant of patient outcome for many cancers, and the identification of subclonal driver events is central to better patient stratification.^{1,2} Aberrations in *TP53* are recognized to be one of most important markers of poor prognosis in MM.³ These are secondary driver events with variable subclonal distribution, with *TP53* typically being deleted and point mutations being relatively rare.⁴ However, defining the prognostic association of subclonal deletion of *TP53* in MM at diagnosis with a cutoff for diagnostic purposes has been problematic as a result of the technical challenges of using interphase fluorescence in situ hybridization (iFISH) in MM to quantify subclonal populations.⁵ To assess the prognostic relevance of subclonal *TP53* deletion at diagnosis, we profiled 1777 MM trial patients using multiplex ligation-dependent probe amplification (MLPA), which is readily applicable in diagnostic settings.

Methods

Myeloma IX and XI trial patients

We studied 1777 patients with MM enrolled in the UK NCRI Myeloma XI trial, and a subset from MRC Myeloma IX and Myeloma XI underwent comparison of MLPA and iFISH (supplemental Methods, available on the *Blood* Web site).

Copy number, translocation calling, and mutation detection

Bone marrow aspirates were processed as detailed in supplemental Methods. Details about iFISH profiling of Myeloma IX and Myeloma XI have been published previously and are described in supplemental Methods.⁶ Myeloma XI cases were profiled for copy number by MLPA and translocations determined by quantitative PCR, as previously reported.⁷

The MLPA P425 probemix (MRC-Holland) interrogates *TP53* exons 4, 7, and 10. *TP53* was considered deleted when

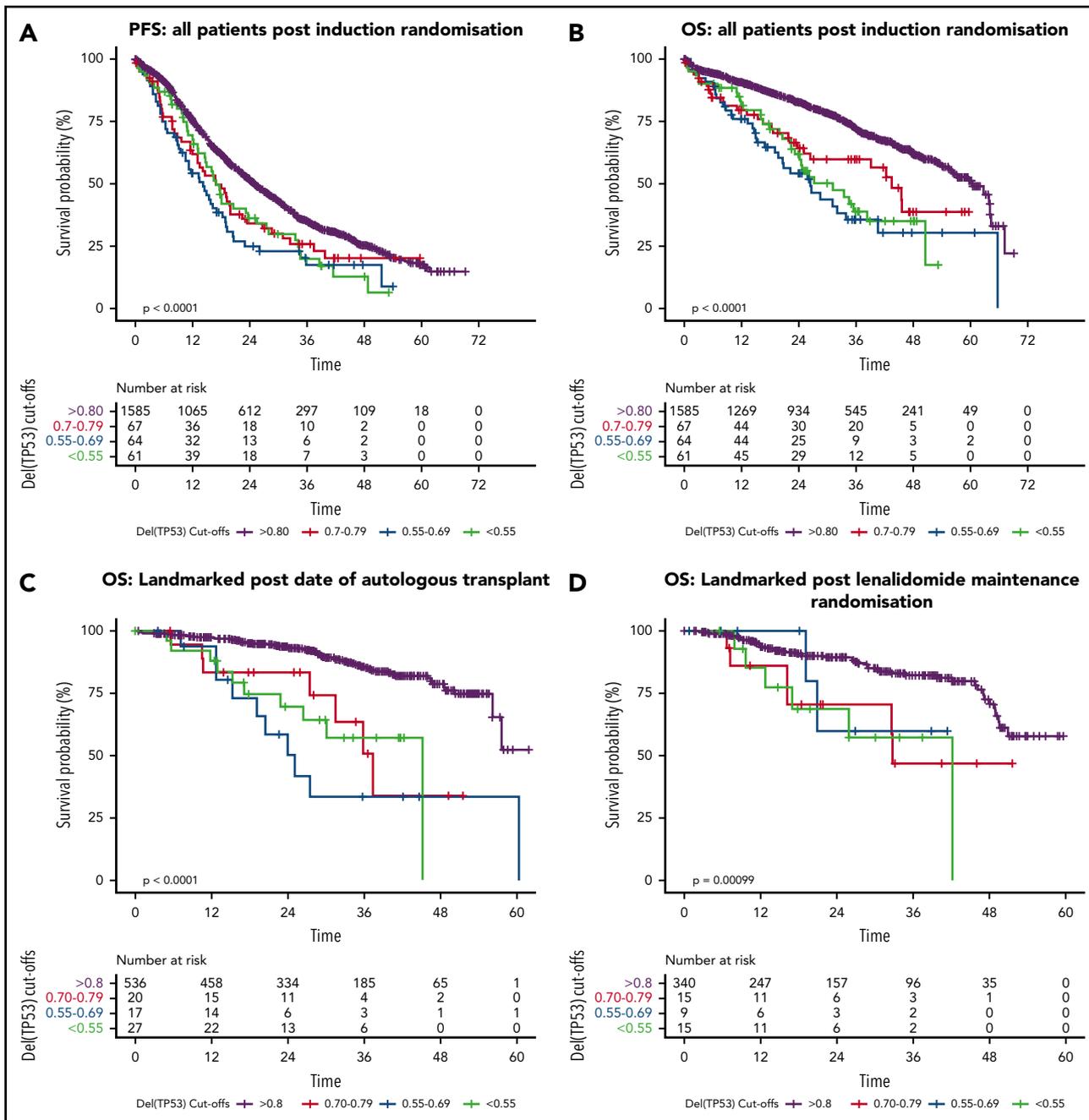


Figure 1. Association between subclonal and clonal TP53 deletion and survival in newly diagnosed myeloma. Kaplan-Meier survival curves showing progression-free survival (A) and OS (B) of 3 approximately equal-sized TP53-deleted clonal subgroups vs no TP53 deletion in 1777 patients in the Myeloma XI trial. OS evaluation of the above subgroups in landmarked analysis from the time of high-dose melphalan and autologous stem cell transplant (C) and from the time of maintenance randomization (D).

normalized copy number values of 2 of 3 MLPA probes were below the defined cutoff. A total of 1357 patient tumors was further analyzed with probemix X073, covering all exons of TP53. Previously published exome sequencing was available for 463 patients.⁴

Statistical analysis

Statistical analyses were performed in R (version 3.4.1) using subroutines survival, survC1, and survivalROC. Progression-free survival was defined as time from randomization to progression or death, and overall survival (OS) was defined as time from randomization to death. To define the optimal prognostic

normalized MLPA cutoff value for TP53 deletion calling, we analyzed subgroups defined by descending (0.05 steps from 1.0 [equivalent to normal diploid copy number]) normalized MLPA value using time-dependent receiver operator curve AUCi (integrated area under the curve) estimates for OS for each cutoff.⁸

Cox proportional hazards regression was used to estimate univariate and multivariable hazard ratios (HRs) and 95% confidence intervals (CIs). Kaplan-Meier survival curves were generated, and homogeneity between groups was assessed using the log-rank test. The association between categorical variables was examined using the Fisher exact test, and the association

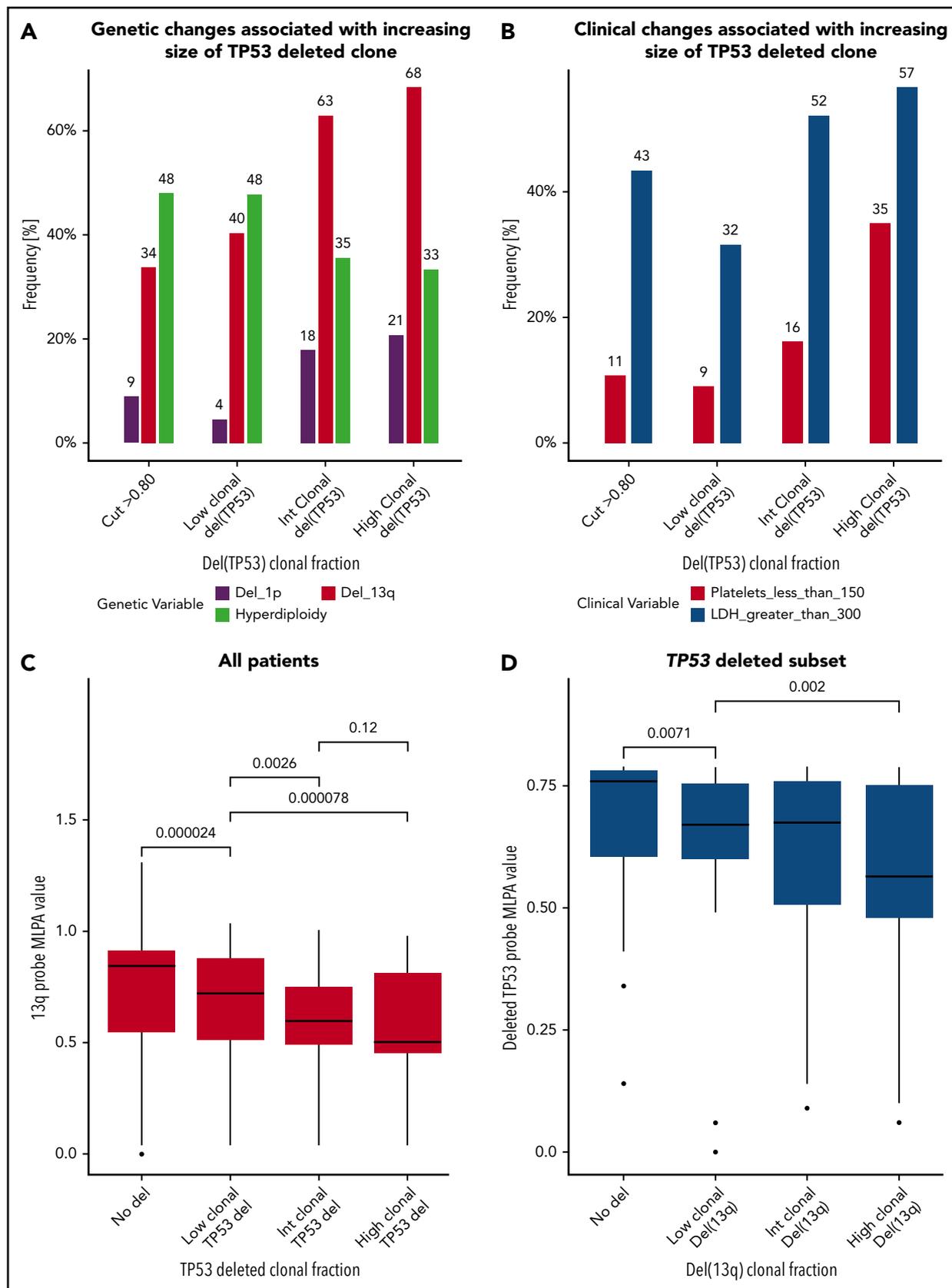


Figure 2. Relationship between subclonal and clonal TP53 deletion and clinical and genetic characteristics of myeloma. Percentage frequency of genetic changes (A) and clinical changes (B) associated with low, intermediate, and high deletion of TP53 clone. (C) MLPA values normalized for 13q probes in the same patients with low, intermediate, and high deletion of TP53 clone. (D) MLPA values across the subset of patients with del(TP53) and increasing size of del(13q) clone. Lower MLPA values represent increasing size of deleted clone.

between continuous variables was determined using the Wilcoxon signed-rank test. A 2-sided P value $<.05$ was considered significant.

Results and discussion

To identify the clinically relevant threshold for subclonal *TP53* deletions, we interrogated step-wise increasing fractions of *TP53* deletion by MLPA using the time-dependent receiver operating characteristic curve analysis method (AUCi) for OS.⁹ We identified a normalized *TP53* MLPA value <0.8 as the cutoff providing optimal prognostic power, identifying 192 of 1777 (10.8%) tumors as *TP53* deleted (supplemental Figure 1). These results were consistent in intensively (transplant eligible) and nonintensively treated patients (nontransplant eligible) (supplemental Table 1). The optimized <0.8 MLPA cutoff is equivalent to 10% to 20% subclonal 17p deletion, MLPA levels <0.6 were equivalent to clonal deletions with $\geq 50\%$ tumor fraction, and MLPA values <0.5 were equivalent to fully clonal (95% to 100%) del(17p) compared with iFISH in a matched data set from the Myeloma IX⁶ and Myeloma XI trials (supplemental Figure 2B). The distribution of MLPA-normalized values for *TP53* probes across 1777 Myeloma XI tumors is shown in supplemental Figure 2. Inclusion of subclonal deletions using an MLPA cutoff <0.8 was confirmed as prognostically most informative by univariate Kaplan-Meier log-rank testing ($P = 6.7 \times 10^{-15}$), Cox regression (Wald $P = 4.1 \times 10^{-14}$), and C-statistic by Uno et al¹⁰ (supplemental Table 2). A limitation of the study is the lack of a validation trial data set.

Treatment allocation, key demographics, and induction response were comparable between patients with *TP53*-deleted and nondeleted tumors, as defined by MLPA <0.8 . However, patients with *TP53* deletion showed features of advanced disease and associated morbidity, specifically reduced platelet counts $<150 \times 10^9/L$ ($P = 5.1 \times 10^{-4}$) and poorer performance status (World Health Organization [WHO] performance status ≥ 2) ($P = .0012$) (supplemental Table 3). Although WHO was independently associated with shorter survival, the association between WHO and *TP53* deletion suggests an interrelationship with genetic and clinical features that are normally thought of as patient related rather than disease related.

To characterize the features of subclonal vs clonal deletion, *TP53*-deleted tumors were divided into 3 equal-sized subgroups based on MLPA values: subclonally deleted tumors ($n = 67$; MLPA cutoff $\geq 0.7 < 0.8$), intermediate clonal tumors ($n = 64$; MLPA $\geq 0.55 < 0.7$), and clonally *TP53* deleted tumors ($n = 61$; MLPA <0.55). All 3 groups were independently associated with OS, with a subclonally deleted HR of 1.8 (95% CI, 1.2-2.8; $P = .01$), an intermediate-deleted HR of 2.9 (95% CI, 1.9-4.4; $P = 5.6 \times 10^{-7}$), and a clonally deleted HR of 2.2 (95% CI, 1.4-3.2; $P = .0002$) (Figure 1A-B; supplemental Table 4). Landmarked analyses from autologous stem cell transplant and lenalidomide maintenance randomization show consistent results for all 3 groups (Figure 1C-D; supplemental Table 4).

Correlating clinical characteristics, patients with clonal, rather than subclonal, *TP53* deletion had markers of high disease burden, specifically reduced platelet counts ($<150 \times 10^9/L$, 35% vs 9%; $P = .00047$) and high LDH levels (>300 U/L, 57% vs 32%; $P = .012$) (Figure 2B). Clonal vs subclonal deletion of *TP53* was associated with higher rates of del(13q) (68% vs 40%; $P = .002$)

and/or del(1p) (21% vs 4%; $P = .006$) (Figure 2A). The rate of *TP53* mutations was increased in clonal deletions (3/18) vs subclonal deletions (1/21). Although MLPA cannot comprehensively assess clonal architecture, an association between *TP53* deletion clonality and an increasing size of del(13q) clone ($P = .002$) (Figure 2C-D; supplemental Figure 5) raises the possibility of coevolution of these lesions. Deletion of *TP53* and *RB1* on chromosome 13q have been shown to be important in cell cycle¹¹ and senescence,¹² suggesting possible mechanisms for how their codeletion may confer a competitive advantage.

Clonal homozygous *TP53* deletions defined by MLPA values <0.25 were present in 9 of 1777 tumors (0.5%) analyzed with the P425 MLPA probemix for exons 4, 7, and 10 of *TP53*. To identify patterns of focal homozygous deletions, all 11 *TP53* exons were analyzed using a specifically designed X073 MLPA probemix in 1357 patients. Homozygous deletion frequency was low (0.6%), and deletions were focal and not restricted to the DNA-binding domain of *TP53*. Homozygous *TP53* deletion was associated with a very short median OS of 22.4 months and an HR for OS of 3.7 (95% CI, 1.5-8.9; $P = .004$) (supplemental Figure 3; supplemental Table 5). Most patients with homozygous *TP53* deletions had markers of clinically and molecularly advanced disease, with elevated LDH (>300 U/L) in 67%, reduced platelet counts ($<150 \times 10^9/L$) in 67%, and del(13q) in 88%. Exome-sequencing data were available for 422 of the tumors profiled using MLPA. Of these, 10 tumors (2.4%) had biallelic (mutation+deletion) *TP53* loss of function, and 47 tumors (11.1%) had monoallelic loss. Biallelic and monoallelic *TP53* loss was independently associated with inferior survival (supplemental Figure 3, supplemental Table 5).

In summary, we demonstrate an independent association between subclonal *TP53* deletions and MM outcome. Detection of subclonal *TP53* deletion by MLPA is readily applicable within diagnostic settings and could enable stratified treatment approaches aimed at preventing subsequent rapid disease evolution.

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Authorship

Contribution: V.S., D.C.J., R.S.H., and M.F.K. conceived and designed the study; all authors acquired data; V.S., D.A.C., R.S.H., and M.F.K. analyzed data; and V.S., R.S.H., and M.F.K. wrote the manuscript.

Conflict-of-interest disclosure: V.S. received travel support from Sanofi and Janssen. S.S. is an employee of MRC-Holland. M.W.J. acted as a consultant for Janssen, Takeda, Amgen, Celgene Corporation, and Novartis; received travel support and honoraria from Janssen, Takeda, and Amgen; received honoraria from Celgene Corporation and Novartis; and received research funding from Janssen and Celgene Corporation. M.T.D. has equity ownership in and serves on the board of directors of Abingdon Health. R.G.O. received honoraria from Takeda and Celgene Corporation; received travel support from Takeda and Janssen; acted as a consultant for Janssen and Celgene Corporation; and received research funding from Celgene Corporation. G.J.M. received research funding from Janssen and Celgene Corporation and acted as a consultant for and received research funding from Bristol-Myers Squibb, Takeda, and Celgene Corporation. W.M.G. received research funding from Celgene Corporation, Amgen, Merck Sharp and Dohme; acted as a consultant for Celgene Corporation; received honoraria from Janssen and Abbvie. F.E.D. acted as a consultant for and received honoraria from Amgen, AbbVie, Takeda, Janssen, and Celgene Corporation. G.C. acted as a consultant for and received honoraria from Takeda, Glycomimetics, Sanofi, Celgene Corporation, Janssen, Bristol-Myers Squibb, and Amgen; received research funding from Takeda, Celgene Corporation, Janssen, and Amgen; and is a member of the speakers bureau for Sanofi, Celgene Corporation, Janssen, and Amgen. D.A.C. received research funding from Celgene Corporation, Amgen, and Merck Sharp and Dohme. G.J. acted as a consultant for and received honoraria from Roche, Amgen, Janssen, Merck Sharp and Dohme, Celgene Corporation, and Takeda; received travel support from Celgene Corporation and Takeda; received research funding from Takeda; and is a member of the speakers bureau for Roche, Amgen, Janssen, Merck Sharp and Dohme, Celgene Corporation, and Takeda. M.F.K. acted as a consultant for Bristol-Myers Squibb, Chugai, Janssen, Amgen, Takeda, and Celgene Corporation; received travel support from Bristol-Myers Squibb and Takeda; received honoraria from Janssen, Amgen, and Celgene Corporation; and received research funding from Celgene Corporation. The remaining authors declare no competing financial interests.

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Footnotes

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Appendix: study group members

The members of the National Cancer Research Institute Haematology Clinical Studies Group are: Peter Hillmen, Alan Chant, Gordon Cook, Mhairi Copland, Charles Craddock, Lavinia Davey, Walter Gregory, Sally Killick, Amy Kirkwood, Judith Marsh, Adam Mead, Gillian Murphy, Kikkeri Naresh, Stephen O'Brien, Andy Peniket, Alasdair Rankin, Clare Rowntree, Anna Schuh, Shamyla Siddique, Simon Stanworth, Simon Watt, Kwee Yong.



Multiple myeloma gammopathies

Predicting ultrahigh risk multiple myeloma by molecular profiling: an analysis of newly diagnosed transplant eligible myeloma XI trial patients

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The prognosis for newly diagnosed multiple myeloma (NDMM) has improved with the advent of new agents, but outcome in some patients remains very poor. Identifying patients with high-risk disease early opens up the prospect of stratified treatment [1–3].

Biomarkers including chromosomal aberrations t(4;14), t(14;16), and t(14;20) translocations, gain of 1q and deletion of 17p, detected by fluorescence in situ hybridization (FISH) or multiplex ligation-dependent probe amplification (MLPA) and qRT-PCR-based translocation detection, have been associated with adverse outcome and co-occurrence of ≥2 such aberrations (a double-hit) is predictive of especially aggressive MM [4, 5]. Multiple gene expression profiles (GEP) have been reported to be associated with outcome,

but so far only EMC92 and UAMS GEP70 have been developed into validated clinical tests, marketed as SKY92 MMProfiler and MyPRS, respectively [6–9].

To examine the combined predictive value of high-risk chromosomal abnormalities and SKY92 risk GEP we studied 329 NDMM patients from the NCRI Myeloma XI trial (ISRCTN49407852) who received intensive therapy (Supplementary Table 1) and validated findings in Medical Research Council (MRC) Myeloma IX trial patients (Supplementary Methods) [10, 11]. In both cohorts of patients purified (>95%) CD138-positive tumor cells were immunomagnetically selected and DNA and RNA were extracted using QIAGEN (Hilden, Germany) Allprep kits. Chromosomal aberrations, including high-risk lesions t(4;14), t(14;16), t(14;20), gain(1q), and del(17p), were assessed using qRT-PCR (Thermo Fisher, Darford, UK) and MLPA (MRC Holland, Amsterdam, The Netherlands), as previously reported (Supplementary Methods) [4]. GEP risk status was determined on a diagnostic Affymetrix GeneChip 3000 Dx v2.0 system (Thermo Fisher) using the SKY92 MMProfiler (SkylineDx, Rotterdam, The Netherlands) (Supplementary Methods). Statistical analyses

Members of the NCRI Haematology Clinical Studies Group are listed below Acknowledgements

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Table 1 Univariate and multivariate Cox proportional hazard survival analyses of genetic, gene expression, and clinical risk markers for PFS and OS for 329 representative Myeloma XI NDMM patients from induction randomization.

Univariate analysis			Multivariate analysis		
<i>Progression free survival</i>			<i>Progression free survival</i>		
	HR (95% CI)	Wald <i>P</i>		HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	2.6 (1.96–3.45)	4.08×10^{-11}	SKY92 high-risk	2.14 (1.54–2.96)	0.00000475
Hyperdiploid	0.74 (0.57–0.95)	0.0198	Hyperdiploid	0.93 (0.7–1.24)	0.634
Adverse translocation	2.04 (1.53–2.72)	1.12×10^{-06}	Adverse translocation	1.89 (1.36–2.62)	0.00015
Del(1p) [<i>CDKN2C</i>]	1.47 (1–2.18)	0.0514	Del(1p) [<i>CDKN2C</i>]	1.01 (0.65–1.56)	0.979
Del(17p) [<i>TP53</i>]	1.63 (1.09–2.42)	0.016	Del(17p) [<i>TP53</i>]	1.32 (0.87–2.0)	0.198
Gain(1q)	1.44 (1.11–1.88)	0.00634	Gain(1q)	0.88 (0.65–1.2)	0.425
Age	1.04 (1.02–1.06)	0.00012	Age	1.04 (1.02–1.06)	0.000144
Induction randomization	0.77 (0.59–0.99)	0.0417	Induction randomization	1.2 (0.92–1.55)	0.176
ISS	1.33 (1.12–1.58)	0.0012	ISS	1.13 (0.95–1.36)	0.176
			<i>n</i> = 328, events = 232		
<i>Overall survival</i>			<i>Overall survival</i>		
	HR (95% CI)	Wald <i>P</i>		HR (95% CI)	Wald <i>P</i>
SKY92 high-risk	3.94 (2.73–5.69)	2.54×10^{-13}	SKY92 high-risk	2.72 (1.78–4.16)	0.00000396
Hyperdiploid	0.6 (0.42–0.87)	0.00717	Hyperdiploid	0.91 (0.6–1.37)	0.647
Adverse translocation	2.5 (1.72–3.64)	1.67×10^{-06}	Adverse translocation	1.85 (1.19–2.88)	0.0061
Del(1p) [<i>CDKN2C</i>]	2.38 (1.49–3.79)	0.000271	Del(1p) [<i>CDKN2C</i>]	1.29 (0.76–2.2)	0.343
Del(17p) [<i>TP53</i>]	3.02 (1.87–4.87)	5.76×10^{-06}	Del(17p) [<i>TP53</i>]	2.48 (1.48–4.17)	0.000602
Gain(1q)	2.39 (1.66–3.44)	2.98×10^{-06}	Gain(1q)	1.3 (0.85–1.97)	0.222
Age	0.62 (0.43–0.9)	0.0113	Age	1.02 (0.99–1.05)	0.2
Induction randomization	0.62 (0.43–0.9)	0.0113	Induction randomization	1.31 (0.9–1.91)	0.153
ISS	1.38 (1.08–1.76)	0.0101	ISS	1.09 (0.84–1.43)	0.512
			<i>N</i> = 328, events = 117		

Statistically significant $P < 0.05$ values are in bold.

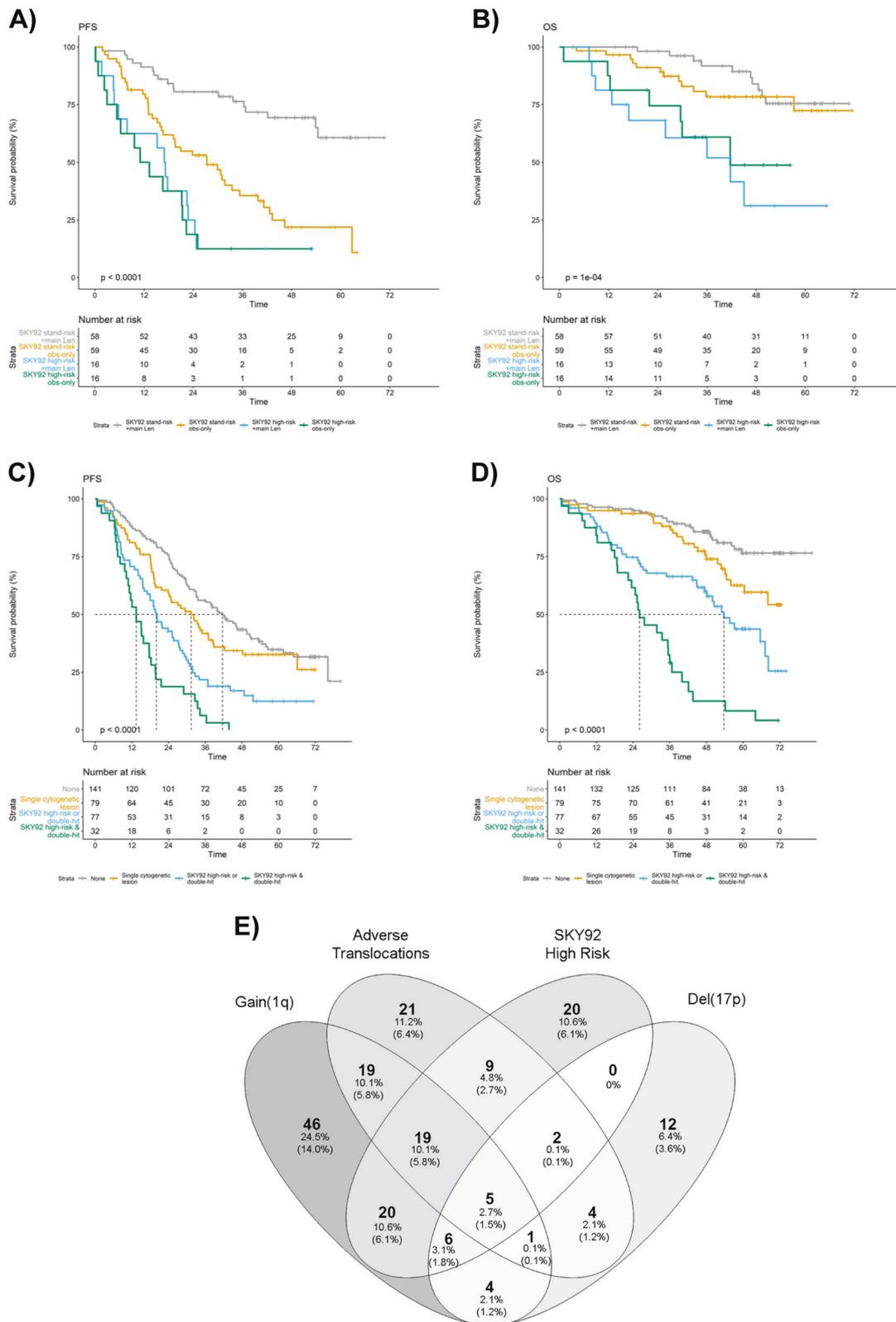
were performed in R (version 3.5.1) as detailed in Supplementary Methods.

MMprofiler assay results by SkylineDx identified 81 of the 329 Myeloma XI trial patients (24.6%) to have a SKY92 high-risk tumor signature (Supplementary Table 2). SKY92 high-risk patients had significantly shorter PFS (median 16.0 vs. 33.8 months; HR 2.6, 95% CI: 2.0–3.5; $P = 4.1 \times 10^{-11}$) and OS (median 36.7 months vs. not reached; HR 3.9, 95% CI: 2.7–5.7; $P = 2.5 \times 10^{-13}$) (Supplementary Fig. 1; Table 1), regardless of induction regimen and posttransplant randomization (Supplementary Figs. 2, 3; Supplementary Tables 3–5). Specifically, patients with SKY92 high-risk disease did not derive statistically significant benefit from lenalidomide single agent maintenance therapy (Fig. 1; Supplementary Fig. 3).

There was partial overlap between patients with GEP or chromosomal high-risk markers (Fig. 1, Supplementary Table 2), with 6.1% (20/329) of patients showing SKY92 positivity but absence of chromosomal high-risk markers. We analyzed prognostic association of molecular and clinical risk markers in a multivariable Cox proportional hazard

model and found presence of SKY92 high-risk (HR 2.7, 95% CI: 1.8–4.2; $P = 4.4 \times 10^{-6}$), adverse translocations (HR 1.8, 95% CI: 1.2–2.9; $P = 0.007$), and del(17p) (HR 2.5, 95% CI: 1.5–4.1; $P = 0.0007$) to be independently associated with shorter OS and SKY92 high-risk (HR 2.1, 95% CI: 1.5–3.0; $P = 4.8 \times 10^{-6}$) and adverse translocations (HR 1.9, 95% CI: 1.4–2.6; $P = 0.0002$) with shorter PFS (Table 1). Results were similar when analyzing GEP risk status with the UAMS GEP70 signature: by multivariable analysis UAMS GEP70 high-risk (HR = 2.54; 95% CI: 1.56–4.13; $P = 1.8 \times 10^{-4}$), presence of del(17p) (HR = 2.22; 95% CI: 1.32–3.72; $P = 0.0025$), and adverse translocation (HR = 2.11; 95% CI: 1.35–3.28; $P = 9.5 \times 10^{-4}$) were independently associated with shorter OS. However, GEP70 was not independently associated with shorter PFS (Supplementary Table 6).

One hundred and sixty-one patient tumors carried no chromosomal high-risk marker, of which 20 (12%) were SKY92 high-risk. The presence SKY92 GEP high-risk in isolation was significantly associated with shorter PFS (HR = 3.18; 95% CI: 1.86–5.46; $P = 2.6 \times 10^{-5}$; median



15.8 vs. 41.7 months) and OS (HR = 2.42; 95% CI: 1.04–5.67; P = 0.04; estimated 4 year OS 55% vs. 86%; Supplementary Fig. 4; Supplementary Table 7).

We have previously demonstrated the adverse prognosis of double-hit tumors, defined by co-occurrence of ≥2 chromosomal high-risk markers [4]. SKY92 and double-hit

◀ **Fig. 1 Patient outcome in context of GEP and chromosomal high-risk markers and their respective frequencies and distribution in Myeloma XI.** Kaplan–Meier plot of Myeloma XI trial patients ($n = 329$) in context of SKY92 risk profiling for (a) PFS, (b) OS from maintenance randomization, with survival curves for patients randomized to lenalidomide or observation plotted separately. Log-rank P values displayed. c, d Kaplan–Meier plots of molecular risk groups defined by absence of any high-risk marker, presence of a single genetic risk marker, presence of either double-hit or SKY92 high-risk or combined double-hit and SKY92 high-risk for c PFS, d OS from induction randomization. e Venn diagram of patients with tumors positive for validated genetic risk markers adverse translocations, gain (1q), del(17p), SKY92 GEP high-risk. % is relative to 188 patients with high-risk lesions, (%) relative to all patients ($n = 329$) in the study. Frequency represented by gray color coding, with darker gray indicating higher frequency.

were independently prognostic by multivariable analysis, with HRs 2.9 (95% CI: 1.9–4.2; $P = 2.6 \times 10^{-7}$) and 2.3 (95% CI: 1.5–3.6; $P = 0.0002$) for OS and HRs 2.0 (95% CI: 1.5–2.8; $P = 6.8 \times 10^{-6}$) and 1.6 (95% CI: 1.2–2.3; $P = 0.005$) for PFS, respectively (Supplementary Table 8). Results were consistent when PFS and OS were measured from maintenance randomization (Supplementary Table 8). We defined four risk groups combining predictive SKY92 and chromosomal high-risk markers: double-hit AND SKY92 (9.7% of pts), double-hit OR SKY92 (23.4% of pts), a single chromosomal high-risk marker (24.0% of pts), and no risk marker (42.9% of pts). Hazard ratios for OS were 11.0 (95% CI: 6.3–19.1; $P < 2.2 \times 10^{-16}$), 3.8 (95% CI: 2.3–6.3; $P = 2 \times 10^{-7}$), and 1.9 (95% CI: 1.1–3.3; $P = 0.03$) compared with those without risk markers, and HRs for PFS were 4.5 (95% CI: 3.0–6.9; $P = 2.3 \times 10^{-12}$), 2.3 (95% CI: 1.7–3.3; $P = 4.4 \times 10^{-7}$), and 1.3 (95% CI: 0.9–1.9; $P = 0.118$), respectively (Fig. 1; Supplementary Table 9). Results were consistent when PFS and OS were measured from time point of ASCT (Supplementary Fig. 5; Supplementary Table 9).

Of note, lenalidomide single agent maintenance markedly extended PFS in patients with a single chromosomal high-risk marker (HR 0.11; 95% CI: 0.03–0.41; $P = 0.0001$) or no risk marker (HR 0.26; 95% CI: 0.12–0.58; $P = 0.001$) when compared with observation. In contrast, those with SKY92 and/or double-hit (HR 0.67; 95% CI: 0.12–1.72, $P = 0.24$; HR 0.67; 95% CI: 0.32–1.37; $P = 0.27$, respectively) did not derive consistent benefit from lenalidomide maintenance (Supplementary Fig. 6).

Patients with combined double-hit and SKY92 high-risk status (9.7%) had poor survival outcomes: all patients (100%) progressed within 48 months from initial randomization and predicted OS at 48 months was 12.5% (Fig. 1). To confirm ultra-high-risk behavior of combined double-hit and SKY92 tumors in an independent trial, we analyzed 116 patients from the intensive, transplant treatment arm of MRC Myeloma IX. Eight (6.9%) patients showed double-hit and

EMC92 ultra-high-risk; all patients progressed within 36 months and died within 48 months. Meta-analysis using a random-effect model showed a HR for OS of 6.0 (95% CI: 4.1–8.9; $P = 4.8 \times 10^{-20}$) and HR of 3.5 (95% CI: 2.5–4.9; $P = 6.9 \times 10^{-13}$) for PFS for patients with combined GEP and double-hit tumors (Supplementary Fig. 7).

After accounting for GEP and chromosomal high-risk status, ISS and serum LDH were not predictive of outcome (Supplementary Fig. 8; Supplementary Table 10). We found significant overlap of these clinical and molecular risk markers: frequency of ISS 3 was higher in SKY92 high-risk vs. non-high-risk (38% vs. 21%; $P = 0.003$; Supplementary Fig. 9) and in those with multiple chromosomal high-risk risk lesions (21.5% without vs. 28.6% with single hit vs. 43.8% with double-hit). Only 15.6% of double-hit tumor patients were ISS 1 at diagnosis (Supplementary Fig. 9), whereas 71% of patients with ISS 3 carried one or more chromosomal or SKY92 high-risk marker. Similarly, baseline LDH was higher in patients with SKY92 or double-hit tumors vs. those without (Supplementary Figs. 9, 10).

We furthermore interrogated a range of risk signatures beyond binary (high-risk/non-high-risk) clinical read-out. Quantitative risk scores were correlated for most clinical signatures, most markedly EMC92 and GEP70 ($r = 0.79$; $P < 0.001$) (Supplementary Fig. 11). The EMC92 score ($r = 0.64$, $P < 0.001$) as well as most others also correlated with the in vitro model derived Proliferation Index (Supplementary Fig. 11).

Extreme copy number abnormalities (CNAs; amplification (≥ 4 copies) or homozygous deletion) have recently been proposed as exclusive drivers of high-risk MM [12], prompting us to investigate the correlation of quantitative CNAs with GEP risk scores. Median EMC92 scores were higher in tumors with gain(1q) vs. those without ($P = 2.1 \times 10^{-8}$) but there was no difference between gain (three copies) and amplification of 1q (≥ 4 copies) ($P = 0.56$; Supplementary Fig. 12), with a wide range of GEP scores in the latter. Tumors with deletion 17p had significantly higher median EMC92 GEP scores than those without deletion. Homozygous del(17p) was rare ($n = 2$), as expected, not allowing for formal comparison (Supplementary Fig. 12). Tumors with high-risk translocations had on average higher EMC92 scores than those without (Supplementary Fig. 13).

Our results demonstrate the prospective prognostic validity of SKY92 profiling in the wider context as a means of identifying patients at diagnosis who have high-risk MM, and show the independent association of SKY92 and high-risk chromosomal aberrations with outcome [9]. Our results highlight the molecular diversity of MM and demonstrate that single time point combined GEP and chromosomal profiling at diagnosis can predict clinical outcome with significant precision, in line with recent findings across multiple solid cancers [13, 14].

We furthermore demonstrate that in context of combined SKY92 and chromosomal profiling, ISS and LDH are not independently predictive. This is perhaps not unexpected, since ISS and LDH are clinical surrogate markers for tumor proliferation, which is assessed by combined GEP and double-hit profiling. Our analysis was, however, limited to younger and fitter, transplant-eligible patients and clinical risk markers such as ISS may have greater and independent relevance in older or frailer patients [15].

Our results demonstrate that patients with double-hit or GEP high-risk status are unlikely to benefit from current treatment approaches, including single agent lenalidomide maintenance therapy. In such patients intensified ongoing therapy with combination agents may be beneficial [3]. Such an assertion will be prospectively assessed in clinical studies such as the risk stratified UK OPTIMUM (MUKnine) trial (NCT03188172).

In conclusion, our findings support the further adoption of molecular biomarkers to stratify NDMM patient therapy.

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Author contributions VS, DAC, and MFK analyzed data. ALS, FC, JK, SE, and AP carried out experiments. VS, MFK wrote the paper. All authors contributed to the final paper.

Compliance with ethical standards

Conflict of interest VS: Sanofi—travel support; Janssen—travel support. MTD: Abingdon Health—equity ownership, membership on board of directors. RGO Takeda—honoraria, travel support; Janssen—consultancy, travel support; Celgene Corporation—consultancy, honoraria, research funding. FED Amgen—consultancy, honoraria; AbbVie—consultancy, honoraria; Takeda—consultancy, honoraria; Janssen—consultancy, honoraria; Celgene Corporation—consultancy, honoraria. GJM: Janssen—research funding; Bristol-Myers Squibb—consultancy, honoraria; Takeda—consultancy, honoraria; Celgene Corporation—consultancy, honoraria, research funding. DAC: Celgene Corporation, Amgen, Merck Sharp and Dohme—research

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