The use of flow cytometry in the diagnosis of the Myelodysplastic Syndromes

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

The Myelodysplastic Syndromes (MDS) are a biologically and clinically heterogeneous group of bone marrow haematopoietic cell disorders that result in ineffective haematopoiesis. Unlike most forms of haematological malignancy, the diagnosis of MDS remains heavily reliant on subjective morphological interpretation which can result in inaccurate and missed diagnoses. The use of flow cytometric immunophenotyping offers a potential solution to aid in the diagnosis of MDS, and numerous flow cytometric scoring schemes have been already been proposed and tested. However, most flow cytometric scoring schemes are user-defined, with simple schemes lacking diagnostic sensitivity, whilst the more comprehensive schemes may be unfeasible to implement in a large-scale diagnostic setting.

The use of machine learning classifiers offered a more subjective approach to the use of flow cytometric data. Therefore, we have tested a series of classifiers both by combining simple immunophenotypic and demographic features, and by utilising a 2 tube-immunophenotyping panel which contained a large array of numerical and immunophenotypic attributes which had been identified as being abnormal in MDS patients.

We have shown that machine learning classifier-based approaches could reproducibly identify patients with definite abnormalities in MDS, and those with normal haematopoietic populations in non-diagnostic, reactive conditions. The classifiers further offered the ability to aid in the triage of patients unlikely to be MDS by providing the basis to a diagnostic confidence score. The application of multiple classifiers also identified a grey-area of MDS patients who were consistently misclassified and who may prove to be challenging to diagnose by flow cytometry, due to an absence of aberrant immunophenotypic features.

Finally, we have also shown that a combination of immunophenotyping and targeted gene mutation analysis provides the potential to identify non-diagnostic cases which may progress to MDS. It is in a combination of these two techniques where the future of MDS diagnosis may lie.

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1 Introduction

1.1 Historic background of MDS

The current diagnostic criteria for the myelodysplastic syndromes (MDS) have a basis both in history and in the *sine qua non* of morphological dysplasia. Although the presence of free iron in sideroblasts in normal and reactive conditions was acknowledged in the 1940's, the first reported MDS-related diagnostic entity was refractory anaemia with sideroblastic normoblasts in 1956 (Kaplan et al., 1954; Bjorkman, 1956). Prior to this publication, patients who would now be classified as MDS, were described as "pre-leukaemic" due to a series of patients with macrocytic anaemia who developed leukaemia (Hamilton-Paterson, 1949). This perception was superseded when it became apparent that most patients succumbed to the complications of cytopenias, rather than to progression to acute myeloid leukaemia (AML).

The first reference to MDS, as we know it, in the context of disease classification arrived with the publication of the 1976 French-American-British (FAB) classification of Acute Leukaemias (Bennett et al., 1976). The production of this document was motivated by a requirement to standardise the naming of myeloid disorders and to define morphological blast cells features according to lineage and maturation. This would provide (i) a means for comparing cases entered into clinical trials and (ii) a reference standard if, or when, new diagnostic tools became available.

Classification according to FAB criteria was based on the morphological examination of both Romanowsky- and cytochemically-stained peripheral blood and bone marrow haematopoietic cells. Two categories of "dysmyelopoietic syndromes" were noted: Refractory Anaemia with Excess Blasts (RAEB) and Chronic Myelomonocytic Leukaemia (CMML). These two were identified as requiring discrimination from other categories of AML. Furthermore, in a throwback to the "pre-leukaemic" label, it was recommended that patients with these two categories should be monitored for transformation to AML.

Six years later, the publication of "Proposals for the classification of the myelodysplastic syndromes" both changed the umbrella term from "dysmyelopoietic syndromes" to "myelodysplastic syndromes" and expanded the classification of MDS from two categories to five (Bennett et al., 1982). The foundation for classification was based primarily on

morphological parameters, with peripheral blood count data used in the diagnosis of Chronic Myelomonocytic Leukaemia (CMML). The 1982 FAB MDS classification scheme is shown in Table 1.1. The 1982 French-American-British Classification

Disorder	**Features**	
	Peripheral Blood	**Bone Marrow**
Refractory Anaemia	Reticulocytopenia Variable dyserythropoiesis Dysgranulopoiesis Blast cells < 1%	Normo/hyperceullular Erythroid hyperplasia and/or dyserythropoiesis Blast cells <5%
Refractory Anaemia with Ring Sideroblasts	As for Refractory Anaemia except the presence of ringed sideroblasts account for >15% of all nucleated cells in the bone marrow	
Refractory Anaemia with Excess Blasts	Abnormalities in all 3 of the erythroid, granulocytic, and megakaryocytic lineages Circulating blasts <5%	Hypercellular Granulocytic or erythroid hyperplasia Dysgranulopoiesis, dyserythropoiesis and/or dysmegakaryopoiesis Ringed sideroblasts may be present Blast cells 5-20%
Chronic Myelomonocytic Leukaemia	Absolute monocytosis (>1 x 10^9/l) with or without dysgranulopoiesis Blast cells < 5%	Similar to RAEB but an increase in monocyte precursors Variable blast count (up to 20%)
Refractory Anaemia with Excess Blasts 'in transformation'	Those not fitting above categories or AML subtypes, and:	
	5% or more blast cells	20 – 30% blast cells Presence of Auer rods in granulocytic precursors

Table 1.1. The 1982 French-American-British Classification of MDS.

Table created with data from (Bennett et al., 1982)

However, despite the classification being widely adopted and used, there appeared to be no underlying biological rationale for the boundaries between the subgroups within the classification. The 5% cut-off for the blast cell percentage appears to have originated from a definition of remission in acute leukaemia. There was also no apparent basis for the 30% blast cell boundary between Refractory Anaemia with Excess Blasts 'in transformation' and Acute Myeloid Leukaemia (reviewed by Lichtman, 2013 (Lichtman, 2013)). A boundary that would, however, be addressed in later classification schemes.

A further feature of this classification system was the reliance on morphology in the enumeration of blast cells and monocytes and in the evaluation of dysplasia. This reliance remains the keystone for subsequent classification schemes, and for entry into clinical trials. Furthermore, due to the origins in both peripheral blood full blood count parameters and morphological parameters, multiple ambiguities have become apparent in MDS classification schemes.

1.2 Why MDS can be diagnostically challenging

At presentation, approximately 80-85% of MDS patients are anaemic with normocytic or macrocytic erythrocytes, 40% are neutropenic, and 30-45% are thrombocytopenic (Steensma and Bennett, 2006). However, none of these cytopenic features are unique to MDS. A large meta-analysis study reported the prevalence of anaemia as 17% in the over 65 year olds (Gaskell et al., 2008). This percentage varied from 12% in the community to 47% in nursing homes and 40% in a hospital-based setting. The prevalence was further correlated with increasing age and the male gender, and was associated with nutritional deficiencies, renal insufficiency, and inflammation (Bach et al., 2014). A population-based study, in a well-defined geographical region, reported that thrombocytopenia, in the form of Idiopathic Thrombocytopenia Purpura (ITP), has an annual incidence rate of 2.64 per 100,000, which increases with age (Frederiksen and Schmidt, 1999).

Once congenital and acquired conditions are excluded, the presence of cytopenia(s), erythrocyte macrocytosis, or leucocyte dysplasia may prompt analysis of the bone marrow for evidence of MDS. A disadvantage of this wide-ranging, albeit indiscriminate, approach was highlighted in a hospital study of 245 patients with unexplained cytopenia, erythrocyte macrocytosis, or monocytosis (Beloosesky et al., 2000). Bone marrow analysis resulted in a diagnosis of MDS in only 15% of patients. This figure translates into a large number of potentially unnecessary and non-diagnostic, invasive bone marrow aspiration procedures.

Although this is a relatively safe procedure and associated adverse events are reportedly rare (Bain, 2005).

The reliance on morphology for a diagnosis of MDS can be problematic for a wide range of reasons. Erythroid, granulocyte, and megakaryocytic dysplasia can all be seen as features in normal and non-MDS individuals (Bain, 1996; Ramos et al., 1999; Parmentier et al., 2012). Dysplastic change may result from mineral deficiency or immunocompromise (Karcher and Frost, 1991; Gregg et al., 2002). Conditions giving rise to “stress erythropoiesis”, such as auto-immune haemolytic anaemia, can show dyserythropoiesis (Bessman, 1977). Vitamin B12 and folate level evaluation can be used to help distinguish macrocytosis from megaloblastic anaemia (Cafolla et al., 1998). Yet, to complicate matters, MDS has been reported as co-existing with megaloblastic anaemia (Drabick et al., 2001).

Morphological identification of MDS in the bone marrow failure syndromes can be problematic. The distinction between aplastic anaemia and hypoplastic MDS is challenging due to the common presence of pancytopenia with a hypocellular bone marrow in both conditions (Barrett et al., 2000). The presence of paroxysmal nocturnal haemoglobinuria (PNH) clonal haematopoiesis which is characterised by loss of GPI-anchored proteins due to the acquired mutations in the *PIG-A* gene and which develops on a background of bone marrow failure, can cause further ambiguity. Not only can PNH patients show dysplastic bone marrow features, but PNH has also been shown to occur in MDS patients, both clonally and non-clonally-related to the underlying dysplasia (Longo et al., 1994; van Kamp et al., 1994; Araten et al., 2001; Raza et al., 2014).

MDS diagnostic concordance can be complicated further by subjective morphological interpretation. An inter-observer effect was noted in studies where the morphological diagnosis of MDS was evaluated in central review or in a large multi-centre trial (Miller et al., 1992; Cantù Rajnoldi et al., 2005). In both these reports, diagnostic concordance was reported as approximately 50%. This inter-observer effect has prompted guidelines and publications as how best to morphologically recognise and enumerate cells in the context of MDS (Mufti et al., 2008; Goasguen et al., 2009; Goasguen et al., 2014).

To overcome ambiguity in determining whether a patient is eligible to be returned with a diagnosis of MDS, a consensus proposal for minimal diagnostic criteria was produced (Valent et al., 2007). This included, amongst other criteria, definitions for cytopenia; percentage dysplasia required; requirement for cytogenetic analysis, and guidance on immunophenotyping, molecular methods, and colony-forming assays (Valent et al., 2007). These guidelines were subsequently updated in 2012 with an increased emphasis on the requirement for cytogenetic analysis (Platzbecker et al., 2012). More recently, a

morphological diagnostic scoring scheme has been developed by defining thresholds for minimal bone marrow dysplasia (Della Porta et al., 2015a) In spite of this guidance, a reproducible MDS diagnosis cannot always be achieved (Font et al., 2013; Senent et al., 2013; Font et al., 2015).

1.3 Can other techniques be used to aid in the diagnosis of MDS?

1.3.1 The 2001 WHO classification of Myeloid Neoplasms

Unlike the FAB classification, the 2001 World Health Organisation (WHO) classification system for haematopoietic and lymphoid neoplasms advocated the use of morphology in conjunction with other laboratory findings. Genetic information, immunophenotypic data, cytochemical findings, and clinical features were incorporated in the definition of diagnostic subgroups across a range of myeloid disorders (Vardiman et al., 2002).

For MDS classification, the WHO wanted to incorporate cytogenetic findings as well as other features identified subsequent to the 1982 FAB recommendations. The classification is shown in Table 1.2 and the diagnostic criteria for CMML can be seen in Table 1.3. However, not everyone agreed with these changes and the classification drew criticism from the Members of the International MDS Study Group (Greenberg et al., 2000).

There were also conceptual changes from the FAB classification. These included transfer of CMML to a MDS/MPD subgroup, and inclusion of a myelodysplastic/myeloproliferative disorder, unclassifiable category (MDS/MPD-U). This, latter, “overlap” category includes patients with features of MDS but in the presence of either a raised platelet count of ≥600x10^9/L or a raised leucocyte count of ≥13x10^9/L. Again, the cut-off for the platelet count originates from an arbitrary point used as a diagnostic threshold for Essential Thrombocythaemia (Barbui et al., 2015) However, it is unclear from where the boundary for a leucocyte count of ≥13x10^9/L originates. It is possible that it originates from the WHO classification for *Atypical chronic myeloid leukaemia, BCR-ABL1 negative* which states that the WBC for this category is always >13x10^9/L and references guidelines to distinguish CML, aCML, and CMML (Bennett et al., 1994). However, these guidelines only mention a leucocyte count of 13x10^9/L when discussing the behaviour of CMML patients, and not as an arbitrary cut-off for diagnosis nor in reference to the reference range (Bennett et al., 1994).

The FAB entity RAEB-T was removed, with an accompanying reduction in the blast threshold for classifying AML to 20% blast cells. The latter changed on the basis of results suggesting that, when controlled for karyotype and age, the blast cell percentage did not show prognostic difference (Estey et al., 1997).

However, the biggest paradigm shift occurred with the first and, so far only, step towards a molecular classification of MDS: the creation of a subgroup for patients with an isolated chromosome 5q-deletion. This is a distinct disorder with well-defined features (Van den Berghe et al., 1985; Nimer and Golde, 1987; Boultwood et al., 1994; Giagounidis et al., 2004).

The changes from FAB to WHO classification resulted in prognostic changes. Germing *et al.* reported significant prognostic differences within subgroups in a 1600 patient, pre-validation study of the proposed WHO subgroups (Germing et al., 2000). Further *post hoc* validation confirmed these prognostic findings and extended them to report differences in response between the newly-defined WHO subgroups (Howe et al., 2003).

The new classification, however, did not fully resolve the underlying dependence on morphology for diagnosing MDS. To re-iterate the problematic nature of the morphological diagnosis, guidance was again provided to define how to identify a morphological “blast cell” or “blast equivalent”. Furthermore, the number of nucleated cells to be counted to obtain a blast cell percentage was also designated. In peripheral blood, this was designated as 200 cells, whilst a 500 cell differential should be performed on a bone marrow aspirate sample. The 95% confidence intervals for a 5% morphological blast count on 100 cells is 1.6 – 11.3% and this reduces for a 500 cells differential to 3.3 – 7.3% (Bennett and Orazi, 2009). Although this increase from a 100 to 500 cell differential reduces the uncertainty of identifying 5% blast cells, it does not exclude it (Vollmer, 2009).

Disease	Blood Findings	Bone Marrow Findings
Refractory anemia (RA)	Anemia No or rare blasts	Erythroid dysplasia *only* < 5% blasts < 15% ringed sideroblasts
Refractory anemia with ringed sideroblasts (RARS)	Anemia No blasts	Erythroid dysplasia *only* ≥15% ringed sideroblasts < 5% blasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenias (bicytopenia or pancytopenia) No or rare blasts No Auer rods < 1×10^9/L monocytes	Dysplasia in $\geq$ 10% of cells in 2 or more myeloid cell lines < 5% blasts in marrow No Auer rods < 15% ringed sideroblasts
Refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS)	Cytopenias (bicytopenia or pancytopenia) No or rare blasts No Auer rods < 1×10^9/L monocytes	Dysplasia in $\geq$ 10% of cells in 2 or more myeloid cell lines ≥15% ringed sideroblasts < 5% blasts in marrow No Auer rods
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenias < 5% blasts No Auer rods < 1×10^9/L monocytes	Unilineage or multilineage dysplasia 5% to 9% blasts No Auer rods
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenias 5% to 19% blasts Auer rods ± < 1×10^9/L monocytes	Unilineage or multilineage dysplasia 10% to 19% blasts Auer rods ±
Myelodysplastic syndrome, unclassified (MDS-U)	Cytopenias No or rare blasts No Auer rods	Unilineage dysplasia in granulocytes or megakaryocytes < 5% blasts No Auer rods
MDS associated with isolated del(5q)	Anemia < 5% blasts Platelets normal or increased	Normal to increased megakaryocytes with hypolobated nuclei < 5% blasts No Auer rods Isolated del(5q)

Table 1.2. The 2001 World Health Organisation Classification and Criteria for MDS

(Table reproduced from Vardiman et al., 2002)

Diagnostic criteria for chronic myelomonocytic leukaemia
Persistent peripheral blood monocytosis greater than 1×10^9/L No Philadelphia chromosome or *BCR/ABL* fusion gene Fewer than 20% blasts* in the blood or bone marrow Dysplasia in one or more myeloid lineages. If myelodysplasia is absent or minimal, the diagnosis of CMML may still be made if the other requirements are present and: • an acquired, clonal cytogenetic abnormality is present in the marrow cells, or • the monocytosis has been persistent for at least 3 months and all other causes of monocytosis have been excluded Diagnose CMML-1 when blasts fewer than 5% in blood and fewer than 10% in bone marrow Diagnose CMML-2 when blasts are 5% to 19% in blood, or 10% to 19% in marrow, or if Auer rods are present and blasts are fewer than 20% in blood or marrow Diagnose CMML-1 or CMML-2 with eosinophilia when the criteria above are present and when the eosinophil count in the peripheral blood is greater than 1.5×10^9/L
*In this classification of CMML, blasts include myeloblasts, monoblasts, and promonocytes.

Table 1.3. The 2001 World Health Organisation Criteria for the Diagnosis of Chronic Myelomonocytic Leukaemia

(Table reproduced from Vardiman et al., 2002)

1.3.2 Update and refinement to the 2001 WHO classification of MDS

The 2008 revision to the WHO classification scheme was published with the aim of incorporating new information to refine the diagnostic criteria, introduce new entities, and acknowledge the number of dysplastic lineages (Vardiman et al., 2009). Two changes included the assimilation of RCMD-RS into the RCMD subgroup and the creation of a new childhood entity, refractory cytopenia of childhood, due to the perceived biological differences between childhood and adult MDS.

The revised classification also saw the creation of a unilineage dysplasia category, Refractory Cytopenia with Unilineage Dysplasia (RCUD). This contained 3 subgroups: refractory anemia (RA), refractory neutropenia (RN), and refractory thrombocytopenia (RT). These were defined on the basis of peripheral blood uni- or bi-cytopenia and single lineage

dysplasia in the bone marrow. This is distinct from the pancytopenia and unilineage dysplasia of MDS-U. However, this can lead to an almost contradictory scenario whereby the dysplasia is evident in the non-cytopenic lineage, a scenario reported in 18 cases by Verburgh *et al.* in a study of 221 low-risk MDS cases (Verburgh et al., 2007).The cytopenia- and dysplasia-affected lineages were, however, unreported. However, classification as RCUD is rare and it is probable that these cases would have fallen into the RA category. This is based on the findings of a large, collaborative study (Gyan et al., 2016). This study examined 1445 MDS patients who presented with isolated cytopenia and reported that the incidence of RN and RT occurred in 2 and 1 patients, respectively, whilst 54 patients could be diagnosed with RA.

Changes to the classification of the "overlap" syndromes also occurred. Firstly, there was a category name change from Myelodysplastic/myeloproliferative disorder (MDS/MPD) to Myelodysplastic/myeloproliferative neoplasm (MDS/MPN), in line with changes to the nomenclature of the Myeloproliferative Disorder category itself to Myeloproliferative Neoplasm. Within this category there was also change to the Myelodysplastic/myeloproliferative neoplasm, unclassifiable category. A platelet count cut-off of ≥450x10^9/L was now adopted in-line with diagnostic criteria for Essential Thrombocythaemia. This cut-off was defined based on the statement that it "exceeds the 95th percentile for normal platelet counts" (Swerdlow et al., 2008). However, a leucocyte count cut-off of 13x10^9 for this classification remained in place.

The 2008 classification also included a provisional entity within the MDS/MPN-U category: Refractory anaemia with ring sideroblasts associated with marked thrombocytosis (RARS-T). This was defined as having features of RARS with a platelet count of >450 x 10^9/L

Lastly, further emphasis was placed on the use of cytogenetic karyotyping to aid in the diagnosis of MDS with the introduction of a category for patients who are lacking morphological evidence of MDS but have one of the cytogenetic abnormalities outlined in Table 1.4. Karyotypic abnormalities which are absent from the list but are also presumptive are: Idic(X)(q13); t(11;16)(q23;p13.3); t(3;21)(q26.2;q22.1); t(1;3)(p36.3;q21.1); t(2;11)(p21;q23); t(6;9)(p23;q34); and a complex karyotype (3 or more chromosomal abnormalities) involving one or more of the above abnormalities or abnormalities in red in Table 1.4 Recurrent cytogenetic abnormalities are reported in patients who are morphologically normal yet are cytopenic, with both the cytopenias and cytogenetic abnormalities usually persisting (Steensma et al., 2003).

1.4 Does the presence of cytogenetic abnormalities aid in the diagnosis of MDS?

The presence of clonal cytogenetic abnormalities is a recurrent feature of MDS and aids the diagnosis. Approximately 50% of patients show identifiable cytogenetic abnormalities (Solé et al., 2005; Haase et al., 2007; Pozdnyakova et al., 2008). The incidence and array of cytogenetic abnormalities is shown in Table 1.4.

Anomaly	Total		Isolated		With one additional abnormality		As part of complex abnormalities	
	No of cases	%	No of cases	%[a]	No of cases	%[a]	No of cases	%[a]
5q−	312	15.1	146	47.0	52	17.0	114	36.0
−7/7q−	230	11.1	86	37.5	31	13.5	113	49.0
+8	173	8.4	81	46.8	37	21.4	55	31.8
−18/18q−	78	3.8	3	3.8	2	2.6	73	93.6
20q−	74	3.6	36	48.6	10	13.5	28	37.8
−5	69	3.3	1	1.4	4	5.8	64	92.8
−Y	58	2.8	41	70.7	5	8.6	12	20.7
+21	45	2.2	5	11.1	18	40.0	22	48.9
−17/17p−	42	2.0	1	2.4	1	2.4	40	95.2
inv/t(3q)	41	2.0	16	39.0	8	19.5	17	41.5
−13/13q−	40	1.9	5	12.5	6	15.0	29	72.5
+1/+1q	37	1.8	3	8.1	6	16.2	28	75.7
−21	33	1.6	3	9.1	4	12.1	26	78.8
+11	28	1.4	6	21.4	4	14.3	18	64.3
−12	26	1.3	0	0.0	2	7.7	24	92.3
12p−	25	1.2	7	28.0	6	24.0	12	48.0
t(5q)	24	1.2	6	25.0	3	12.5	15	62.5
11q−	23	1.1	8	34.8	4	17.4	11	47.8
9q−	23	1.1	8	34.8	3	13.0	12	52.2
t(7q)	22	1.1	6	27.3	6	27.3	10	45.5
−20	22	1.1	0	0.0	0	0.0	22	100.0

[a]Of cases with the respective abnormality

Table 1.4. The incidence of chromosomal abnormalities in 2072 MDS patients.

The abnormalities shown in red are recurrent cytogenetic abnormalities considered as presumptive evidence of MDS in the absence of morphological features of MDS. (Table created with data combined from Haase et al., 2007 and Vardiman et al., 2009)

Although the presence of a solitary 5q deletion is a WHO diagnostic subgroup in itself and accounts for 7% of cases in this cohort, a deletion of 5q- is frequently accompanied by other cytogenetic abnormalities. An isolated 5q- is also not the only, recurrent, isolated karyotypic abnormality, trisomy 8, and monosomy 7/7q deletion are both recurrently identified in MDS. However, unlike the isolated 5q-, these are not afforded a WHO subgroup of their own.

The finding that 50% of MDS patients demonstrate a karyotypic abnormality should aid in a confident diagnosis of MDS. However, this finding creates different diagnostic challenges. Firstly, the frequency and presence of an identifiable cytogenetic abnormality varies between the WHO subgroups (Haase et al., 2007; Pozdnyakova et al., 2008). A normal karyotype is found more frequently in the WHO subgroups RA and RARS, than in the RCMD and RAEB subgroups (Haase et al., 2007; Pozdnyakova et al., 2008). It is the RA and RARS groups which are most diagnostically challenging as they show the least inter-observer concordance when assessed morphologically (Font et al., 2013; Font et al., 2015). In addition, none of the common chromosomal abnormalities are unique to MDS as all can be found in cases of AML and some myeloproliferative disorders. This is, perhaps, unsurprising due to the arbitrary boundaries and degree of overlap between the three categories. Moreover, 3 of the most frequent, recurrent cytogenetic abnormalities in MDS (del (20q), trisomy 8, and –Y) are excluded from the WHO list for presumptive evidence of MDS, in the absence of definitive morphological features, due to the presence of these abnormalities in aplastic anaemia (Maciejewski et al., 2002). Lastly, the number of patients who do not have a cytogenetic result due to failed or missing cytogenetic analysis is not well reported. The cytogenetic failure rate for the biggest MDS cohort was 3.3% (Haase et al., 2007). The number of cytogenetic failure cases in the three other, largest, MDS karyotypic studies is unreported (Greenberg et al., 1997; Solé et al., 2005; Pozdnyakova et al., 2008). The diagnostic and prognostic implication of a failed cytogenetic analysis in MDS is unknown. In AML, cytogenetic failure rates of 2.1% and 6% have been cited and both reports associated failure with a poor prognosis (Medeiros et al., 2014; Lazarevic et al., 2015). The proportion of cases in which no sample was sent for cytogenetic analysis in MDS is unknown but, in AML, this has been reported as 20% (Medeiros et al., 2014; Lazarevic et al., 2015).

1.5 Can patients with persistent cytopenia but no dysplasia, or *vice versa*, be classified as MDS?

One category mentioned in the revised WHO guidelines, but not adopted as an entity, was that of idiopathic cytopenia of undetermined significance (ICUS). This term was first used to describe in a series of patients who presented with a prolonged (>6 month) cytopenia with,

predominantly, a normal karyotype, and no, or insufficient, morphological evidence of dysplasia to diagnose MDS (Wimazal et al., 2007). This heterogeneous group of patients with ICUS have been reported as progressing to other myeloid disorders including both MDS and to AML (Wimazal et al., 2007; Schroeder et al., 2010; Valent et al., 2012).The exact percentage of patients with ICUS who progress is unclear due to patient to patient variability in the follow-up timeframe. However, in those patients who did progress, the timeframe to progression was variable and ranged from 4 months to 186 months (Wimazal et al., 2007; Schroeder et al., 2010).

A counterpart to ICUS is the condition termed idiopathic dysplasia of uncertain significance (IDUS). Like ICUS, this condition does not attain minimal diagnostic criteria for MDS and patients have a predominantly normal karyotype (Valent et al., 2011). Unlike its counterpart, IDUS presents with no cytopenia but has dysplastic bone marrow features, the lineage of which varies from patient to patient. Again, the exact percentage of patients who progress to myeloid malignancy and time to progression is unclear due to the variable follow-up timeframe. However, the timeframe for those patients who did progress ranged from 2 years to 6 years (Valent et al., 2004; Valent et al., 2011)

1.6 Is there an underlying biological basis of MDS?

Haematopoiesis can be defined as the self-renewal of haematopoietic stem cells and the production of mature blood cells by a hierarchy of progressively more lineage restricted, differentiated progenitors (Wang and Dick, 2005). The differentiation process combines the loss of self-renewal potential with lineage restriction and functional specialisation. These self-renewal, commitment and differentiation pathways are governed by transcription factors which are influenced by cytokine signals (Zhu and Emerson, 2002). The stage of differentiation is dependent upon specific combinations of genes and their protein products. Therefore, differentiation can be detected using techniques such as gene expression analysis and immunophenotyping of protein expression, as well as by the assessment of morphological changes using conventional cytochemical stains.

In contrast to the detection of differentiation, identification and characterisation of haematopoietic stem cells is more challenging. An *in vivo* functional assay using xenotransplantation of sorted stem cells into immune deficient mice, such as the non-obese diabetic severe combined immunodeficient strain (NOD-SCID), is required to detect the most primitive cell possessing the repopulating abilities attributable to haematopoietic stem cells. These repopulating cells were first identified by Baum *et al.* as expressing CD34 (Baum et al., 1992). Studies further isolated these cells to the $CD34^+$ compartment lacking in CD38

expression ($CD34^+CD38^-$) (Bhatia et al., 1997). In humans, this $CD34^+CD38^-$ compartment was regarded as containing the most primitive bone marrow haematopoietic cell (Terstappen et al., 1991; Rusten et al., 1994). More recent studies by Manz *et al.* and Doulatov *et al.* investigating the expression of CD7, CD10, CD38, CD45RA, CD90, CD123, and CD135 on $CD34^+$ cells have further refined the understanding of haematopoietic progenitor cell hierarchy (Manz et al., 2002; Doulatov et al., 2010). Table 1.5 shows the current human haematopoietic $CD34^+$ population hierarchy.

Group name	Phenotype								% of MNC's	Lineage output
	CD34	CD38	CD90	CD45RA	CD123	CD135	CD7	CD10		
HSC									0.04	All
MPP									0.04	All
MLP7−									0.01	B, T, NK, MDC
MLP7+									0.01	B, T, NK, MDC
CMP									0.15	EMK, G, MDC
GMP									0.05	G, MDC
MEP									0.30	EMK
B-NK									0.05	B or NK

Table 1.5. Progenitor population hierarchy characterised from cord blood and bone marrow.

Red denotes the presence of antigenic expression, whilst white denotes absence of expression. Yellow indicates that expression by this subgroup was unreported.

Abbreviations (Group name): HSC = haematopoietic stem cell, MPP = multipotent progenitor; MLP7- = multilymphoid progenitor $CD7^-$; MLP7+ = multilymphoid progenitor $CD7^+$; CMP = common myeloid progenitor; GMP = granulocyte/macrophage progenitor; MEP=megakaryocytic/erythroid progenitor; B-NK = B-lymphoid and NK-lymphoid progenitor.

Abbreviations (Lineage output): B = B-lymphoid; T = T-lymphoid; NK = Natural killer cells; MDC = Monocytes, Macrophages and Dendritic cells; EMK = Erythroid and Megakaryocyte; G = Granulocytes

Table created from data in Manz et al., 2002; and Doulatov et al., 2010.

Findings in acute myeloid leukaemia (AML) patients have given rise to the concepts of haematopoietic, clonal stem cell disorders and leukaemic stem cells. Similar to normal haematopoiesis, a leukaemia-initiating-cell in the $CD34^+CD38^-$ compartment of AML patients has been demonstrated (Lapidot et al., 1994). The concept of a leukaemic stem cell was further strengthened by the finding of both proliferation/differentiation and self-renewal properties of the $CD34^+CD38^-$cells and functional evidence of organizational hierarchy in AML cells (Bonnet and Dick, 1997).

1.6.1 The role of stem cells in MDS

MDS is commonly referred to as a clonal haematopoietic stem cell disorder. However, proving this is true remains a challenge. The interpretation of *in vitro* colony assays is difficult due both to the presence of non-clonal, non-MDS progenitor cells and to the increased apoptosis of MDS progenitor cells (Asano et al., 1994; Raza et al., 1995). Early NOD-SCID repopulating experiments had limited success and, when successful, showed only transient MDS-engraftment (Thanopoulou et al., 2004). This study by Thanopoulou *et al.* did, however, manage to demonstrate the co-existence of donor-derived, trisomy 8, B-lymphoid and myeloid cells co-existing in a NOD-SCID mouse following xenotransplantation (Thanopoulou et al., 2004). Unfortunately, although the donor patient was classified as MDS (RAEB-T) at the time of publication, under current WHO classification, they would be categorized as AML.

1.6.1.1 Insights into MDS progenitor and stem cell biology using genetic abnormalities

Better evidence of the stem cell involvement in MDS is derived from studies on patients in the 5q- WHO subgroup. These patients offer an attractive model for study due to the potential to track the 5q- abnormality by the use of fluorescent *in-situ* hybridisation (FISH). Firstly, Nilsson *et al.* showed that the 5q deletion was present in over 90% of the cells in the $CD34^+CD38^-$ compartment (Nilsson et al., 2000). Perhaps, more importantly, this study demonstrated that the 5q- abnormality was present in a fraction of $CD34^+CD19^+$ B-cells, which implied that the abnormality resided in a lympho-myeloid stem cell (Nilsson et al., 2000). The same group demonstrated that gene expression profiling of the $CD34^+CD38^-$ stem cells in normal and 5q- patients showed an almost perfect concordance between the two groups of patients (Nilsson et al., 2007). This finding provides support for the 5q- abnormality originating in the $CD34^+CD38^-$ stem cells but, unfortunately, did not reveal any further insight into the underlying pathogenesis of the 5q- abnormality. The ability to identify and track the 5q- abnormality in both the $CD34^+CD38^-$ stem cells and the $CD34^+CD38^+$ myeloid progenitor cells was further exploited for biological and clinical purposes (Tehranchi et al., 2010). Despite the presence of the 5q- in both the $CD34^+CD38^-$ stem cell compartment and the $CD34^+CD38^+$ myeloid progenitor cell compartment at presentation, the two compartments showed differential resistance to lenalidomide therapy with the 5q- persisting at a higher level in the $CD34^+CD38^-$ stem cell compartment which portended cytogenetic progression (Tehranchi et al., 2010).

The use of an underlying (cyto)genetic abnormality to investigate stem cells in MDS was not restricted solely to studies of MDS patients with a 5q-. The TET2 gene was found to be

mutated in various myeloid malignancies and is mutated in 20-25% of all MDS patients (Delhommeau et al., 2009; Solary et al., 2014). In four MDS patients, a TET2 mutation was found in both the $CD34^{+}CD38^{-}$ stem cell compartment and the $CD34^{+}CD38^{+}$ myeloid progenitor cell compartment, albeit in a lesser proportion of $CD34^{+}CD38^{-}$ cells (Delhommeau et al., 2009). A study by Pang *et al.* successfully used blocking of CD47 expression to xenotransplant stem cells harbouring a monosomy 7 from MDS patients into NOD-SCID mice which resulted in chimeric human $CD45^{+}$ cells that demonstrated monosomy 7 (Pang et al., 2013). This study, and a study by Will *et al.*, also demonstrated the presence of perturbed subgroup expansions in the CMP and GMP populations in the different subgroups of MDS in comparison to normal controls (Will et al., 2012; Pang et al., 2013).

Perhaps the most comprehensive study to date was by Woll and colleagues (Woll et al., 2014). By tracking the 5q- abnormality, this study demonstrated that only the $CD34^{+}CD38^{-}$ $CD90^{+}CD45RA^{-}$ MDS stem cells (MDS-HSC) from 5q- patients, and not the CMP's, GMP's, or MEP's, were capable of reconstituting haematopoiesis in mice. Furthermore, genetic mutations which were found within the MDS-HSC compartment in patients were identical to those found in the GMP or MEP populations, and there was no mutation found in the bulk of the cells which was not present in the MDS-HSC population. A similar approach to Woll *et al.* has been adopted more recently using the presence of mutations in the SF3B1 gene in patients with RARS to demonstrate the stem cell origin of this MDS subtype (Mian et al., 2015)

1.6.2 The role of ineffective haematopoeisis in MDS

The bone marrow specimen provides an insight into the disordered and ineffective haematopoiesis in MDS. Assessment of the trephine allows evaluation of bone marrow cellularity, architectural structure of the bone marrow, presence of fibrosis, and permits quantitative evaluation of any accumulation of specific haematopoietic populations. The presence of a normo/hyper-cellular bone marrow alongside dyserythropoiesis, dysmegakaryopoiesis, reticulin fibrosis, and abnormal localisation of immature precursors are all features consistently reported in MDS patients (Tricot et al., 1984; Frisch and Bartl, 1986; Ríos et al., 1990; Mangi and Mufti, 1992). The finding of a normal/hyper-cellular marrow in a cytopenic patient is a key "paradoxical" feature raising the suspicion of MDS. This paradox was resolved by the discovery that patients with MDS have increased apoptosis in the bone marrow with dysregulation of TNFα, FAS and TRAIL all implicated in this process (Raza et al., 1995; Parker et al., 2000; Kerbauy and Deeg, 2007). Whilst MDS patients with the 5q- again providing further insight into MDS with the discovery that the ineffective erythropoiesis and transfusion requirement attributable to this MDS subtype can

be overcome with the administration of lenalidomide which restores erythropoiesis by suppressing the 5q- clone (List et al., 2006)

1.7 What is the incidence of MDS?

Historically, data on MDS has been rarely collection by cancer registries and epidemiological studies were rare. In 2001, the International Classification of Disease for Oncology (ICDO) changed the classification of MDS from /1 (uncertain behaviour) to /3 (malignant, primary site). This change permitted evaluation of MDS diagnoses at the population level and allowed the annual incidence to be calculated.

In the US, the average annual MDS incidence is reported as 5 new diagnoses per 100,000 persons (National Cancer Institute. SEER Cancer Statistics Review 1975-2012.). There is a male: female skew in all diagnostic categories, with the exception of MDS with an isolated 5q- where the inverse occurs. Although MDS can be diagnosed in patients under the age of 40, it is primarily a disease which occurs in elderly patients. This can be seen in the increasing incidence with age which ranges from 0.2 per 100,000 in the under 40 years old to 59.1 per 100,000 in the over 80 years old (National Cancer Institute. SEER Cancer Statistics Review 1975-2012.). The incidence is also higher in whites compared to other racial groups.

European studies have reported similar findings to the US. Pre-2001 and the ICDO3 classification, there were few reported studies on the epidemiological findings of MDS. Those published were in well-defined populations and these reported higher incidences for males than females, increasing incidence with age, and crude annual incidence rates between 3.2 and 4.1 (Aul et al., 1992; Radlund et al., 1995; Maynadie et al., 1996). More recent large-scale publications by Sant *et al.* and Visser *et al.* have re-iterated the male to female skew, increased incidence with age, and, albeit lower than previously reported, overall crude incidences of 1.8 and 1.5, respectively (Sant et al., 2010; Visser et al., 2012). However, the use of the FAB classification of MDS in these European studies has rendered it difficult to determine population-based information regarding incidence and survival associated with specific MDS categories.

The Haematological Malignancy Research Network (HRMN) is a collaboration encompassing 2 UK Cancer Networks which cover 3.6 million people, 14 hospitals, and a single integrated haematopathology laboratory (HMDS), in which the patient data for diagnosis in accordance to WHO classification, prognosis, treatment, and outcome are obtained as well as socio-demographic measures (Smith et al., 2010a). A map of the geographical area covered and the participating hospitals is shown in Figure 1.1.

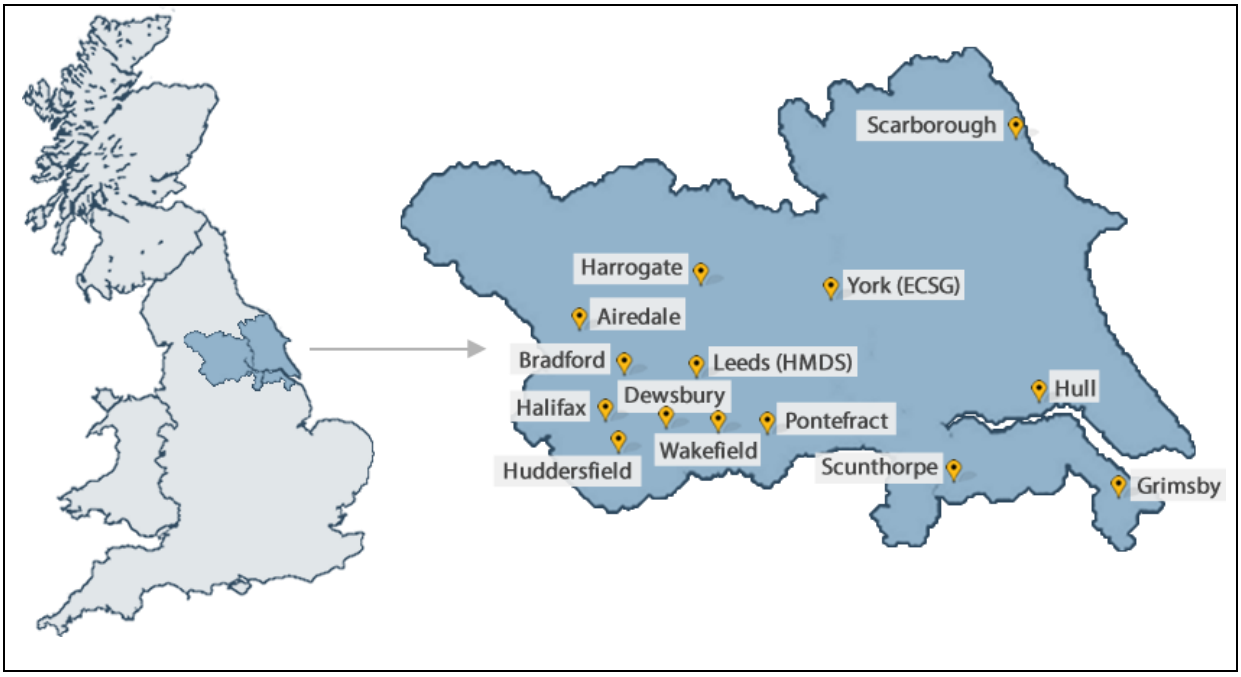


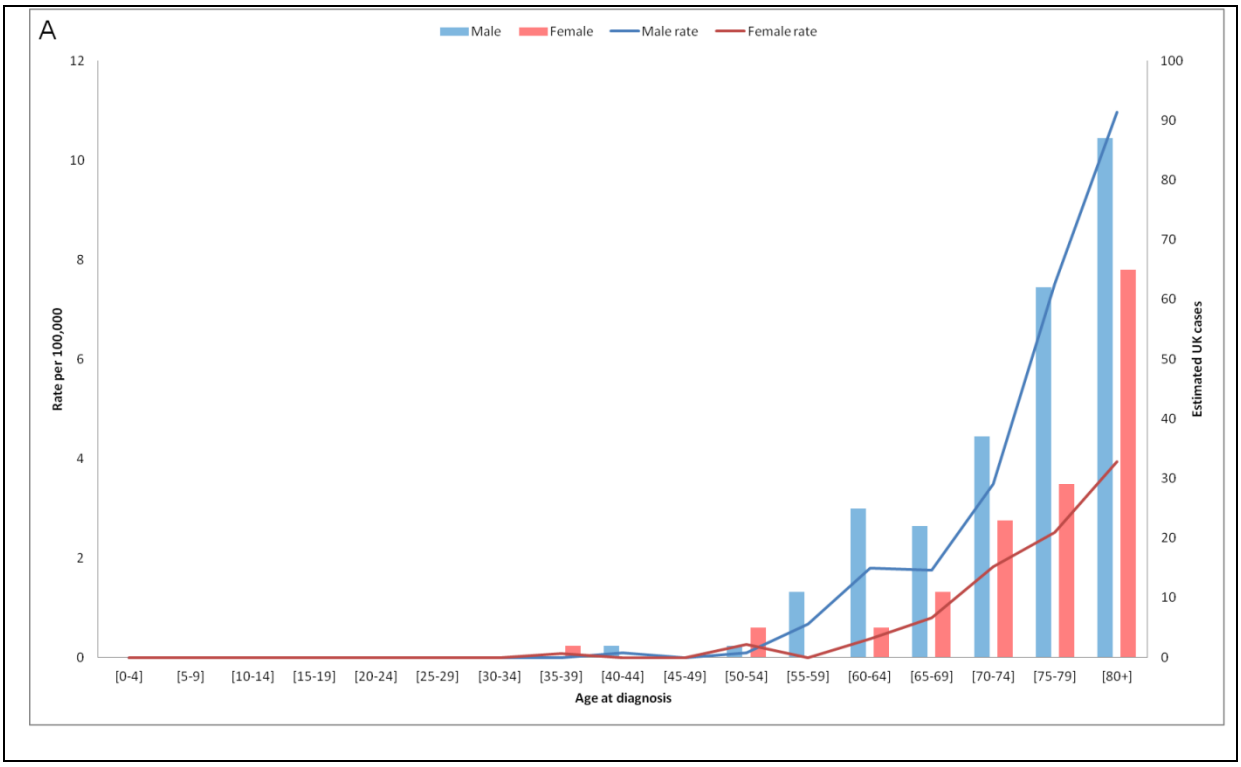
Figure 1.1. The geographical area and 14 hospitals served by HMDS and HMRN (HMRN., 2016).

Work undertaken by HMRN has shown that, overall, MDS accounts for 6% of all haematological malignancies, has a median age at diagnosis of 76 years old, an incidence of 3.7 per 100,000, a male:female rate ratio of 2.09, and no association with deprivation (Smith et al., 2011). From this resource, the incidence, sex ratio rate, median age at diagnosis and expected UK cases per year for RARS, RCMD, RAEB, CMML, and MDS/MPN-U could be calculated from HMRN data obtained between 2004 and 2013. These data are shown in Table 1.6. The incidence with age for RARS, RCMD, and RAEB could also be obtained and this is shown in Figure 1.2.

	Annual Rate per 100,000					Expected UK cases per year		
Disorder	**Total**	**Male**	**Female**	**M:F ratio**	**Median age at diagnosis**	**Total**	**Male**	**Female**
RARS	0.7	0.9	0.5	1.8	77.6	390	250	140
RCMD	1.5	2.3	0.8	2.9	75.7	910	670	240
RAEB	1.4	1.9	1.0	1.9	74.5	830	530	300
5q-	0.1	0.0	0.1	0.2	72.0	NS	NS	NS
CMML	0.8	1	0.5	1.8	77.4	440	280	160
MDS/MPN-U	0.1	0.1	0.1	1.4	77.5	50	30	20

Table 1.6. HMRN incidence data for the 6 available WHO subgroups in the MDS and MDS/MPN categories.

Data was obtained from the HMRN website (HMRN., 2016.)



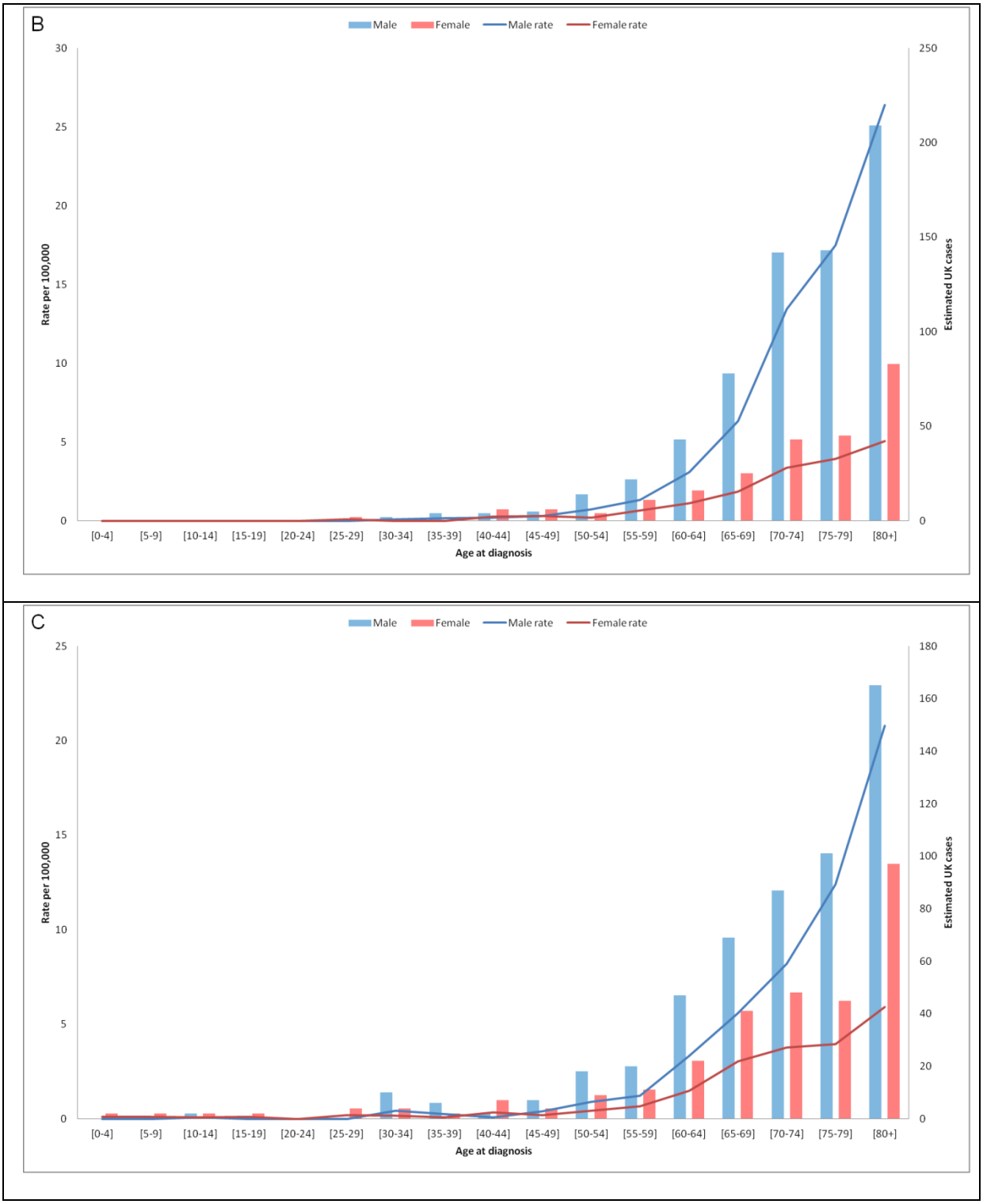


Figure 1.2. Age-specific incidence and estimated UK cases of (A) RARS, (B) RCMD, and (C) RAEB.

These graphs were created from data available from HMRN (HMRN., 2016.). The number of estimated UK cases was calculated by applying HMRN age and sex specific rates to the 2001 UK population census data.

As noted, HMDS receives specimens referred for the investigation of haematological malignancy from all of the centres within the 2 regional cancer networks and, thereby, provides diagnostic classification for the HMRN database. However, HMDS receives specimens from various other referral centres across the UK as shown in Figure 1.3. Some of these centres only provide bone marrow aspirate samples, as the trephine is processed in-house by the local histopathology laboratory. Furthermore, due to the geographical location and logistical arrangements, bone marrow specimens may be received over 24 hours following aspiration. This latter feature, if coupled with a lack of referred bone marrow aspirate smear and unavailability of full patient clinical information, can make accurate diagnosis of MDS and other haematological malignancies challenging.

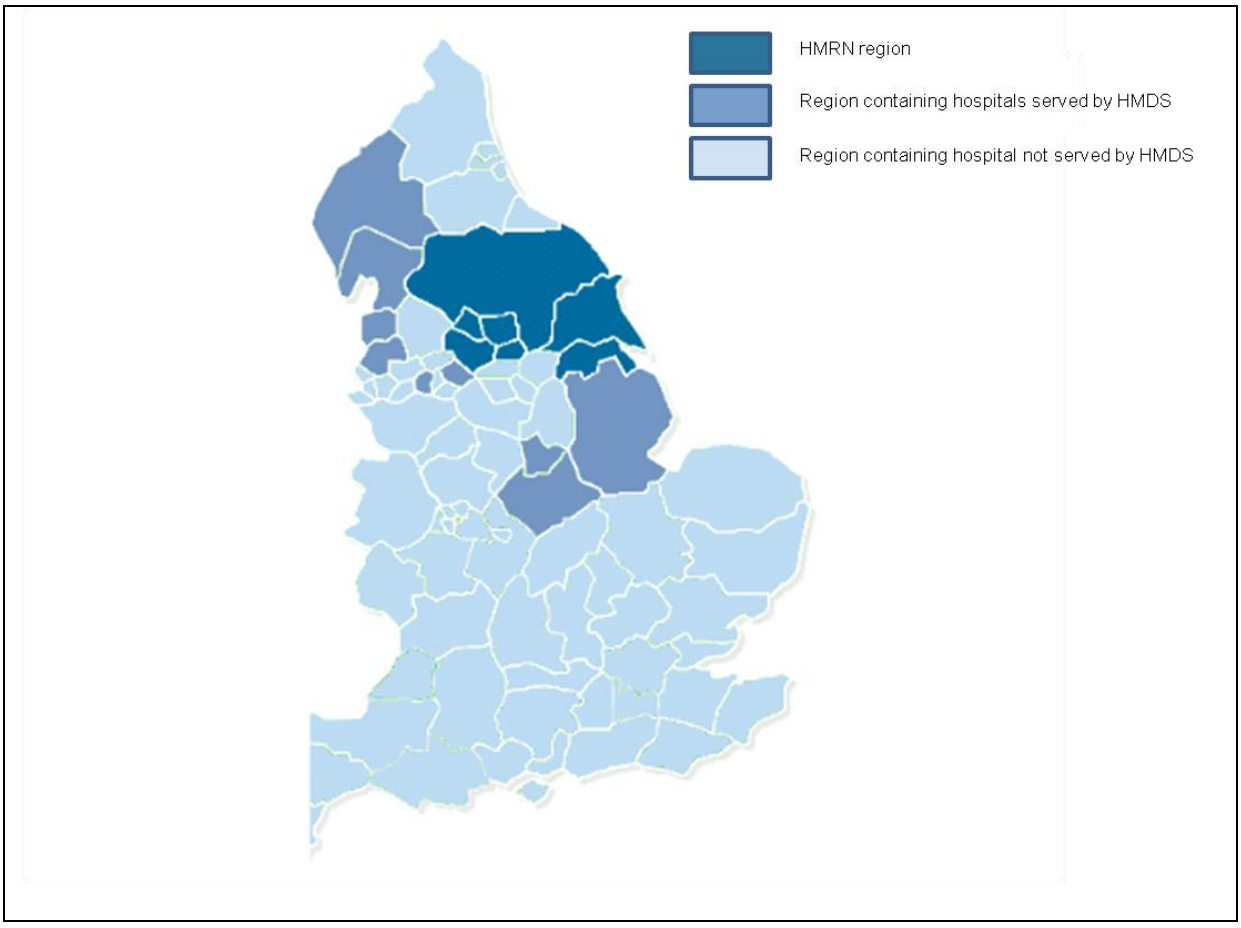


Figure 1.3. Map of the UK indicating the geographical regions containing hospitals which are served by HMDS.

1.8 Does MDS pose a clinical problem?

As there is a variable natural history of MDS due to the biological heterogeneity, one of the first aims following diagnosis is to provide a prognosis for the patient and decide upon

appropriate therapeutic approach based on this assessment. As neither the WHO classification scheme, nor the FAB classification scheme before it, predicts the need for clinical intervention, alternative schemes for generating a prognostic score have been proposed.

1.8.1 The International Prognostic Scoring Scheme

The International Prognostic Scoring System (IPSS) was established in 1997 to make predictions on patient outcome (Greenberg et al., 1997). This stratified patients into 4 risk categories (Low, Intermediate-1, Intermediate-2, and High) on the basis of percentage of bone marrow blast cells, cytogenetic karyotype, and presence and number of cytopenias.

There were limitations to the IPSS. Many patients now considered to have AML were included. There was no distinction between the presence of 2 or 3 cytopenias, nor was the depth of cytopenia taken into account. This latter point is clinically relevant as there is an increased infection rate when the neutrophil count is below $1x10^9$/L, and infection is the leading cause of death in MDS (Pomeroy et al., 1991). Furthermore, the depth of anaemia has a prognostic value for overall survival (Kao et al., 2008). The requirement for blood transfusion was overlooked, with patients requiring blood transfusion having a lower probability of survival (Cazzola and Malcovati, 2005). Lastly, the list of cytogenetic karyotypes defined as intermediate is long and studies have shown variable prognosis within this subgroup (Solé et al., 2000; Solé et al., 2005).

However, despite these criticisms, the stratification into prognostic subgroups allowed the natural history (median survival and time to AML progression) to be evaluated for each of the 4 subgroups as shown in Table 1.7.

Risk Group	Total score	Median survival (years)	Time for 25% to progress to AML (years)
Low	0	5.7	9.4
Intermediate-1	0.5-1.0	3.5	3.3
Intermediate-2	1.5-2.0	1.2	1.1
High	≥2.5	0.4	0.2

Table 1.7. MDS risk category as defined by the IPSS score values.

The table shows the clinical outcome as defined by median survival and risk of developing AML.

1.8.2 The WHO-based Prognostic Scoring System

An alternative scoring system attempted to address some of the criticisms of the IPSS. The WHO-based Prognostic Scoring System (WPSS) was based on the WHO classification, used the same karyotypic subgroups as the IPSS, and added transfusion requirement to classify patients into 5 different risk groups (very low; low; intermediate; high, very high) with different outcomes and progression to AML rates, as shown in Table 1.8 (Malcovati et al., 2007).

Risk Group	Total score	Median survival (years)		AML Progression (Cumulative probability)			
				2 years		5 years	
		Test	Validation	Test	Validation	Test	Validation
Very Low	0	8.6	11.8	0.0	0.03	0.06	0.03
Low	1	6.0	5.5	0.11	0.06	0.24	0.14
Intermediate	2	3.3	4.0	0.28	0.21	0.48	0.33
High	3-4	1.8	2.2	0.52	0.38	0.63	0.54
Very High	5-6	1.0	0.8	0.79	0.80	1.0	0.84

Table 1.8. MDS risk category as defined by the WPSS score values.

The table shows the clinical outcome as defined by median survival and risk of developing AML.

However, like the IPSS, there were some concerns, mainly surrounding the definitions regarding blood transfusion, the inter-observer reproducibility of the WHO classification, and the different ages of the training and validation cohorts (Bowen et al., 2008).

1.8.3 Other prognostic scoring systems

Other scoring systems have attempted to address the inadequacies the IPSS, 2 of which have been produced by the MD Anderson Cancer Centre. The first evaluated those patients in the Low and Intermediate-1 categories of the IPSS and stratified into 3 prognostic groups on the basis of age, cytogenetic karyotype, platelet count, haemoglobin, and blast cell percentage (Garcia-Manero et al., 2008). The second allowed the inclusion of those patients

(CMML, previous therapy, secondary MDS) previously excluded from the IPSS and stratified on the basis of performance status, age, platelet count, haemoglobin, blast cell percentage, leucocyte count, cytogenetic karyotype, and previous transfusion (Kantarjian et al., 2008). The inclusion of age as a prognostic factor is, perhaps, unsurprising given its value as a prognostic factor in AML and the finding that it is a significant variable in univariate analysis in other MDS prognostic schemes (Greenberg et al., 1997; Malcovati et al., 2007; Döhner et al., 2015). Given that epidemiological studies have also shown a sex rate skew in MDS, a large German and Austrian collaborative study incorporated both age and sex with the IPSS to improve the prognostication of MDS (Nosslinger et al., 2010).

1.8.4 Revised International prognostic Scoring System (IPSS-R)

In response to criticism, the IPSS was further refined on a larger cohort of patients to produce the IPSS-R. This generated new cut-offs for assessment of cytopenia and the percentage of blast cells, and included more cytogenetic abnormalities, using a scoring system based on nearly 3,000 patient, which contributed the highest weight to the score (Greenberg et al., 2012; Schanz et al., 2012). Age was included as a feature, but not sex, and this can be used to generate an age adjusted IPSS-R. The outcomes per patient group using the IPSS-R are shown in Table 1.9.

The IPSS-R has been widely adopted and IPSS-R calculators are available on-line and for mobile devices (*Revised International Prognostic Scoring System (IPSS-R) for Myelodysplastic Syndromes Risk Assessment Calculator* | *MDS Foundation*, 2016).

However, criticism of the IPSS still remains. One criticism regards the ability to determine accurate blast cell percentages to the stated cut-offs. One study showed only fair inter-observer concordance for cases with 0.1-2% blast cells (kappa = 0.50) and for cases with >2% but less than 5% blast cells (kappa = 0.28) (Senent et al., 2013). Furthermore, the IPSS-R was formulated for untreated patients, although its validation has been performed for MDS on a single institute cohort, and on patients treated with azacytidine and lenalidomide (Lamarque et al., 2012; Mishra et al., 2013; Sekeres et al., 2014).

Risk Group	Total score	Median survival (years)	Time for 25% to progress to AML (years)
Very Low	≤1.5	8.8	Not reached
Low	2.0-3.0	5.3	10.8
Intermediate	3.5-4.5	3.0	3.2
High	5.0-6.0	1.6	1.4
Very High	>6.0	0.8	0.73

Table 1.9. MDS risk category as defined by the IPSS-R score values.

The table shows the clinical outcome as defined by median survival and risk of developing AML.

1.8.5 Revised WHO-based Prognostic Scoring System

An initial refinement to the WPSS was proposed in 2011 with the inclusion of the haemoglobin threshold level at the expense of transfusion requirement (Malcovati et al., 2011). As the WPSS uses the same cytogenetic abnormalities as defined by the IPSS, there was an obvious requirement for revision to the WPSS following revision to the IPSS. This was performed in 2015 on a large cohort of 5326 patients and incorporated the haemoglobin thresholds alongside the WHO categories and the recently defined IPSS cytogenetic risk group (Della Porta et al., 2015b). The outcomes per patient group using the revised WPSS are shown in Table 1.10. Perhaps unsurprising, given similar use of the karyotypic data, the revised WPSS and the IPSS-R were strongly correlated, although discrepancies were seen between lower risk patients (Della Porta et al., 2015b).

Risk Group	Total score	Median survival (years)	Time for 25% to progress to AML (years)
Very Low	0-1	8.2	Not reached
Low	2	6.3	14.5
Intermediate	3	3.7	6
High	4-5	1.8	1.5
Very High	>5	0.7	0.7

Table 1.10. MDS risk category as defined by the Revised WPSS score values.

The table shows the clinical outcome as defined by median survival and risk of developing AML.

1.8.6 CMML-based scoring schemes

CMML has dysplastic features and shows overlap with features of MDS cases but, despite having a heterogeneous biology and natural history, it was not included for risk assessment in the traditional MDS prognostic scoring schemes. However, it has been included in one MDS prognostic scoring scheme, an MD Anderson scoring scheme, albeit based on FAB criteria (Kantarjian et al., 2008). This is not to say that specific CMML prognostic scoring schemes do not exist, but, mainly, these have been based upon FAB defined CMML criteria (Worsley et al., 1988; Gonzalez-Medina et al., 2002; Onida et al., 2002).

In the WHO era, there have been 2 attempts to produce CMML specific prognostic scoring schemes. Cytogenetic karyotype alone has been used to assign CMML to 3 prognostic categories (Such et al., 2011). Although features previously identified by the FAB CMML prognostic scoring schemes as risk factors were available in this cohort (i.e. blast cell count, haemoglobin level, leucocyte count, platelet count), they were not included in the final scheme (Such et al., 2011). The 3 cytogenetic risk groups were, however, used as a foundation to develop to develop a CMML-specific prognostic scoring scheme (CPSS) (Such et al., 2013). The outcomes per patient group using the CPSS are shown in Table 1.11.

		Median survival (years)		Time for 25% to progress to AML (years)	
Risk Group	**Total score**	**Test Set**	**Validation Cohort**	**Test Set**	**Validation Cohort**
Low	0	6.0	5.1	7.9	4.9
Intermediate-1	1	2.6	2.6	3.3	2.0
Intermediate-2	2-3	1.1	1.3	0.9	1.1
High	4-5	0.4	0.8	0.3	0.3

Table 1.11. CMML risk category as defined by the CPSS score values.

The table shows the clinical outcome of the training and validation cohort, as defined by median survival and risk of developing AML.

A schema comparing and contrasting the features used to generate each prognostic scoring scheme is shown in Figure 1.4.

	IPSS	WPSS	MD Anderson Low Risk MDS	MD Anderson All Patients	Modified IPSS	IPSS-R	Revised WPSS	CPSS
Number of risk groups	4	5	3	4	4	5	5	4
%BM Blast cells	■		■	■		■		
Cytogenetic karyotype	■	■	■	■		■	■	■
Presence of cytopenia	■	■						
Anaemia			■	■		■	■	
Leucocyte count >20x10^9/L				■				
Neutropenia						■		
Thrombocytopenia			■	■		■		
WHO subgroup		■					■	■
FAB subgroup								■
Transfusion parameters				■				■
Age				■	■			
Sex					■			
ECOG performance status				■				
IPSS score					■			

Figure 1.4. A comparison of the components used to define the different prognostic scoring schemes in MDS and CMML.

A blue-coloured cell denotes use of feature. A white-coloured cell denotes non-use of feature.

1.9 Treatment aims

As stated, and as can be seen from the prognostic scoring schemes, MDS poses particular clinical management concerns due to the heterogeneity of the disorder with respect to survival and transformation to AML. As befits the disorder, there is also heterogeneity in treatment aims. At the lower-risk end of the spectrum, the aim may be to give supportive care (i.e. observation, clinical monitoring, quality-of-life assessment, blood transfusions and chelation therapy, erythroid stimulating agents, and antibiotics to control infections), or, ideally, to resolve the ineffective haematopoiesis, especially in the 5q- subgroup with the administration of lenalidomide (reviewed by Fenaux and Adès) (Fenaux and Adès, 2013; Killick et al., 2014). At the other end of spectrum, the aim is to both overcome any differentiation blocks and promote apoptosis of the blast cells, usually by administration of hypomethylating agents (Sekeres and Cutler, 2013). Ultimately, however, the only true curative therapy is allogeneic bone marrow transplantation, albeit with variable results (Stone, 2009).

1.10 Can contemporary genetic-based techniques improve the identification of MDS patients and of new prognostic features?

Given the importance of cytogenetic karyotype in the diagnosis of MDS, and in the prognosis where it carries the highest statistical weight of all components in the IPSS-R, identification of karyotypic abnormalities is critical. Recently, array comparative genomic hybridisation (CGH) has identified copy number changes in 11% of MDS patients with a normal karyotype by conventional cytogenetic analysis, including a complex karyotype (Volkert et al., 2016). However, neither conventional karyotyping nor array CGH can identify uniparental disomy (UPD-also referred to as copy neutral loss of heterozygosity (CN-LOH)), which refers to the inheritance of two copies of chromosome from one parent and which is a feature of many cancers (Tuna et al., 2009).

1.10.1 Single nucleotide polymorphism arrays

The single nucleotide polymorphism array (SNP-array) is a valuable tool in the investigation of haematological malignancies, including myeloid malignancies, as it is the only technique available for assessing copy number changes and CN-LOH (Gondek et al., 2008; Maciejewski and Mufti, 2008; O'Keefe et al., 2010). The use of SNP-arrays in MDS was first reported in an IPSS low-risk cohort of patients of whom 65% had a normal karyotype (Mohamedali et al., 2007). In this study, 18% of patients had abnormalities identified by SNP-array but not by cytogenetic analysis. Statistical comparison with the IPSS revealed a correlation with the frequency of deletions, although not with the frequency of gains/amplifications or with CN-LOH (Mohamedali et al., 2007). Currently, its utility resides in complementing, as opposed to replacing, conventional cytogenetic analysis (Tiu et al., 2011). However, the finding that there was a high concordance between peripheral blood and bone marrow SNP-array karyotype (100% for the pilot study and 95% in a larger cohort) means that it has clinical advantages over conventional cytogenetic karyotyping, which typically requires a bone marrow, and has a potential application in diagnosis using solely a peripheral blood sample (Mohamedali et al., 2013; Mohamedali et al., 2015).

1.10.2 Genetic mutations in MDS

Recurrent areas of LOH can be used to identify regions of interest in myeloid malignancies such as mutations in *CBL* in MPN (Grand et al., 2009). An approach analogous to the Knudson 2-hit model of carcinogenesis, as LOH can be thought of as a second “hit”, with the first “hit” being a somatic point mutation which either causes activation of an oncogene or

loss of a tumour suppressor gene (Knudson, 1971). Once identified, the presence of a recurrent somatic point mutation has been reported as an invaluable diagnostic and/or prognostic tool in such myeloid malignancies as MPD, AML, and systemic mastocytosis (Nagata et al., 1995; Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Thiede et al., 2006; Beer et al., 2008; Klampfl et al., 2013; Nangalia et al., 2013; Linda M. Scott, 2014).

1.10.2.1 Can specific, recurrent genetic mutations be found in MDS?

As if to complement the varied diagnostic features and differing natural history within MDS, a large number of genetic mutations have been identified as associated with MDS. It also seems appropriate, given the heterogeneous nature of the disease, that a variety of techniques and approaches have aided in the identification of these mutations. Early reports used *in vivo* selection assays to identify, and a combination of the polymerase chain reaction (PCR) and DNA sequencing to confirm, *NRAS* mutations (Hirai et al., 1987; Bar-Eli et al., 1989). PCR/single strand conformation polymorphism (SSCP) was used to demonstrate *TP53* mutations in MDS (Sugimoto et al., 1993).

The close, overlapping relationship of MDS to other myeloid malignancies has aided in the discovery of mutated genes in MDS. A mutation in *FLT3* was first discovered as an internal tandem duplication in patients in AML, and then confirmed as present in MDS (Nakao et al., 1996; Yokota et al., 1997). The search for, and discovery of, mutations in *RUNX1* was guided by the involvement of this gene in chromosomal translocations in AML and B-ALL (Osato et al., 1999; Imai et al., 2000). Likewise, the discovery of NPM1 mutations was influenced by the involvement of *NPM1* in chromosomal translocations and identified via the use of immunocytochemical methods (Falini et al., 2005). SSCP and DNA sequence analysis was used to demonstrate an Asp816 *KIT* (aka *cKIT*) mutation in patients with systemic mastocytosis with associated haematological malignancy (Nagata et al., 1995). Both Asp816 and mutations in previous unreported codons were subsequently reported in MDS patients (Lorenzo et al., 2006).

Techniques borrowed from cytogenetic analysis aided in the identification of other genetic mutations. Mutations in *TET2* and *EZH2* were both discovered through detection of areas of LOH on chromosomes 4 and 7, respectively (Langemeijer et al., 2009; Ernst et al., 2010). Mutations in *ASXL1* were discovered via a similar approach to LOH, only by use of CGH (Gelsi-Boyer et al., 2009).

However, the advent of so-called Next Generation Sequencing (NGS) has superseded the use of the above approaches for mutation discovery. It has also made the cost of sequencing an entire genome relatively now affordable and almost under $1000. This can be seen in Figure 1.5 which shows the decreasing cost of DNA sequencing and was plotted

using data obtained from the NIH National Human Genome Research Institute website (Wetterstrand, 2016). The biggest drop in cost in 2007 was attributable to the switch from traditional Sanger sequencing to Next Generation Sequencing methods.

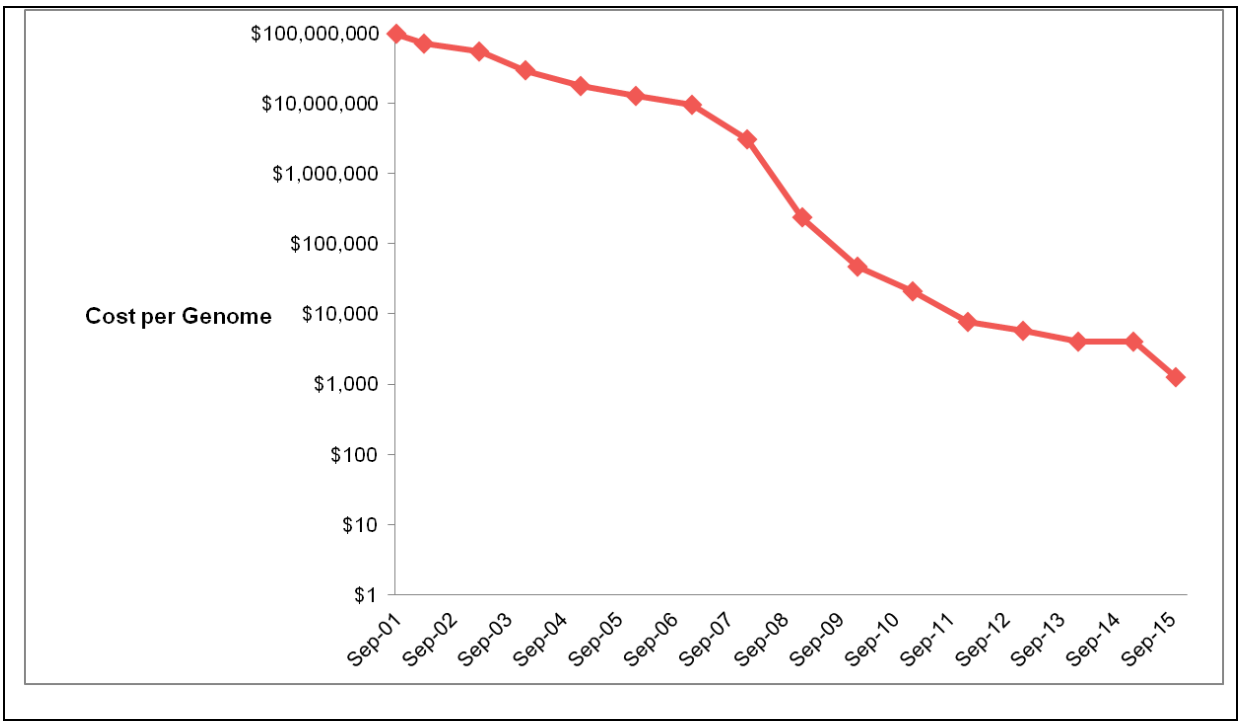


Figure 1.5. The decreasing cost of sequencing the human genome with time.

The cost per genome is plotted using a logarithmic scale. Graph created with data obtained from NIH National Genome Research Institute (Wetterstrand, 2016)

This affordability has enabled the identification of numerous genes which are found to be mutated in MDS. Ley *et al.*, in Washington, sequenced the whole genome of a patient with AML with a normal karyotype in a bid to identify cancer-specific genetic mutations (Ley et al., 2008). A repeated approach by this group on a second patient resulted in the identification of somatic mutations in *IDH1* and a demonstration that this gene was recurrently mutated in additional AML patients (Mardis et al., 2009). The same group then retrospectively identified a *DNMT3A* mutation in the first AML patient and, again, demonstrated recurrent mutations of this gene in additional AML patients (Ley et al., 2010). Both IDH1 and DNMT3A were subsequently shown to be mutated in MDS (Thol et al., 2010; Walter et al., 2011). The recurrence of somatic mutations within specific genes in cancer has given rise to the concept of a “driver” and “passenger” mutations. A driver mutation is a somatic mutation in a gene in a cell with self-renewal abilities which leads to selection advantage and gives rise to a mutated clone, whilst a “passenger” mutation has no impact upon neoplastic clone formation (Stratton et al., 2009). Although passenger mutations in sub-populations of cancer cells can

become driver mutations with the introduction of selection pressure in the form of therapy (Stratton et al., 2009). However, it is possible that the delineation of driver and passenger mutations may only be resolved with the analysis of incidence of recurrent genetic mutations in large scale cancer studies (Greenman et al., 2007).

The *whole genome/exome sequence* approach was subsequently applied to MDS patients with predictably novel findings and biological insights. Whole exome sequencing and targeted re-sequencing identified recurrent genetic mutations in the RNA splicing gene *SF3B1* (Papaemmanuil et al., 2011). Moreover, there was a strong correlation between the presence of a somatic mutation in *SF3B1* and the presence of ring sideroblasts (Papaemmanuil et al., 2011). The finding of a somatic mutation in a RNA splicing gene was not restricted to just *SF3B1*. Whole exome/genome sequencing identified mutations in other genes involved in splicing, including *SRSF2*, *ZRSR2*, and *U2AF1* (Yoshida et al., 2011; Graubert et al., 2012).

However, the true extent of the number of genes found to be mutated in MDS and the proportion of patients who are affected was to be revealed by large scale studies. The first study, by Bejar *et al.*, applied a targeted gene panel approach to 439 MDS patients (Bejar et al., 2011). The authors identified a somatic mutation in 18 genes with the finding that 52% of MDS patients demonstrated the presence of at least one mutation, whilst mutations in two or more genes were noted in 18% of patients. *TET2* was the most frequently mutated gene (mutated in 20.5% of cases) and 26% of patients with a *TET2* mutation had two distinct mutations. Furthermore, as a portend of things to come, 13 out of the 18 genes sequenced were each found to be mutated in less than 5% of MDS cases.

Two similar, European, collaborative studies were then published which expanded on the findings of Bejar *et al*. Papaemmanuil *et al.* targeted 104 genes on 738 MDS patients for mutational analysis, whilst Haferlach *et al.* targeted 111 genes on 944 patients (Papaemmanuil et al., 2013; Haferlach et al., 2014). Each study identified somatic mutations in 43 and 47 genes, respectively, with the top 5 mutated genes (*SF3B1*, *TET2*, *SRSF2*, *ASXL1*, *DNMT3A*) the same in both publications, albeit in different orders. In neither study was a mutation identified in every single case, with Papaemmanuil reporting 74% of patients having at least one mutation, and Haferlach reporting 89.5%. Like Bejar, both studies reported cases showing multiple genes harbouring mutations with Papaemmanuil reporting 10% of cases showing the presence of 4-8 mutations. Furthermore, both studies reported significant correlations between genes, with the finding of both positive correlations and mutual exclusivity between mutated genes.

The mutual exclusivity between genes occured mainly for genes within the specific biological pathway. For example, mutations in genes involved in the RNA splicing pathway (*SF3B1, SRSF2, ZRSR2, U2AF1*) were mutually exclusive. This extended to those involved with DNA methylation (*TET2, DNMT3A, IDH1, IDH2*); chromatin modification (*ASXL1, EZH2*) transcription factors (*RUNX1, NPM1, BCOR*), or signalling (*FLT3, NRAS, KRAS, CBL, cKIT, JAK2, MPL, CSF3R*). However, mutual exclusive mutations between genes were also shown to exist between genes in different pathways. These findings imply that there is both a functional redundancy and underlying biological basis to the mutations found in MDS (Papaemmanuil et al., 2013).

Finally, and to underline the heterogenous nature of MDS, both studies showed the presence of a Pareto-type distribution with respect to the number of genes mutated in MDS – described as a so-called “long tail” which indicates the small number of genes mutated in approximated 10% of MDS patients and a “long tail” of approximately 50 genes mutated in less than 5% of patients.

1.10.2.2 How can mutation analysis be incorporated into the WHO MDS diagnostic classification scheme?

A WHO-based classification is based on a combination of morphological, phenotypic, cytogenetic, and molecular features. Therefore, the incorporation of distinct genetic entities with specific disease biology and therapeutic options is an attractive prospect which fits with the WHO classification ideology. Currently, the 5q- syndrome is the only cytogenetic/molecular-based defect in the WHO MDS category. However, the associated of an SF3B1 mutation with the presence of ring sideroblasts makes this mutation an obvious candidate. Malcovati *et al.* showed that *SF3B1* could identify a subset of MDS patients with similar genotypic and phenotypic features and a good prognosis (Malcovati et al., 2014).

Incorporation of genetic mutations into a diagnostic classification scheme may not be straightforward. The findings from the 2 European large scale studies showed that somatic mutations within the 5 most frequently mutated genes were found across most WHO MDS categories (the Bejar *et al.* study used the FAB classification). Simply overlaying the underlying genetic mutation or mutations onto the current framework of pre-existing WHO MDS entities would subdivide each category into increasing numbers of subcategories and create an even more diverse group of disorders. It is possible that patients could be defined by the pathway affected by the mutation i.e. RNA splicing (*SF3B1*, *SRSF2*, *ZRSR2*, *U2AF1*); DNA methylation (*TET2*, *DNMT3A*, *IDH1*, *IDH2*); chromatin modification (*ASXL1, EZH2*) transcription factors (*RUNX1*, *NPM1*, *BCOR*), or signalling (*FLT3*, *NRAS*, *KRAS*, *CBL*, *cKIT*, *JAK2*, *MPL*, *CSF3R*). Alternatively, a *mutation barcode* approach which takes into account

all MDS-related driver mutations could be used. This would overcome the difficulty in classifying those patients who demonstrate mutations in multiple pathways (Papaemmanuil et al., 2013; Haferlach et al., 2014).

However, not every patient tested was found to harbour a mutation in the genes analysed. Although patients were investigated with targeted panels which, for example, did not include mutation analysis in gene regulatory elements. The percentage of MDS patients with non-mutated genes was reported as 48.5%, 22%, and 10.5% in the 3 large scale studies (Bejar et al., 2011; Papaemmanuil et al., 2013; Haferlach et al., 2014). It remains to be seen whether the application of whole genome sequencing to MDS patients, as opposed to targeted gene sequencing, in combination with traditional karyotypic studies would identify genetic abnormalities in all patients.

1.10.2.3 Can mutation analysis be incorporated into MDS prognostic scoring schemes?

The use of the mutation analysis may also be suited to prognostication as well as to disease classification. Statistical analysis for the construction of the IPSS-R identified the cytogenetic chromosomal abnormality component as having the highest weighting of all the features (Greenberg et al., 2012). It is thought that chromosomal abnormalities are secondary events following an initial driver mutation (Cazzola et al., 2013). Therefore, it is unsurprising that prognostic scoring schemes are already being generated which attempt to include genetic mutations.

Studies in CMML patients preceded those in MDS and gave a good overview of the potential of genetic mutation analysis in a prognostic setting, although with some conflicting results. A study by Kosmider *et al.* showed that a *TET2* mutation was an independent prognostic factor for overall survival in CMML (Kosmider et al., 2009). However, this finding did not extend to an independent cohort containing both MDS and CMML patients (Smith et al., 2010b).

In numerous, multi-gene studies of outcome in CMML, an *ASXL1* mutation consistently appears to have prognostic implications. The presence of a mutation in *ASXL1* was reported as having a significantly lower time to AML progression, has also been shown to be a significant feature in multivariate analysis of overall survival OS, and has been incorporated into a prognostic models (Gelsi-Boyer et al., 2010; Itzykson et al., 2013; Cui et al., 2015; Padron et al., 2015). One group initially reported no significance in univariate analysis of overall survival with the presence of mutation in *ASXL1* or in the spliceosomes *SF3B1*, *SRSF2*, and *U2AF1*, before reversing their findings for *ASXL1* using a larger cohort (Patnaik et al., 2013; Patnaik et al., 2014).

Various groups have also studied the impact of interactions between genes on outcome. Although not initially prognostic for overall survival in multivariate analysis, subgroup analysis of *SRSF2* in the presence or absence of a *RUNX1* mutation showed a difference in overall survival (Meggendorfer et al., 2012). Damm *et al.* and Patnaik *et al.* reported similar findings of a difference in overall survival when assessing *ZRSR2* and *ASXL1* mutations, respectively, in the presence or absence of *TET2* mutations (Damm et al., 2012; Patnaik et al., 2016).

The publication of these latter 3 studies highlights a potential issue in the use of multiple correlated and mutually exclusive molecular combinations in either diagnostic classification or prognosis. Namely, a “combinatorial explosion” associated with evaluating multiple genetic mutations. The reported outcomes may highlight a “subgroup within subgroup” approach to prognostication, akin to *NPM1* and *FLT3* in AML (Thiede et al., 2006).

The 3 large scale studies Bejar *el al*, Papaemmanuil *et al.* and Haferlach *et al.* all showed the potential prognostic implications of mutations in MDS patients (Bejar et al., 2011; Papaemmanuil et al., 2013; Haferlach et al., 2014). Bejar *et al.* showed that mutations in 5 genes (*TP53*, *EZH2*, *ETV6*, *RUNX1*, and *ASXL1*) were associated with worse overall survival (Bejar et al., 2011). Whilst Haferlach *et al.* confirmed the independent prognostic value of a mutation in 3 of these genes in multivariate analysis (*ASXL1*, *RUNX1*, and *TP53*) and included all 5 genes in a 14 gene prognostic model which generated 4 significantly different prognostic risk groups (low, intermediate, high, and very high) (Haferlach et al., 2014). Finally, Papaemmanuil *et al.* demonstrated that the number of mutations inversely correlated with leukaemia free survival rate, and that this also held true for each of the IPSS prognostic groups (Papaemmanuil et al., 2013).

Lastly, it is unknown whether there is any clinical relevance in the position of mutation within a specific gene. This may not be obvious until the advent of targeted therapy for specific genetic mutations in MDS patients. In this respect, the paradigm would be the treatment of chronic myeloid leukaemia in which mutations in the chimeric *BCR/ABL* sequence cause resistance to specific tyrosine kinase inhibitors (Redaelli et al., 2009).

1.10.2.4 Can the presence of mutation aid in the diagnosis of MDS?

Although the integration of mutation analysis data into diagnostic classification and prognostic scoring schemes may be challenging, it might be assumed that the presence of a driver mutation could be used as a simple diagnostic tool to aid in overcoming the difficulties involved in the diagnosis of MDS. However, the presence of a genetic abnormality does not always translate into malignancy. For example, loss of chromosome Y, or the presence of the *BCR/ABL* or the *BCL2/IGH* chimeric fusions gene can occur, albeit at a low frequency, in

normal individuals without evidence of CML or follicular lymphoma, respectively (Biernaux et al., 1995; Limpens et al., 1995; Dumanski et al., 2015).

Recently, large scale studies have shed light as to whether this phenomenon can be extended from chimeric fusion genes and karyotypic abnormalities to driver mutations. The first study performed whole exome sequencing on 17182 unselected persons (Jaiswal et al., 2014). Whilst a second study performed exome sequence analysis on 12380 unselected persons (Genovese et al., 2014). Mutations in driver genes were rare in persons under the age of 40, but the frequency of mutations increased with age, with Genovese *et al.* reporting 10.4% of those of 65 years old demonstrated a mutation, whilst Jaiswal reported a constant increase of driver mutations with age, ranging from 5.6% in 60-69 years old up to 18.4% of those aged 90 and above (Genovese et al., 2014; Jaiswal et al., 2014). Furthermore, although other genes were found to be mutated, both studies demonstrated that the mutations primarily occurred in *ASXL1*, *TET2* and *DNMT3A*, 3 of the top 5 mutated genes in MDS (Papaemmanuil et al., 2013; Genovese et al., 2014; Jaiswal et al., 2014; Haferlach et al., 2014). These findings of mutated genes in individuals without haematological malignancy have led to the concept of clonal haematopoiesis of indeterminate potential (CHIP), whereby patients have mutations in MDS driver genes but do not have diagnostic criteria for haematological malignancy (Steensma et al., 2015).

Jaiswal *et al.* also examined the full blood count parameters of a subset of 3107 persons within their cohort. Aside from a slight difference in red cell distribution width, there were no significant differences in full blood count parameters between those with mutations and those without (Jaiswal et al., 2014). However, those persons with multiple cytopenias were more likely to have a mutation (Jaiswal et al., 2014).

To investigate the frequency of mutations in patients referred for the investigation of cytopenia(s), targeted sequencing using a 22 myeloid gene panel was performed on a cohort of 144 patients presenting with at least one cytopenia (Kwok et al., 2015). In the 24 patients diagnosed with MDS, 71% demonstrated at least one mutation; in the 21 patients with cytopenia and present of morphological dysplasia, but which was insufficient to diagnose MDS, 62% demonstrated a mutation; in the 99 patients with cytopenia and no morphological dysplasia, 20% of patients had mutations (Kwok et al., 2015).

Currently, there is no guidance as how to monitor those patients who demonstrate mutations in known myeloid genes, but do not meet the criteria for MDS. Kwok *et al.* reported that, from their cytopenic patient database, only 8% of those referred for investigation are diagnosed with MDS, with 30% having an alternate diagnosis and 62% not meeting any diagnostic criteria (Kwok et al., 2015). Overall, 27% of non-MDS cytopenic patients reported by this

group had a mutation; the monitoring of such patients may require some effort and resources.

However, monitoring these patients should ultimately identify those mutations with a higher risk of progression. Already it has been shown that an increase in the variant allelic fraction (VAF) and accumulation of additional mutations occurs with cytopenic patients who progress to myeloid malignancy (Cargo et al., 2015). This finding mimics that of progression of MDS to a higher risk subgroup, or from MDS to AML (Pellagatti et al., 2016).

The coexistence of suspected MDS and another haematological malignancy, or in the investigation of therapy-related MDS, could complicate the interpretation of the presence of a driver mutation. Panels were designed from using cancer-genome studies and some driver mutations are not unique to MDS, or indeed to myeloid malignancies. *NRAS* and *KRAS* mutations can be found in multiple myeloma (Chapman et al., 2011). *TET2*, *IDH1*, *IDH2*, and *DNMT3A* mutations can be found in T-cell lymphomas (Cairns et al., 2012; Couronné et al., 2012). Mutations in *EZH2* have been reported in patients with follicular lymphoma and with diffuse large B-cell lymphoma (Morin et al., 2010). The splicesome *SF3B1* is found to be recurrently mutated in chronic lymphocytic leukaemia (CLL) patients (Rossi et al., 2011; Wang et al., 2011; Quesada et al., 2012). Mutations of *SF3B1* have also been reported in patients with monoclonal B-cell lymphocytosis, a condition with monoclonal circulating CLL-like phenotype cells not satisfying the criteria for CLL (Greco et al., 2013; Ojha et al., 2014). This latter study by Ojha *et al.* used CD19-selected B-cells to prove the existence of the SF3B1 mutation was present in the B-cells and determining the cell of origin may be a prerequisite for cases of suspected MDS co-existing with another malignancy (Ojha et al., 2014).

A cell of origin approach may not be suitable for all cases presenting with suspected MDS. Patients with aplastic anaemia are difficult to distinguish from MDS (Bennett and Orazi, 2009). However, targeted and whole exome genetic studies have shown that 5-36% of aplastic anaemia patients harbour somatic mutations, particularly in *BCOR*, *ASXL1*, *DNMT3A*, and also in the *PIG-A* gene which is associated with development of PNH clones (Lane et al., 2013; Heuser et al., 2014; Kulasekararaj et al., 2014; Yoshizato et al., 2015)

Currently, all these genetic mutation analysis approaches are being performed in specialised laboratories with skilled scientists. The applicability of these methods in a routine diagnostic laboratory will require additional resources and, although the cost of sequence analysis is decreasing, whole genome analysis still costs over $1000 and, in its current guise, is not applicable to the majority of laboratories. A targeted approach which sequences a smaller number of myeloid genes may be better suited to a diagnostic setting.

1.11 Can flow cytometry be used as a primary technique in the investigation of MDS?

1.11.1 Introduction to flow cytometry

The last few decades has seen advancement in flow cytometry technology with 8-colour flow cytometers now a standard installation in routine clinical laboratories. This technological advance has driven, and is driven by, an increase in the availability of antibodies and fluorochromes, and improvements in analytic software. These features have combined to lead to an increase in the routine use of flow cytometry in the investigation of normal, reactive, and malignant haematopoiesis. The outcome of these features is twofold: the publication of consensus guidelines for medical indications which support the use of flow cytometry in the analysis of haematological malignancies (Davis et al., 2007); and the incorporation of flow cytometry into clinical trials to monitor minimal residual disease and to direct therapy (Santacruz et al., 2014; Rawstron et al., 2015; AML18 Trial UK Clinical Trials Gateway).

1.11.2 Overview of the use of flow cytometry in the investigation of MDS

Differentiation pathways from haematopoietic stem cells to mature peripheral blood haematopoietic cells result from an orderly, programmed process of differential gene expression. The receptors for these haematopoietic cytokines are cell surface proteins and any differentiation is accompanied by alterations in genes encoding other, functionally important, cell surface proteins. Morphological dysplasia in MDS reflects the visual integration of organisational and functional abnormalities across the differentiating myeloid lineages.

Therefore, morphological abnormalities should be reflected in changes in protein expression and function. It is likely that these changes will be accompanied by abnormal patterns of surface protein expression, therefore providing a potential means to identify abnormal populations. Precedent for this phenomenon is ample, with aberrant phenotypes defining neoplastic haematopoietic populations in both lymphoid malignancies and AML(Craig and Foon, 2008) .

The utility of flow cytometry in assessing haematopoietic populations within samples referred for the investigation of MDS resides in 3 features:

- Identification of cell lineage
- The ability to enumerate discrete populations

- Comprehensive immunophenotypic profiling to determine the stage of differentiation

All 3 features are used in assessing the presence of potential dysplasia in suspected MDS cases. Furthermore, these 3 features can all be improved by increasing the number of antibodies available per individual tube. This improvement can be illustrated by the progress made over time in identifying the population containing haematopoietic stem cells (HSC) from the $CD34^+$ population through to the $CD34^+CD38^-$ population and, finally, identification within the $CD34^+CD38^-CD45RA^-CD90^+$ population (Baum et al., 1992; Bhatia et al., 1997; Manz et al., 2002; Doulatov et al., 2010).

1.11.3 Recommendations for the use of flow cytometry in the investigation of MDS

There is a broad acceptance in the utility use of flow cytometry in the study of MDS. The 2008 WHO classification acknowledged the role of flow cytometry with the recommendation that if 3 or more abnormalities were found then the term “suggestive” of MDS could be used, but that these features were not diagnostic of MDS (Vardiman et al., 2009). The European LeukemiaNet guidelines for the diagnosis and treatment of MDS recommends the use of flow cytometry in the diagnosis of MDS, but recommends that this is performed in accordance with the ELN Working Group for Flow Cytometry in MDS guidelines (Westers et al., 2012; Malcovati et al., 2013). A 2014 ELN Working Group for Flow Cytometry in MDS publication has also provided further guidance in the integration of flow cytometry in the diagnosis of MDS (Porwit et al., 2014). In the United States, flow cytometric assessment is considered “helpful” in the evaluation of suspected MDS, albeit for the exclusion of PNH or large granular lymphocytosis (Greenberg et al., 2013).

1.11.4 Characterisation of normal pathways

The characterisation of antigenic expression at the different stages of haematopoiesis provides a framework for recognition of normal differentiation pathways. Early immunophenotypic studies identified basic differentiation stages in the granulocytic, monocytic, erythroid, and megakaryocytic lineages (Loken et al., 1987; Terstappen et al., 1990; Terstappen and Loken, 1990). Elghetany reviewed these, and other studies, and constructed a table to indicate the changes in antigen expression through the neutrophil differentiation stages (Elghetany, 2002) These changes in antigen expression with differentiation can be seen in Figure 1.6. More complex, multicolour flow studies, have confirmed these studies and have shown reproducible patterns of antigen expression during myeloid maturation (Kussick and Wood, 2003; Elghetany et al., 2004; van Lochem et al.,

2004). The culmination of these studies has been the multi-centre validation of a standardised protocol for data analysis and the production of a colour-coded, reference atlas to define antigen differentiation patterns in normal bone marrow (Arnoulet et al., 2010).

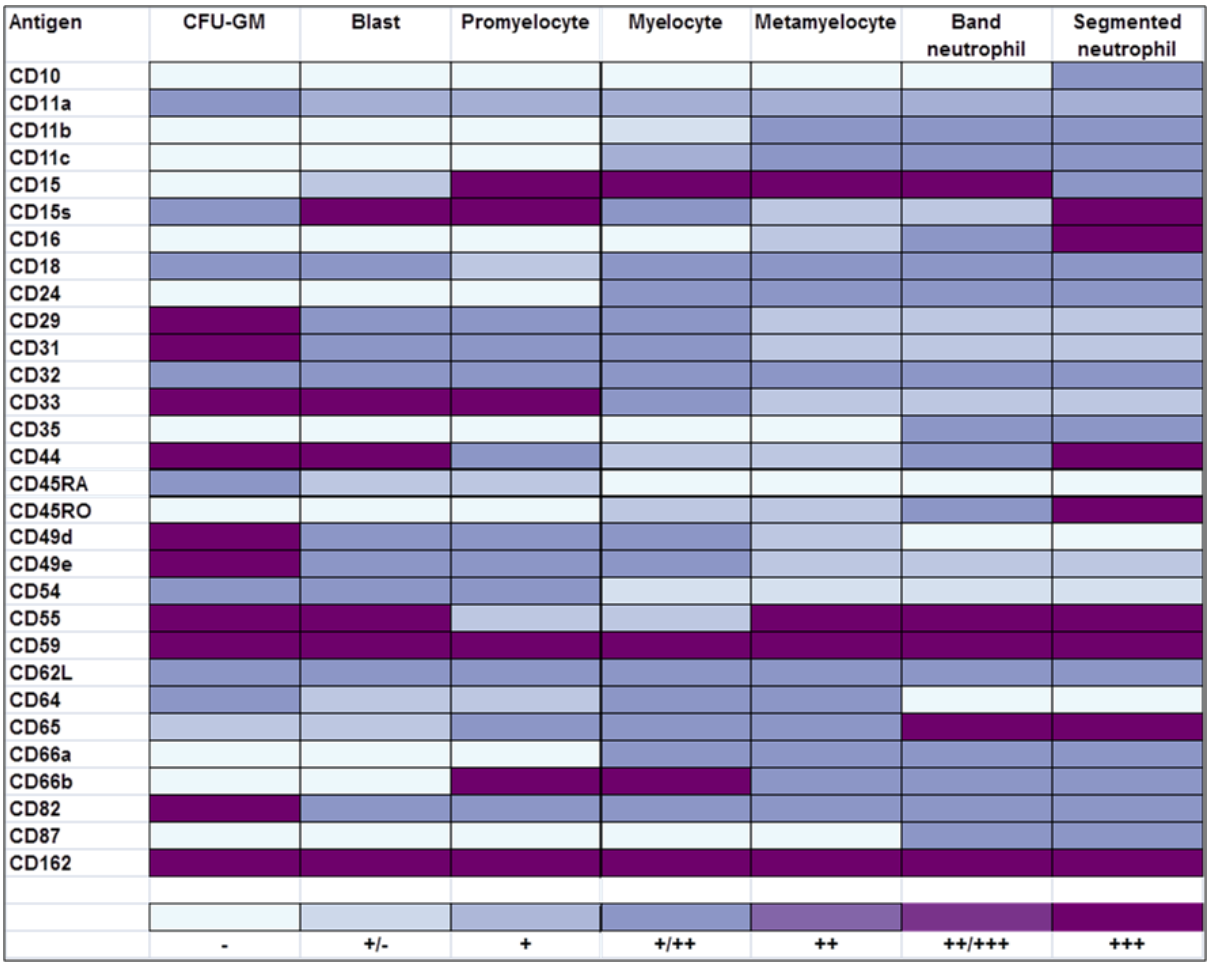


Figure 1.6. Expression of antigens through differentiation stages during neutrophil maturation.

Figure created from data in table 1 of *Surface antigen changes during normal neutrophilic development: a critical review* (Elghetany, 2002).

These antigenic differentiation frameworks are, therefore, essential for identifying any deviation from normal which may be found in MDS. These deviations can be used to identify aberrancies in MDS in two ways: (i) single antigen aberrancies on individual populations and (ii) the identification of asynchronous antigen expression within differentiation pathways.

1.11.5 Population specific immunophenotypic abnormalities in MDS

Immunophenotypic abnormalities in patients with MDS have been reported for many years. A review of the literature conducted by Elghetany in 1998 showed that single, surface antigen abnormalities in patients with MDS was a frequently finding by both flow cytometry and immunohistochemistry (Elghetany, 1998). Furthermore, analogous to the multi-lineage

dysplastic morphological features, flow cytometric analysis of the different haematopoietic lineages and populations revealed that antigenic abnormalities could be found in most lineages across MDS patients.

1.11.5.1 Progenitor cell abnormalities

CD34 recognises haematopoietic progenitor cells in the bone marrow and, as might be expected, patients with MDS can demonstrate increased proportions of bone marrow $CD34^+$ cells in comparison to controls (Civin et al., 1984; Del Cañizo et al., 2003; Malcovati et al., 2005). Despite this finding, the WHO is reluctant to allow the use of the percentage of $CD34^+$ cells as a surrogate for blast cell as "*not all leukaemic blast cells express CD34, and hemodilution and processing artefacts can produce misleading results*", although, paradoxically, CD34 staining of the trephine biopsy is judged to be useful if the aspirate is poorly cellular (Vardiman et al., 2009). A gating strategy using CD34, CD45, CD117, and HLA-DR has, however, been shown to correlate well with the morphological blast count in patients with MDS and correlates better than $CD34^+$ cells alone (Sandes et al., 2013).

Although the evidence suggests that MDS is a stem cell disorder, it has not yet been possible to distinguish malignant MDS haematopoietic stem cells on the basis of immunophenotype. Expression of CD123 has been suggested to distinguish malignant $CD34^+CD38^-$ stem cells from normal $CD34^+CD38^-$ stem cells in AML and variable expression has been reported in MDS (Jordan et al., 2000; Florian et al., 2006; Xie et al., 2010). The expression of the C-type lectin-like molecule-1 (CLL-1) has been reported to be present on the $CD34^+CD38^-$ cells in AML patients and in a variable proportion of MDS patients, but is absent in the normal and regenerating bone marrow $CD34^+CD38^-$ stem cell compartment (Bakker et al., 2004; van Rhenen et al., 2007).

There is a caveat in the use of a $CD34^+CD38^-$ to identify stem cells in MDS. Goarden *et al.* reported the use of a reduction in the fluorescent intensity of CD38 expression on $CD34^+$ cells in MDS as both a biological feature and part of a scoring scheme, a reduction which was independent of the $CD34^+CD38^-$ population (Goardon et al., 2009). Whilst Monreal *et al.* reported that there was increased proportion of $CD38^-$ cells in the $CD34^+$ population of high risk MDS and AML (Monreal et al., 2006). If these two reports are related then studies examining proposed haematopoietic stem cells in MDS may be, in reality, assessing a population of committed myeloid progenitor cells with abnormal down-regulation of CD38. If this down-regulation of CD38 explains the findings of Monreal *et al.* in high-risk MDS cases, then this may be a reason why the most successful stem cell studies have occurred mainly with the low-risk 5q- and RARS MDS subgroups (Tehranchi et al., 2010; Woll et al., 2014; Mian et al., 2015).

An increase in the percentage of $CD34^+$ progenitors is not the only numerical abnormality found in the progenitor compartment. Gene expression micro-array analysis of selected $CD34^+$ cells identified a reduction in B-lymphoid associated genes in patients with MDS, which translated into the common finding of a reduction of B-lymphoid progenitors in the bone marrow of patients with MDS (Sternberg et al., 2005). Whilst monocytic, plasmacytoid dendritic cell, erythroid, and basophil precursors were all found to be decreased in a significantly higher proportion of MDS patients (Matarraz et al., 2008)

In additional to numerical differences in the $CD34^+$ cells, numerous immunophenotypic aberrancies can also be noted in patients with MDS. Aberrant expression of the lymphoid antigens CD2, CD5, CD7, and CD56 has been described in MDS patients (Ogata et al., 2002). In a series of 104 MDS patients, the expression of CD7 and CD56 was found to be more frequency than the expression of CD2 and CD5, whilst CD3 and CD19 expression was not found (Ogata et al., 2002). The same group also reported asynchronous expression of CD11b and CD15 on the $CD34^+$ cells in MDS patients (Ogata et al., 2002).

Up- and down-regulation of normal myeloid antigens has also been reported as a feature of the $CD34^+$ progenitor cells in MDS. As previously noted, CD38 expression can be down-regulated (Goardon et al., 2009). Expression of the myeloid CD13 and CD117 has also been reported as differing significantly between MDS and control groups, with both antigens being overexpressed (Matarraz et al., 2008).

CD117 is recognised as expressed by bone marrow myeloid progenitor cells, with approximately half of the $CD117^+$ cells also found to express CD34 (Escribano et al., 1998). A finding attributable to the down-regulation of CD34 occurring before down-regulation of CD117 during myeloid differentiation (van Lochem et al., 2004). An increase in the $CD34^-$ $CD117^+$ myeloid progenitor population has been found in MDS patients in comparison to normal and reactive patients (Matarraz et al., 2010).

1.11.5.2 Granulocyte abnormalities

As would be expected due to the morphological granulocytic dysplasia, immunophenotypic abnormalities can be found in the granulocyte series. One of the most frequently reported abnormalities in patients with MDS is a decreased side scatter (SSC) expression of neutrophils, a feature which mirrors the visual hypogranularity noted in this population (Stetler-Stevenson et al., 2001). Decreased CD10 expression and expression of CD36 by granulocytes have both been reported as abnormalities in MDS patients (Chang and Cleveland, 2000; Lacronique-Gazaille et al., 2007). Expression of CD56 has been reported on granulocytes in MDS, however expression has also been reported in non-MDS, non-malignant conditions (Stetler-Stevenson et al., 2001; Wells et al., 2003; Malcovati et al.,

2005). Visual assessment of the differentiation pattern of CD13 and CD16, and CD11b and CD13 on granulocytes has been reported as a frequent abnormality in MDS patients (Stetler-Stevenson et al., 2001; Wells et al., 2003). However, there are two caveats with this visual assessment approach. Firstly, inter-observer reproducibility remains unproven. Secondly, on granulocytes, CD16 is a GPI-linked antigen and is absent on PNH-clone derived granulocytes (Kawakami et al., 1990). It is, therefore, unclear how the CD13/CD16 differentiation pattern would be interpreted in the context of a sizeable PNH clone.

1.11.5.3 Monocyte abnormalities

Like precursor cells and granulocytes, the monocytic compartment in MDS and in CMML can show a number of differences from their normal counterparts. These include numerical differences, down-regulation of expressed antigens, expression of lymphoid antigens, and perturbed differentiation patterns. An increased percentage of monocytes (>10%) can be found in the peripheral blood of MDS patients who do not fulfil the criteria for CMML (Rigolin et al., 1997). Down-regulation of the expression of CD13, CD14, CD16, CD36, CD64, and HLA-DR has been reported as a feature in MDS (Wells et al., 2003; Xu et al., 2005; Matarraz et al., 2010). However, as antigenic expression levels vary throughout differentiation, it is unknown whether this down-regulation is a consequence of a block in the monocytic maturation stage (van Lochem et al., 2004). However, it must be noted that, analogous to CD16 on granulocytes, CD14 is also a GPI linked antigen therefore absent expression can occur due to the presence of a PNH clone (Kawakami et al., 1990). Expression of the lymphoid antigens, CD2 and CD56, can occur on monocytes on CMML and MDS (Xu et al., 2005; Lacronique-Gazaille et al., 2007; Matarraz et al., 2010). Although both of these antigens can be expressed by patients with a reactive monocytosis (Xu et al., 2005). Lastly, a perturbed HLA-DR/CD11b differentiation pattern can be seen in the monocytes in MDS, although the same caveat regarding inter-observer reproducibility applies here as it does for assessment of the granulocyte CD13/CD16 differentiation pattern.

1.11.5.4 Erythroid abnormalities

Both numeric and immunophenotypic abnormalities can be seen in the erythroid compartment in MDS. Furthermore, the evaluation of immunophenotypic features in this lineage has given rise to a paradigm shift in flow cytometric assessment of suspected MDS. Numerically, a higher overall percentage of nucleated red cells in the bone marrow has been noted in the bone marrow of MDS patients (Malcovati et al., 2005). Numerical changes in specific erythroid populations can also be seen. A decreased percentage of $CD34^{-}CD117^{+}$ erythroid precursors, and an increased number of $CD117^{+}$ erythroid cells within the erythroid compartment, have both been reported (Matarraz et al., 2010; Westers et al., 2012).

Immunophenotypic abnormalities have also been reported with loss of the erythrocyte blood group antigens A, B, and H, increased CD105 expression, and decreased expression of CD36 and of CD71 (Kuiper-Kramer et al., 1997; Bianco et al., 2001; Malcovati et al., 2005; Della Porta et al., 2006; Matarraz et al., 2010).

A further study which assessed the immunophenotypic properties of CD36 and CD71 expression gave rise to a previously unreported approach to evaluate antigen expression in MDS patients. Mathis *et al.* reported that the coefficient of variation (CV) of CD36 and CD71 was higher in MDS patients than control samples (Mathis et al., 2013). Although this is the only reported study to formally use this feature so far, the use of the CV offers another approach in identifying immunophenotypic differences in MDS patients. The CV is calculated by dividing the standard deviation by the mean and indicates the variability of a population. This can be useful as two different populations can shown the same mean but different CV's as shown in Figure 1.7.

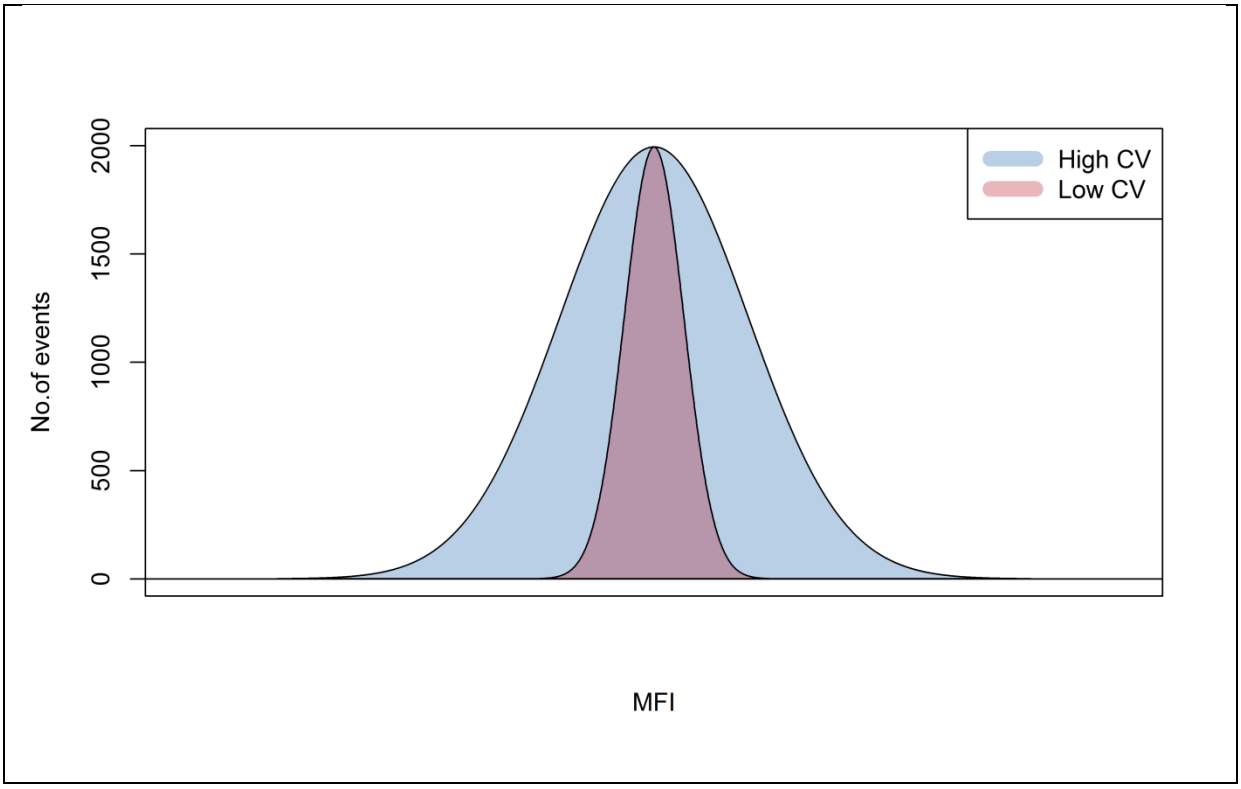


Figure 1.7. 2 Populations showing the same MFI but with different Coefficient of Variations.

Both populations show normal distribution and have the same MFI. However, the population shown in blue has a higher standard deviation than that shown in purple and, therefore, has a higher CV.

1.11.5.5 Immunophenotypic studies of other haematopoietic lineages

There have been only limited studies of the megakaryocytic lineage. This is due to the technical issues involving clumping and adhesion of platelets to monocytes. One study has shown a decreased expression of *MPL*, glycoprotein IIb/IIIa, and glycoprotein Ib on the platelets of MDS patients compared to normal controls (Izumi et al., 2001). Whilst Sandes *et al.* showed both increased and decreased expression of platelet glycoproteins, as well as light scatter abnormalities, in patients with MDS (Sandes et al., 2012). However, it is unclear whether immunophenotypic studies of platelets are suitable for widespread use. Both these studies used peripheral blood samples and guidelines recommend use sodium citrate as the anticoagulant and performing platelet studies within 4 hours, both features which may preclude uptake as a routine diagnostic procedure (Harrison et al., 2011)

There is limited published evidence for the presence of abnormalities in the remaining, minor leucocyte populations: basophils, mast cells, and plasmacytoid dendritic cells (pDC). Conflicting results have been published as to whether the percentage of pDC's in the bone marrow of MDS patients significantly differs (Matarraz et al., 2010; Saft et al., 2013). Matarraz *et al.* reported no difference in percentage of pDC's or of basophils or mast cells in the bone marrow of MDS patients (Matarraz et al., 2010). In contrast, Saft *et al.* reported a significant decrease in pDC's, as well as a significant decrease in myeloid dendritic cells in MDS patients (Saft et al., 2013). Although peripheral blood basophils were reduced in MDS patients in comparison to normal controls, these did not show immunophenotypic differences (Fureder et al., 2001). A phenotypic exception is mast cells in systemic mastocytosis, in which there is an association/overlap with MDS and which have well characterised immunophenotypic abnormalities of CD2, CD25, CD59 and HLA-DR (Escribano et al., 2004; Jabbar et al., 2014).

1.11.6 Guidelines for the use of flow cytometry in MDS

Unlike new molecular sequencing methods, flow cytometry is a well-established technique which is used daily in both clinical and research settings. Laboratory to laboratory variation does, however, occur and inconsistency of both technical and reporting approaches was noted as a feature when Elghetany reviewed the literature in search of antigenic abnormalities in MDS (Elghetany, 1998). To address these issues, the European LeukemiaNet MDS Flow Cytometry Working Group has produced two publications which address the issues of sample handling and the lineages and antigens to be assessed by immunophenotyping (van de Loosdrecht et al., 2009; Westers et al., 2012).

1.11.7 Can immunophenotypic abnormalities be applied to the diagnosis of MDS?

As has been shown, MDS demonstrates heterogeneity with respect to clinical features, laboratory parameters, morphological features, genetic and cytogenetic features, and, now, immunophenotypic features. This diversity of immunophenotypic features was first exploited as an aid to MDS diagnosis by Stetler-Stevenson *et al.* in a study published in 2001(Stetler-Stevenson et al., 2001). In this publication, multiple immunophenotypic abnormalities in the granulocytic, erythroid, and myeloid lineages were assessed in an attempt to confirm the diagnosis of MDS. This study also introduced other important concepts which were adopted by further studies. Firstly, there was the selection of Reactive, or so-called pathological control, cases as a comparison. Secondly, there was an indication that immunophenotypic abnormalities were not solely restricted to MDS and could be found in other normal and reactive conditions. A feature the authors tied to the notion that the number of abnormalities could be used to discriminate between MDS and other conditions. Finally, there was the use of the pattern-recognition approach to identify abnormalities (Stetler-Stevenson et al., 2001).

This approach by Stetler-Stevenson *et al.* shifted the emphasis from simply reporting the finding of novel immunophenotypic abnormalities in MDS, to testing whether immunophenoptypic abnormalities could distinguish MDS from Reactive conditions. Many studies were subsequently published which comparing Reactive cases to MDS cases and all showed slight variations in this approach in the use of immunophenotyping. The variations in approach included: use of antibody pattern recognition (Kussick et al., 2005; Stachurski et al., 2008); use of peripheral blood (Cherian et al., 2005); use of a single immunophenotypic feature (Goardon et al., 2009); use of single lineage immunophenotypic abnormalities (Della Porta et al., 2006); use of bi-lineage immunophenotypic abnormalities (Malcovati et al., 2005; Truong et al., 2009); use of tri-lineage immunophenotypic abnormalities (Lorand-Metze et al., 2007); use of CV and red cell blood count parameters to produce a RED-score (Mathis et al., 2013).

In 2003, Wells *et al.* applied this approach to define a scoring scheme for MDS patients and determine whether it correlates with outcome post-transplantation (Wells et al., 2003). A number of immunophenotypic features were assessed and patients were allocated points depending upon the type of abnormality present. These points were subsequently converted into a flow score (FCSS) which was used to classify patients as mild, moderate, or severe. These three classes correlated with IPPS scores and significantly differed in outcome post stem-cell transplant (Wells et al., 2003). Although this scoring score was initially applied to

patients in a post-transplant setting, further validation of the FCSS was performed on a cohort of both MDSS and Reactive patients to show its utility as an aid to MDS diagnosis (Chu et al., 2011). Minor modifications to this panel were also reported in 2011 (Cutler et al., 2010). However, the FCSS does have limitations. It discriminated the classes on the basis of the sum of abnormalities, yet applied different, user-assigned, scores for different immunophenotypic features. The cut-offs for defining the different MDS class were user-defined and appeared arbitrary. Furthermore, its applicability in a routine setting may be challenging due to the number of features requiring assessment and the use of a visual *deviation from normal* approach, which requires prior user experience.

Other publications and flow cytometry scoring schemes, attempted to overcome some of these limitations. Matarraz *et al.* evaluated 83 attributes obtained from the global assessment of bone marrow haematopoietic populations (Matarraz et al., 2010). These numerical and immunophenotypic MFI attributes were compared to normal expression and scored according to number of standard deviations from normal. The number of points was summed and converted into an immunophenotypic score (IS). The IS classified patients into mild, intermediate, or severely altered classes, depending upon overall score (Matarraz et al., 2010). This dispensed with a visual approach and was, therefore, applicable to laboratories with lesser experience with identifying visual deviations from normal. However, the points allocated for deviation from normal were user-defined, as were the boundaries for class membership. A simpler, standardised flow cytometry approach was published in 2009. Ogata and colleagues defined reference ranges for myeloid progenitor cell parameters, some of which were implemented into a flow cytometry scoring scheme (FCM) which was based on the low inter-observer variability of 4 parameters: These 4 parameters were: percentage of CD34 myeloid progenitors, CD45 expression on the CD34 myeloid progenitors, proportion of B-lymphoid progenitors within the $CD34^+$ cells, and granulocyte SSC (Ogata et al., 2006; Satoh et al., 2008; Ogata et al., 2009). Each parameter was allocated a score of 1 if outside a reference range and a point score of 2 or more was considered suggestive of MDS. The validity and reproducibility of this method was then further tested in a multicentre study (Della Porta et al., 2012). However, despite its simplicity and general applicability, each parameter was weighted the same, despite the authors showing logistic regression coefficients which ranged from 1.76 to 2.59 for the 4 parameters (Della Porta et al., 2012). It must be noted that two of the parameters are reported as ratios (CD45 expression and granulocyte SSC). The use of ratios in regression analysis can result in spurious correlations (Kronmal, 1993; Curran-Everett, 2013). As noted elsewhere, 2 of the parameters may show collinearity, with a decrease in the percentage of B-progenitors in the

$CD34^+$ compartment attributable to an increase in myeloid progenitors in the same compartment (Westers et al., 2012).

The performance metrics of the FCM were broadly similar for the 4 tested cohorts (Japanese cohort/Italian cohort/Training cohort/Validation cohort) across both publications, with a high specificity (98/90/92/93%) and a lower sensitivity (65/89/70/69%) (Ogata et al., 2009; Della Porta et al., 2012). There are some studies which report high sensitivity (>95%) with a high specificity (>90%) (Della Porta et al., 2006; Goardon et al., 2009). However, a sensitivity and specificity similar to those results obtained with the FCM is a hallmark of most of the flow cytometry scoring schemes (Cherian et al., 2005; Stachurski et al., 2008; Truong et al., 2009; Kern et al., 2010; Xu et al., 2010; Chu et al., 2011; Xu et al., 2012; Mathis et al., 2013; Xu et al., 2013).

The best approach to improve the sensitivity of flow cytometry scoring schemes is unknown. Bardet *et al.* showed that addition of the lymphoid-related antibodies CD5, CD7, and CD56 to the FCM improves sensitivity, but this showed only a marginal improvement for low-risk MDS patients (Bardet et al., 2015). However, Mathis *et al.* noted that a score suggestive of MDS by either the RED score *or* the FCM score resulted in a sensitivity of 88%, compared to 81% for the RED score alone and 49% for the Ogata score alone (Mathis et al., 2013).

There may be different reasons for these low(er) sensitivities. Since each scoring scheme classifies on the basis of different attributes, the immunophenotypic composition of each misclassified group may be expected to be different and group composition would be dependent upon the scoring scheme itself. Therefore an *either/or* combination of scoring schemes, as described by Mathis *et al.*, may further improve sensitivity. Alternatively, the lower sensitivities may imply that there are subsets of MDS patients who will be misclassified irrespective of the scoring scheme. This may occur as a result of expression of immunophenotypic features which are indistinguishable from control samples. Indeed, this feature can be seen when unsupervised hierarchical clustering of immunophenotypic and numerical attributes is performed. Using 32 attributes for cluster analysis, Matarraz *et al.* showed that, although the majority of normal bone marrow samples and the majority of high risk MDS cases formed discrete clusters, low risk RA and RCMD and Reactive cases were intermingled and clustered together (Matarraz et al., 2008). If this is a universal feature of certain MDS cases, the sensitivity for scoring schemes on lower-risk patients would not be expected to improve substantially simply by the inclusion of additional features. Indeed, Bardet *et al.* reports little benefit to sensitivity by inclusion of CD5, CD7, and CD56 (Bardet et al., 2015). Whilst simply increasing the number of aberrancies required for a flow scoring scheme to be suggestive of MDS understandably decreases sensitivity with little gain in specificity (Kern et al., 2010)

One further issue which may account for variability between the performance metrics of each scoring scheme is the choice of control samples and composition of the control group. Although the use of Reactive, or so-called pathological, controls is widespread, there may be immunophenotypic differences between the commonly used diagnostic entities which form control groups. Some studies have predominantly used ITP, Aplastic Anaemia, Anaemia of Chronic Disease (ACD), Megaloblastic Anaemia, and Iron-deficiency Anaemia cases as the Reactive control group (Goardon et al., 2009; Truong et al., 2009; Chu et al., 2011). Other studies have used unspecified anaemia or non-clonal cytopenias as a control group (Ogata et al., 2009; Della Porta et al., 2012; Mathis et al., 2013). The ELN Working Group for Flow Cytometry in MDS also proposes the use of well-described haematological malignancies including AML, MPN, PNH, and systemic mastocytosis (van de Loosdrecht et al., 2009). Since there are different underlying biological mechanisms which give rise to conditions such as ITP or Anaemic of Chronic Disease or Megaloblastic Anaemia, it is unknown whether these biological differences manifest as immunophenotypic differences. As immunophenotypic differences between the control and MDS groups dictate the cut-off boundaries for attributes and, therefore, ultimately, a scoring scheme itself, any differences in control groups may affect the performance metrics. Indeed, it could be argued that a control group containing predominantly uni-lineage diagnostic cases such as ITP and ACD cases is not the correct control for cases which may have bi- or tri-lineage cytopenia. Ideally, a control group would be patients with bi- or tri-lineage cytopenia without evidence of dysplasia, who have no evidence of clonal haematopoiesis (CHIP), and who are clinical monitored and shown not to develop MDS. However, attempting to obtain cases for such a control group would be impractical for most routine diagnostic laboratories.

1.11.8 The utility of immunophenotyping in the prognosis of MDS

In addition to diagnostic studies, flow cytometric immunophenotyping has been shown to have prognostic impact in MDS. An early study showed that CD7 expression on myeloid progenitor cells was a poor prognostic indicator in MDS (Ogata et al., 2002). However, this was not validated in an independent study, possibly due to the respective MDS subgroup composition of each cohort (Font et al., 2006). However, patients with aberrant myeloid progenitors which express either CD7, CD5 or CD56 have been reported to have poorer response to erythropoietin and G-CSF (Westers et al., 2010). Furthermore, immunophenotypic abnormalities in the myeloid and monocytic lineages, including CD7, can

be found in patients with in the RA unilineage dysplasia category (van de Loosdrecht et al., 2008).

The use of flow cytometry scoring schemes has also been shown to have an impact on the prognosis of MDS patients. The FCSS was constructed for the assessment of post-transplant MDS patients and was reported as an independent prognostic factor within the IPSS Int-1 MDS subgroup in this setting (Wells et al., 2003; Scott et al., 2008). In a non-transplant setting, the FCSS, or modified FCSS, has been reported to correlate with transfusion dependency and with the IPSS, IPSS-R, and WPSS, and has been demonstrated as an independent prognostic factor within specific IPSS and IPSS-R risk subgroups (van de Loosdrecht et al., 2008; Chu et al., 2011; Alhan et al., 2014). Likewise, the FCM score has been shown to have prognostic significance in patients classified as very low or low risk by the IPSS-R (Della Porta et al., 2014). Both Matarraz *et al. and* Kern *et al.* have shown a correlation between number of immunophenotypic abnormalities and the IPSS score (Matarraz et al., 2008; Kern et al., 2010). Whilst, in a series of patients referred with suspected MDS, the number of immunophenotypic abnormalities was associated with overall survival (Kern et al., 2015).

Although flow scoring schemes are composed of multiple variables, identification of the individual variable or variables within the schemes with prognostic significance is no well reported. The Kern group, in both 2010 and 2015 publications, identified 3 features from univariate analysis which, when at least one of these features was present, resulted in a worse overall survival. These three features were myeloid progenitor count greater than 5%, ≥3 aberrant antigens, and a granulocyte side scatter:lymphocyte side scatter ratio (Kern et al., 2010; Kern et al., 2015). There was a similar finding from Alhan *et al.* who reported that a new scoring scheme composed of CD117 expression on myeloid progenitors, CD13 expression on monocytic cells, and myeloid progenitor:lymphocyte side scatter ratio showed differences in overall survival overall, and within the IPSS-R low-risk group (Alhan et al., 2015). In both groups' publications, it was assumed that the side scatter ratio was a surrogate marker for granularity in the granulocyte and myeloid progenitor populations, respectively. However, due to its use in a ratio to lymphocytes, any differences in MDS cases may also reflect the underlying scatter characteristics of the lymphocyte population. Dysregulation of lymphopoiesis is a feature of MDS with both a decrease in B-lymphoid progenitors and an expansion in regulatory T-cells reported (Sternberg et al., 2005; Kordasti et al., 2007; Kahn et al., 2015). These alterations in the lymphocyte populations may manifest as differences in lymphocyte scatter characteristics in MDS patients. This may then affect the calculation of the side scatter ratio of the myeloid populations. The side scatter of monocytes has also been reported to differ between control and MDS patients (van de

Loosdrecht et al., 2008). This was also calculated as a ratio and this finding further implies that there are either abnormalities in the scatter characteristics of three major myeloid populations in MDS (progenitors, granulocytes, and monocytes) or in the denominator (the lymphocytes), or it is a combined effect of the two populations used in the ratio.

1.11.9 Can flow cytometry identify those patients at risk of developing MDS

As noted by Kwok *et al.*, over 60% of patients referred for the investigation of cytopenia do not have a confirmed diagnosis, MDS or otherwise (Kwok et al., 2015). As a proportion of these patients will progress to MDS and other myeloid malignancies, it was investigated whether the presence of immunophenotypic abnormalities could identify those at risk of progression. Firstly, Kern *et al.* reported the findings from a cohort of 142 cytopenic patients with no, or insufficient evidence, for a morphological diagnosis of MDS. 5 of these patients could be diagnosed with MDS due to the presence of a cytogenetic abnormality presumptive of MDS. The remaining patients were classified as probable MDS, possible MDS, or not MDS according to a flow cytometry scoring scheme. Of the 47 patients who developed MDS, 40 patients, at initial referral, were either probable (30) or possible (10) MDS by flow cytometry (Kern et al., 2013). Cremers *et al.* performed a similar analysis of 379 consecutive cytopenic patients (Cremers et al., 2016). Of the 164 patients who were reported as non-diagnostic, 5 developed MDS of which, at initial referral, 1 was classed as MDS by flow cytometry and 1 had minimal features of MDS by flow cytometry. The remaining 3 patients who developed MDS had no identified features of MDS by flow cytometry.

Both studies highlight multiple independent and common points. Firstly, the Kern *et al.* study highlights that cytogenetic analysis is not particularly helpful in trying to identify patients without morphological dysplasia who are at risk of developing MDS with a lack of morphological features. This study also highlights the diagnostic difficulty in identifying patients who may have MDS with a third of patients without morphological MDS developing MDS. Both studies show that there is utility in assessing immunophenotypic abnormalities in patients referred for the investigation of cytopenia, although the sensitivity of each scoring scheme differed between studies. The true specificity of each scoring scheme is difficult to assess. The median timeframe for patient follow-up in both studies was 9 months and 12 months, respectively, and both studies, and other studies, demonstrate that patients can develop MDS many years after the initial referral (Kern et al., 2013; Cargo et al., 2015; Cremers et al., 2016). Finally, a number of patients in both cohorts who developed MDS were not identified by immunophenotypic aberrancies. This might implies that the patient has

not developed MDS at the time of initial referral, that, as mentioned previously, there may be a group of MDS patients whose immunophenotypic features are too similar to non-MDS patients, or that there is a potential for sampling error in the process of bone marrow investigation and the aspirate and/or trephine is not truly representative of the entire bone marrow.

1.12 Summary, hypotheses, and aims of the thesis

When the National Institute for Clinical Excellence (NICE) published the “Improving outcomes in Haematological Cancers” document in October 2003, it stated “Improving the consistency and accuracy of diagnosis is probably the single most important aspect of improving outcomes in haematological malignancy” (NICE, 2003). Whilst the late diagnosis of cancer has been shown to impact on survival (Richards, 2009). However, a consistent and accurate diagnosis in MDS has been shown to be elusive and current methods fail to identify some patients who progress to MDS.

Flow cytometry is a key component in the investigation of suspected MDS cases via its ability to identify normal and abnormal immunophenotypic features and it has proven its utility in both diagnostic and prognostic settings. The overall aim of this PhD was to determine the feasibility of using flow cytometry immunophenotyping as a primary technique in the investigation of suspected MDS. This was based on the hypothesis that flow cytometry immunophenotyping can provide an objective means for MDS classification, thereby reducing the inherent subjectivity currently employed due to morphological assessment and improving clinical effectiveness. Various flow cytometric scoring schemes have previously been proposed. However, their implementation in a large-scale diagnostic laboratory such as HMDS is challenging due to the size and cost of the antibody panel, the time required for analysis, or sample integrity requirements. As HMDS is mentioned within the 2007 Department of Health Cancer Reform Strategy to be representative of the current paradigm for regional service provision within the NHS, evaluation and development in this setting is of particular importance for impacting on patient care pathways in the UK (NHS, 2007).

To achieve the overall aim of determining the feasibility of the use of flow cytometry in the investigation of MDS, this study will:

- Investigate whether simple immunophenotypic features could be combined with demographic details to develop a test to identify MDS or aid in its exclusion.
- Identify key immunophenotypic features predictive of MDS by an extended assessment of antigens across all haematopoietic lineages and combine these features in an immunophenotypic panel for further testing on cytopenic cases.
- Develop methods to produce and test an independent classifier which was capable of dealing with the results from the potentially high number of attributes assessed by the immunophenotypic panel.

2 Materials and Methods

2.1 Ethical approval, overview of patient selection and study design

All patients were referred for investigation to the Haematological Malignancy Diagnostic Service based at St. James's University Hospital, Leeds. The use of waste clinical samples at HMDS was approved by the National Research Ethics Service Committee (reference number: 14WS0098).

All patients for retrospective investigation were identified through the use of the Haematological Malignancy Diagnostic Service Laboratory Information Management System (HILIS). Patients for prospective studies were identified on the basis of clinical details or morphological features. The diagnosis was subsequently confirmed through HILIS following normal diagnostic reporting procedures.

Although categorized in the WHO overlap MDS/MPN-U group, for the purposes of this thesis, patients within the diagnostic categories CMML, MPDS/MPN-U, and RARS-T were considered for inclusion and, when used, were included as a class within the MDS patient group.

With respect to flow cytometry studies, the progenitor cell screen was routinely performed as a component of normal diagnostic investigation. Therefore, acquisition, analysis, results checking, and reporting onto the HILIS database was performed by all members of the HMDS flow cytometry team, including the author. For all other flow cytometry studies, the samples were processed, incubated, acquired and analysed by the author alone.

For results Chapter 1, a retrospective cohort study was used to assess the clinical and laboratory features of MDS patients and compare to those noted in Reactive patients. The development of a predictive logistic regression model and classifier testing both utilised a case control study approach.

2.2 Retrospective patient identification and selection

2.2.1 Determining the incidence of MDS in patients referred with cytopenia

All patient request forms are scanned and uploaded onto HILIS and are available for viewing. A simple Structural Query Language (SQL) search was performed to identify all patients who were referred to HMDS in the calendar month of January 2010. This list of patients was imported into Microsoft Excel and filtered to include only those patients on whom a bone marrow aspirate (with or without trephine biopsy) was received. For each case identified, the request form on HILIS was examined to determine the clinical details/reason for referral and the reported diagnosis was recorded.

2.2.2 Determining the proportion of MDS patients who have previous been referred for the investigation of cytopenia.

A simple SQL search on HILIS was performed to identify all patients who had a diagnosis of RARS (including RARS-T), RCMD, RAEB, or CMML in the year 2014. Each patient specimen number was re-examined on HILIS to determine (a) whether each diagnosis was a presentation or whether MDS had been previously diagnosed and the sample was being referred for monitoring or disease progression, and (b) whether the patient had been previously referred for the investigation of cytopenia. Patients in whom the sample was not the initial diagnostic sample or represented disease progression were excluded. If the patient had been previously referred for investigation of cytopenia and had been classified as non-diagnostic, the duration between the first investigation and MDS diagnosis was recorded.

2.2.3 Identification of patients on whom a flow cytometric progenitor cell screening tube had been performed

A HILIS search was performed to identify all patients on whom a flow cytometric progenitor cell screen (see Materials and Methods section 2.4.3 for flow cytometry details) was performed between January 2007 and September 2010. This search was restricted to bone marrow aspirate samples (with or without trephine biopsy. The following laboratory data was recorded: Patient age and sex; Percentage $CD34^+$ cells (of leucocytes) and percentage $CD19^+$ cells (of $CD34^+$ cells); Morphological and diagnostic comments; Diagnosis.

4756 samples were identified from this search. These records were exported as a .csv file and imported into Microsoft Excel for further data clean-up. To obtain a two class dataset of MDS patients and Reactive controls, exclusion criteria were applied. These exclusions were:

- Any sample from a patient with a confirmed non-MDS haematological malignancy or secondary infiltration of the bone marrow by metastatic carcinoma.
- Follow up samples from patients with known, non-MDS haematological malignancies.
- Cases with a diagnosis of “Unsuitable specimen” following morphological evaluation.

For patients who were referred on multiple occasions for the investigation of cytopenia and were non-diagnostic, only the initial sample was included. Similarly, in cases of recurrent samples on MDS patients who remained in the same WHO subgroup, only the results for the initial sample were included. For patients with progressive MDS who changed WHO subgroup, both results were included. This occurred in 4 patients.

The above patients were used to construct the logistical regression model for the training set. The same process was applied to records with the timeframe records from September 2010 until April 2013 to obtain the data for the test set for validation of the model.

2.3 Sample selection for flow cytometry studies

2.3.1 Overview of routine diagnostic samples received in HMDS

All bone marrow samples received in HMDS are drawn into EDTA-containing tubes. Due to sample transport logistics, HMDS receives samples which can be over 24 hours old. Some referral centres do not provide a trephine biopsy or a peripheral blood sample for FBC analysis, nor do they always provide comprehensive FBC results on the request form.

2.3.2 Comparison of MDS and normal control bone marrow samples

Samples for the comparison of immunophenotypic features of normal and MDS haematopoietic populations were selected based on the following criteria:

Normal control group	**MDS group**
FBC parameters within the normal reference range	Unambiguous evidence of dysplasia in one or more lineage
Referral for the staging of low-grade B-cell or T-cell lymphoma	
No morphological evidence of bone marrow involvement	
A normal B-cell Kappa:Lambda light chain ratio or normal T-cell subsets	
A cellular aspirate which yielded at least 20 x 10^6 leucocytes following red cell lysis	

Table 2.1. Criteria for selection of bone marrow aspirate selection for the comparison of normal control and MDS samples.

2.3.3 Generation of a classifier from MDS and Reactive bone marrow samples

Samples for this study were chosen on the basis of either a referral for the staging of low-grade B-cell or T-cell lymphoma or for the investigation of cytopenia, with an emphasis on testing patients with unambiguously dysplastic morphological features. A cellular aspirate was not required as only 2 x 10^6 leucocytes were required following red cell lysis. There was no requirement for the sample to be less than 24 hours old.

2.3.4 Classifier testing and evaluation against other flow cytometry MDS scoring schemes and targeted gene mutation analysis

Samples for the classifier testing and comparison against other methods of evaluating presence of dysplasia or clonal haematopoiesis were selected based on the following criteria:

Normal	**Cytopenic group**
FBC parameters within the normal reference range	Presence of a cytopenia in one or more lineages
Referral for the staging of low-grade B-cell or T-cell lymphoma	Clinical details which do not indicate the presence of a paraprotein or B-lymphoid symptoms
No morphological evidence of bone marrow involvement	Sufficient sample remaining for DNA extraction for targeted gene mutation analysis
A normal B-cell Kappa:Lambda light chain ratio or normal T-cell subsets	
Presence of a trephine biopsy	
A cellular aspirate which was less than 24 hours old and which yielded at least 13 x 10^6 leucocytes following red cell lysis, and the presence of a trephine biopsy	

Table 2.2. Criteria for selection of bone marrow aspirate selection for classifier testing and evaluation against other flow cytometry scoring schemes and against targeted gene mutation analysis.

2.4 Flow cytometry studies

2.4.1 Machine Set-up

2.4.1.1 Defining instrument voltages and instrument quality control measures

To define the optimal photomultiplier tube (PMT) voltages for each detector, Cytometer Setup and Tracking (CS&T) Beads (BD Biosciences, Oxford, UK) were used. The beads were subsequently used to run day-to-day performance checks to ensure consistency of the data obtained from the flow cytometer. Rainbow Calibration Particles (Spherotech, Lake Forest, Chicago) were also used to monitor flow cytometer performance. This is a solution of eight different 3.0μm particles, each of which has a discrete fluorescent intensity (peak). One drop of beads was incubated with 350μl of FACSFlow. The strongest fluorescent peak was used to monitor the CV and MFI in each detector. As the PMT voltage remained fixed, there should only be slight day-to-day variation in the CV and MFI per detector. A referral to BD Technical Support for further advice would be indicated by a variation of greater than 15% for the target MFI or a persistent drift in the CV value for Rainbow Beads, or a consistent fail alert from the CS&T day-to-day performance check.

2.4.1.2 Compensation

Flow cytometry compensation is the process by which we correct for spectral overlap of fluorochromes which are measurable in more than one detector. This calculation relies upon the ratio of the fluorescent intensities between the negatively- and positively-stained events. Classically, this is calculated using antibodies to peripheral blood lymphocyte subsets, with non-antigen expressing lymphocytes as the negative. However, as some of the antibodies conjugated to the tandem dyes e.g. CD34 PerCp-Cy5.5 and CD117 PC7 are not expressed by peripheral blood lymphocytes, and as a standard approach was preferred, calculation was performed using antibody capture beads (Bangs Laboratories, Fishers, USA). As different fluorochromes and different antibodies were used in the B670, B780/60, R780/60, V450/50, and V530/30 detectors in each different panel, experiment specific antibodies were used for these detectors, whilst an anti-CD8 antibody was used for FITC, PE, and APC. The generic compensation set-up experiment for all experiments is shown in Table 2.3.

Detector name	B530/30	B585/42	B670LP	B780/60	R660/20	R780/60	V450/50	V530/30
Fluorochrome(s) detected	FITC	PE	PerCp-Cy5.5	PE-Cy7, PC7	APC	APC-Cy7, APC-H7	Pacific Blue, BV421	Pacific Orange, V500
Antibody used	CD8	CD8	Panel specific	Panel specific	CD8	Panel specific	Panel specific	Panel specific
Antibody volume (µl)	5	5	2.5	0.5	2.5	5	0.5	5

Table 2.3. Fluorochromes, antibodies and antibody volumes required for the compensation experiment for each panel.

FITC, PE and APC antibodies were all anti-CD8. The antibodies used to compensate spectral overlap for the B670LP, B780/60, R780/60, V450/50 and V530/30 were determined by which antibodies were employed in the panel

2.4.2 Sample preparation

2.4.2.1 Red cell lysis and antibody incubation

Leucocytes were isolated by incubating bone marrow aspirate sample with a 10-fold excess of ammonium chloride (8.6g in 1 litre distilled H_2O) for 10 minutes at 37^oC and washing twice with 10ml of FACSFlow containing 0.3% bovine serum albumin (BSA). A full blood count was performed to obtain a nucleated cell count.$1x10^6$ nucleated cells were then pipetted into each microtitre plate well and stained with of the appropriate volume of antibody combination cocktail before incubation for 20 minutes in the dark at 4^0C. The wells were then washed twice with 200µl of FACSFlow/BSA and re-suspended in 200µl FACSFlow ready for acquisition.

All antibodies for all immunophenotypic studies were used at a final volume of 5µl per fluorochrome per test. Whilst the majority of antibodies were used undiluted, certain antibodies required dilution to either appear on scale or to optimise signal-to-noise. Details of antibody dilutions, clones, reagents, and manufacturer details can be seen in Appendix Table 2.1 and Appendix Table 2.2 .

2.4.2.2 Flow cytometry data acquisition and analysis

Samples were acquired on a FACSCanto II analyser (BD Biosciences, Oxford, England). Analysis of raw flow cytometry data was performed using FacsDiva software (BD Biosciences, Oxford, UK) or Infinicyt^{TM} (Cytognos).

For the SCS, the samples would be acquired on any of 3 given FACSCanto II analysers. Due to a standardised reporting template, analyser-to-analyser variability would not affect the percentage results. For all other flow cytometry studies the same FACSCanto II analyser

was used for panel acquisition. This eliminated the potential analyser-to-analyser variation which can be seen due to differences in laser output, detector settings, and signal to noise ratios between the different cytometers.

2.4.3 Flow cytometry evaluation of the progenitor cells using the SCS

For all samples requiring enumeration and lineage evaluation of progenitor cells, a progenitor cell screen (SCS) is performed which uses 30µl of the antibody combination shown in Table 2.4. A minimum of 100,000 events is acquired for this panel.

Fluorochrome	**FITC**	**PE**	**PerCP-Cy5.5**	**PE-Cy7**	**APC**	**APC-Cy7/APC-H7***
Antibody	CD15	CD117	CD19	CD3	CD34	CD45

Table 2.4. The fluorochrome and antibody combination used for the evaluation of progenitor cells in the SCS.

The CD45 fluorochrome was changed from APC-Cy7 to APC-H7 in March 2009.

This combination allows the identification of myeloid ($CD45^{dim+}CD34^{+}CD19^{-}CD117^{+}$) and B-lymphoid ($CD45^{dim+}CD34^{+}CD19^{+}CD117^{-}$) progenitors and the calculation of the percentage of total $CD34^{+}$ cells and the percentage of $CD19^{+}$ B-progenitors within this compartment. This is shown in Figure 2.1 below. Patients with greater than 5% B-progenitors were classed as having “B-progenitors present”, whilst those with less than 5% were classed as having “decreased B-progenitors”.

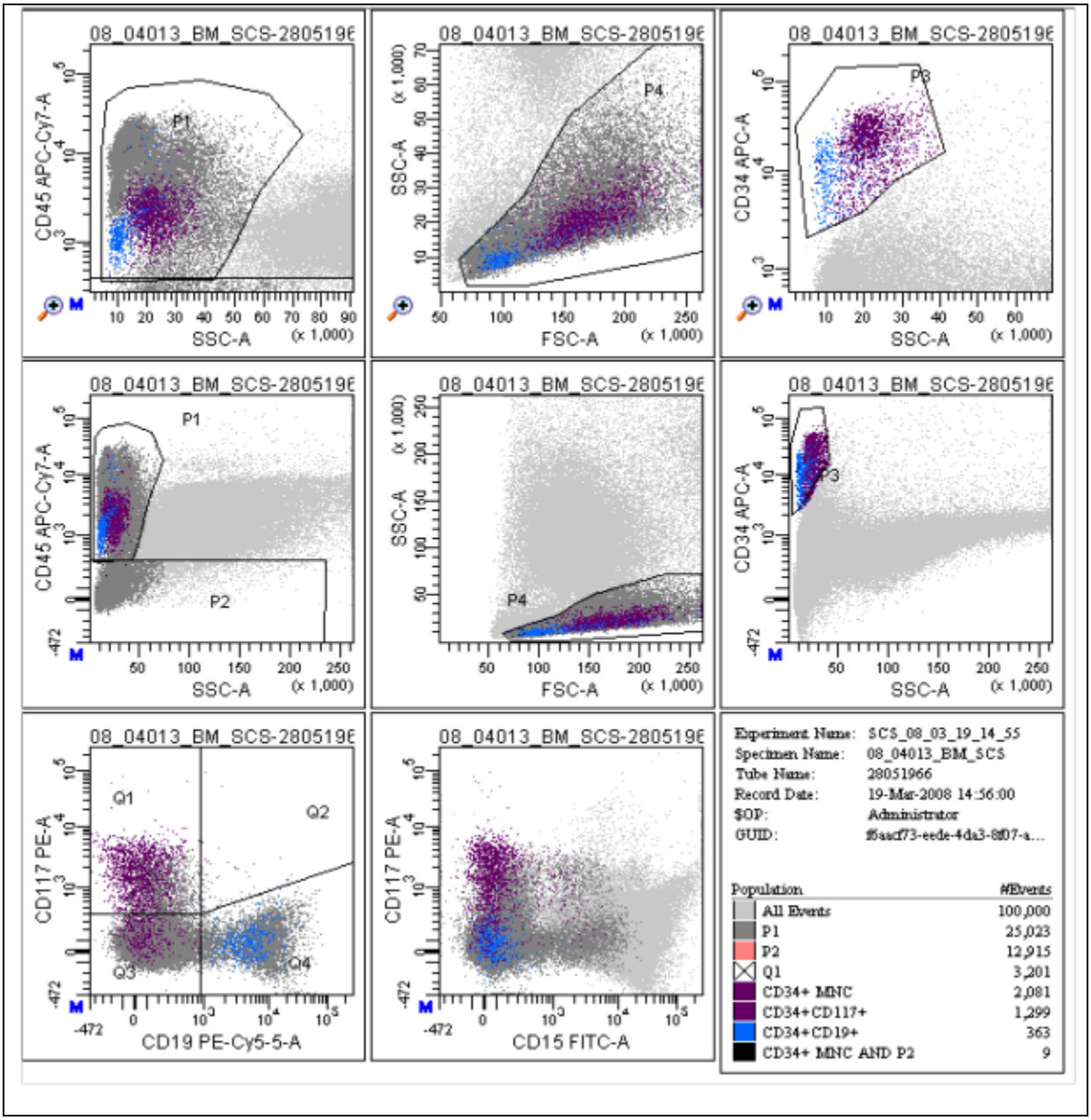


Figure 2.1. Gating strategy and data analysis for the SCS.

CD34+ cells are identified on the basis of CD45 (P1) and CD34 (P3) expression. CD34+ B-lymphoid progenitors (blue) are identified on the basis of positive CD19 expression (Q4). $CD34^+$ myeloid progenitors are identified on the basis of CD117 positivity and/or CD19 negativity (Q1 and Q3). Non-leucocytes are identified on the basis of CD45 negativity (P2). The percentage of CD34 is calculated as a percentage of the total number of events minus the $CD45^-$ events. Whilst the percentage of B-progenitors is calculated as the percentage of $CD34^+CD19^+$ events of total $CD34^+$ events.

2.4.4 Flow cytometry studies for the comparison between MDS and normal control sample haematopoietic populations

2.4.4.1 Antibody panel and acquisition

A 5-antibody backbone of CD34, CD38, CD45, CD117, and HLA-DR was present in all 20 tubes in this panel. These backbone antibodies were selected as this combination recognises all myeloid progenitors and allows evaluation of myeloid differentiation pathways (Matarraz et al., 2010; Sandes et al., 2013). The fluorochrome conjugation for these 5 backbone antibodies was CD34 PerCp-Cy5.5, CD38 APC-H7, CD45 Pacific Orange, CD117 PC7, and HLA-DR Pacific Blue. The backbone antibodies were configured to allow the FITC, PE, and APC fluorochromes to be available for tube specific antibodies. This is due to the majority of antibodies being available on these fluorochromes, whilst availability on the other fluorochromes may be limited. The antibody composition and configuration of the FITC, PE and APC antibodies for each tube is shown in Table 2.5. 40µl of the antibody combination was used per tube and a minimum of 500,000 events were acquired per tube.

2.4.5 Flow cytometry studies for the generation of a classifier from MDS and Reactive bone marrow samples

2.4.5.1 Antibody panel and acquisition

A 6-antibody backbone of CD19, CD34, CD38, CD45, CD117, and HLA-DR was present in both tubes in this panel. CD19 was added to ensure better discrimination between myeloid and B-lymphoid progenitors than side scatter alone. The backbone antibody fluorochromes were slightly altered from the previous study to accommodate the inclusion of CD19. The antibody composition and configuration of each tube is shown in Table 2.6. 40µl of the antibody combination was used per tube and a minimum of 500,000 events were acquired per tube.

Tube Number	FITC	PE	APC
1	CD16	CD13	CD11b
2	CD14	CD64	CD300e
3	CD61	CD42b	CD25
4	CD71	CD105	CD5
5	CD36	CD95	CD33
6	CD45RA	CD13	CD45RO
7	CD90	CD133	CD28
8	CD13	CD150	CD43
9	CD7	CD62L	CD2
19	CD9	CD154	CD123
11	CD4	CD203	CD22
12	CD24	CXCR4	CD10
13	CD59	CD84	CXCR5
14	CD18	CD82	-
15	CD49d	CD86	-
16	CD11a	CD106	-
17	CD48	CD19	CD56
18	CD81	CD122	-
19	CD75	CD163	CD15

Table 2.5. FITC, PE, and APC conjugated antibodies used to compare MDS and normal control sample haematopoietic populations

Tube Number	FITC	PE	PerCp-Cy5.5	PC7	APC	APC-Cy7	BV421	V500
1	CD64	CD123	CD38	CD117	CD34	HLA-DR	CD19	CD45
2	CD16	CD13	CD38	CD117	CD34	HLA-DR	CD19	CD45

Table 2.6. The fluorochrome and antibody combination used to generate a classifier for distinguishing MDS and Reactive samples

2.4.6 Flow cytometry studies for classifier testing and comparison against other flow cytometry MDS scoring schemes and targeted gene mutation analysis

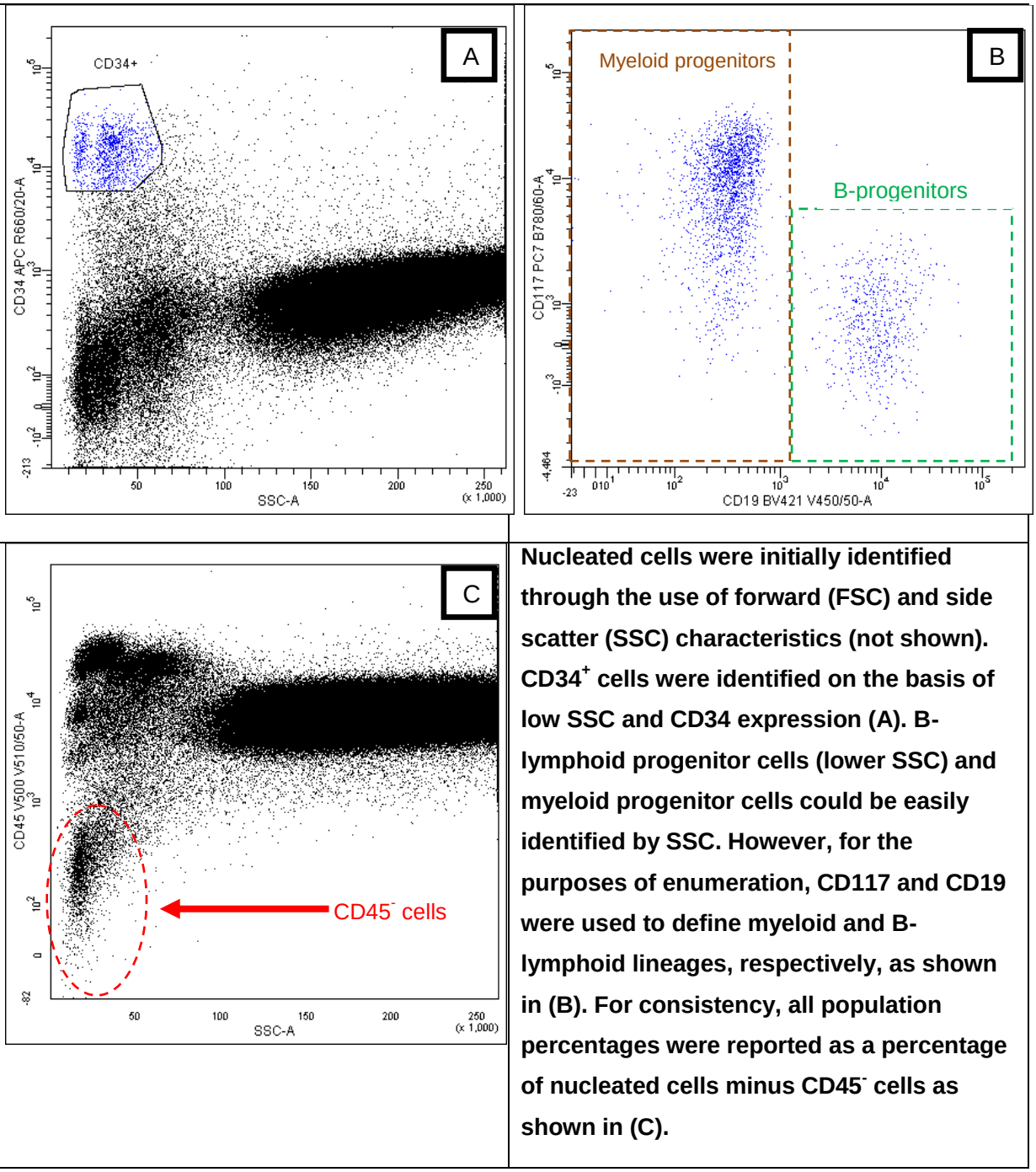
To allow evaluation of the classifier against other flow cytometry MDS scoring schemes, a comprehensive 13-tube flow cytometry panel was assessed against a series of normal and cytopenic patients. This panel contained all the antibodies which were used in the study to generate a classifier. The panel also contained the majority of antibodies recommended by the ELN guidelines and the majority of antibodies present in the FCSS. The antibody composition and configuration of each tube is shown in Table 2.7. 40µl of the antibody combination was used per tube and a minimum of 500,000 events were acquired per tube.

Tube Number	FITC	PE	PerCp-Cy5.5	PC7	APC	APC-Cy7	BV421	V500
1	CD123	CD13	CD38	CD117	CD34	HLA-DR	CD19	CD45
2	CD45RO	CD45RA	CD38	CD117	CD34	HLA-DR	CD19	CD45
3	CD49d	CD84	CD38	CD117	CD34	HLA-DR	CD19	CD45
4	CD18	CD133/2	CD38	CD117	CD34	HLA-DR	CD19	CD45
5	CD81	CD62L	CD38	CD117	CD34	HLA-DR	CD19	CD45
6	CD71	CD123	CD38	CD117	CD34	HLA-DR	CD19	CD45
7	CD59	CD43	CD38	CD117	CD34	HLA-DR	CD19	CD45
8	CD14	CD64	CD34	CD117	CD300e	HLA-DR	CD19	CD45
9	CD16	CD13	CD34	CD117	CD11b	HLA-DR	CD19	CD45
10	CD36	CD105	CD34	CD117	CD71	HLA-DR	CD19	CD45
11	CD24	CD95	CD34	CD117	CD10	HLA-DR	CD19	CD45
12	CD15	CD86	CD34	CD117	CD33	HLA-DR	CD19	CD45
13	CD2	CD7	CD5	CD56	CD34	CD4	CD19	CD45

Table 2.7. The fluorochrome and antibody combination used for classifier testing and comparison against other flow scoring schemes

2.4.7 Flow cytometry gating strategies

A consistent flow cytometry gating strategy using the backbone antibodies CD34, CD45, CD117, and HLA-DR was used for the immunophenotypic panels used in 2.4.4, 2.4.5, and 2.4.6. The backbone antibodies were used to gate the following populations: CD34 progenitors, $CD34^{-}CD117^{+}$ committed myeloid progenitors, and granulocytes (as shown in Figure 2.2, Figure 2.3, and Figure 2.4). The inclusion of the other antibodies within the panels allowed subpopulation analysis to be performed as described in each figure.



Nucleated cells were initially identified through the use of forward (FSC) and side scatter (SSC) characteristics (not shown). $CD34^{+}$ cells were identified on the basis of low SSC and CD34 expression (A). B-lymphoid progenitor cells (lower SSC) and myeloid progenitor cells could be easily identified by SSC. However, for the purposes of enumeration, CD117 and CD19 were used to define myeloid and B-lymphoid lineages, respectively, as shown in (B). For consistency, all population percentages were reported as a percentage of nucleated cells minus $CD45^{-}$ cells as shown in (C).

Figure 2.2. Gating strategy for the identification and enumeration of $CD34^{+}$ cells.

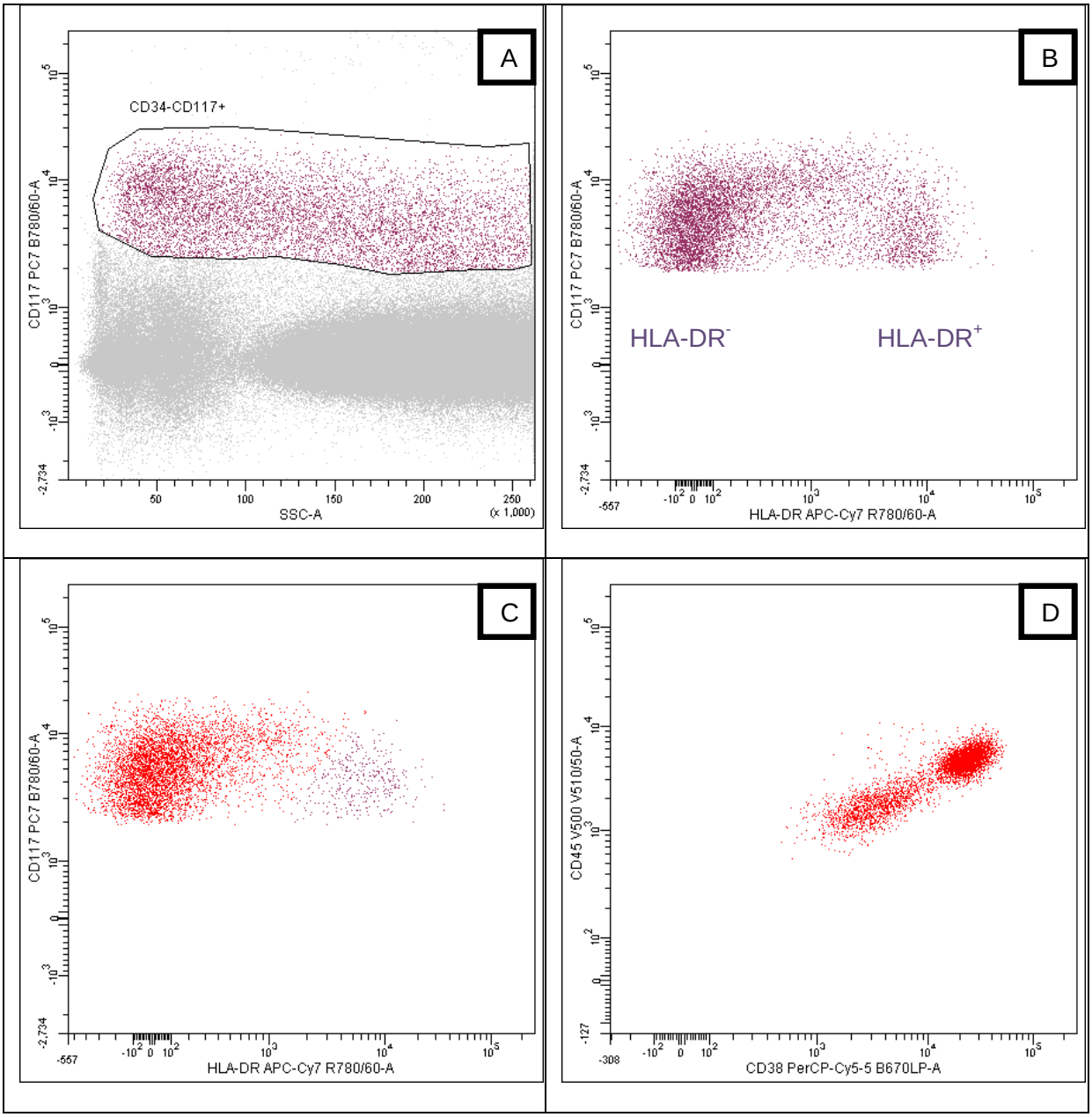
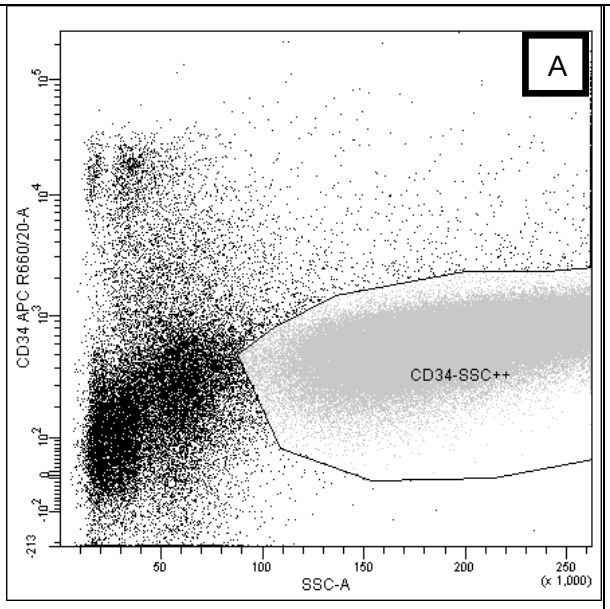
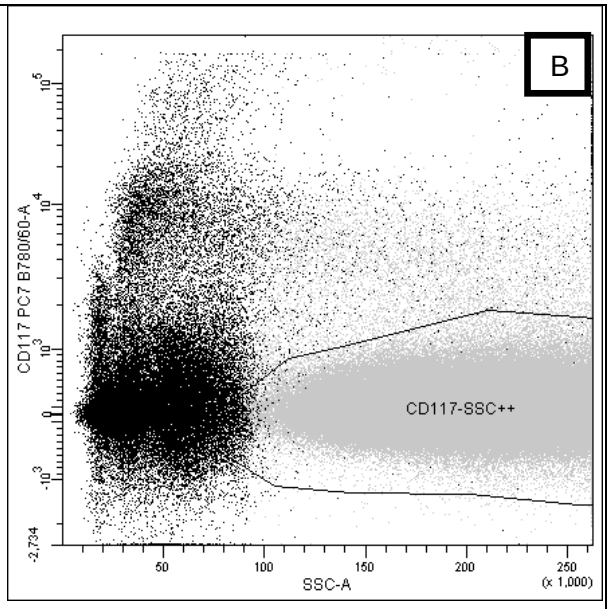
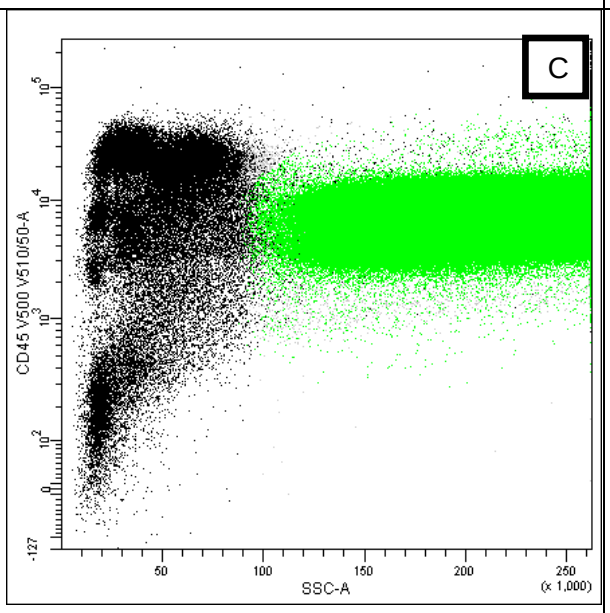


Figure 2.3. Gating strategy for the identification and enumeration of the CD34$^-$CD117$^+$ population and subpopulations.

CD34 expressing cells were first excluded, as per the gating strategy in Figure 2.2. CD117 expressing cells were gating on the basis of CD117 expression and SSC characteristics (A). Subpopulations within the CD117 expressing cells were identified on the basis of HLA-DR expression as shown in (B). The HLA-DR expressing cells were monocytic precursors. The HLA-DR$^-$ cells shown in red in (C) could be further divided by differential expression of CD38 and CD45 into CD38$^+$(weak)CD45$^+$(weak) erythroid precursors and CD38$^+$CD45$^+$ granulocytic precursors.







Granulocytes were identified on the basis of medium to high SSC and absence of CD34 (A) and CD117 (B). CD45 expression was evaluated (C) to confirm the gating strategy

Figure 2.4. Gating strategy for the identification and enumeration of granulocytes

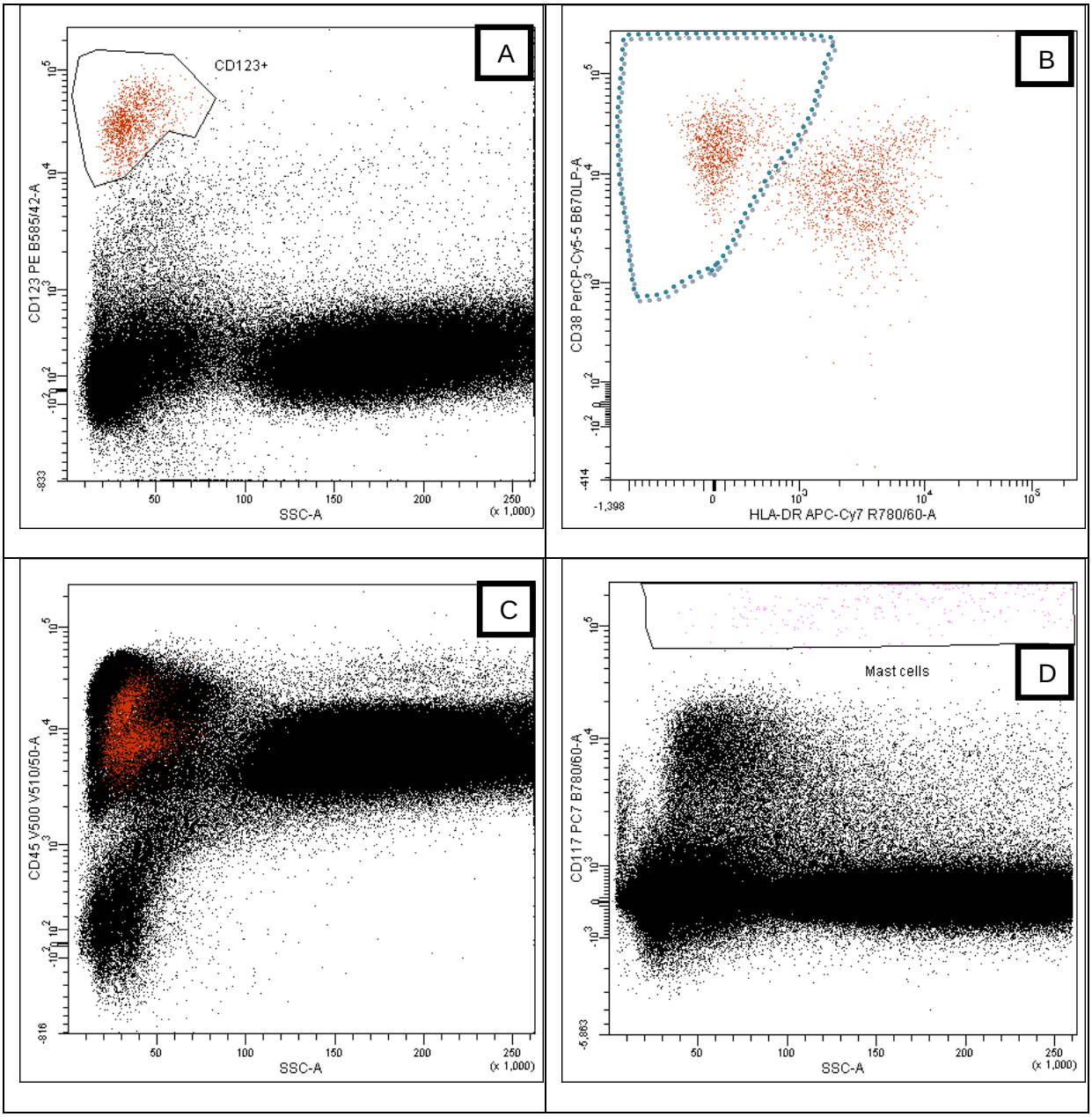
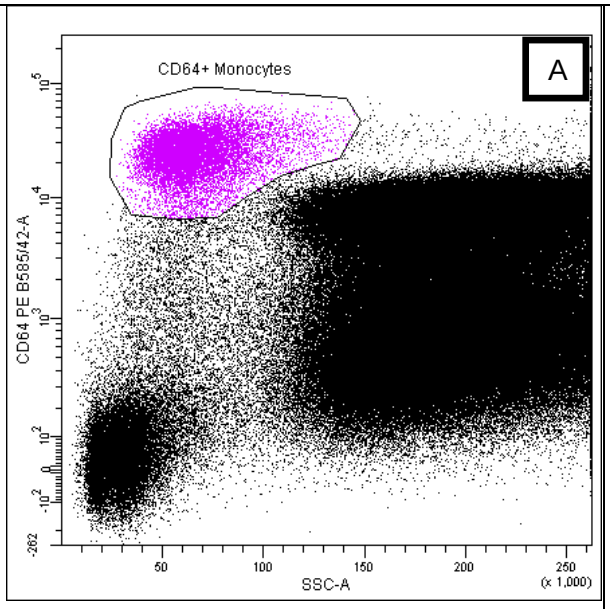
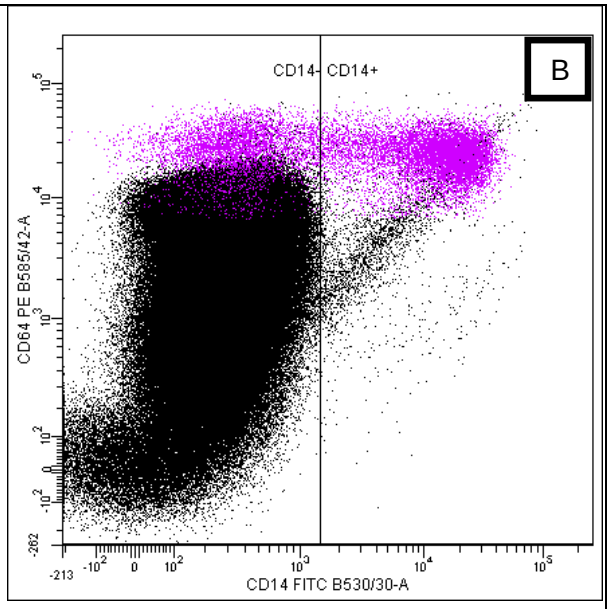
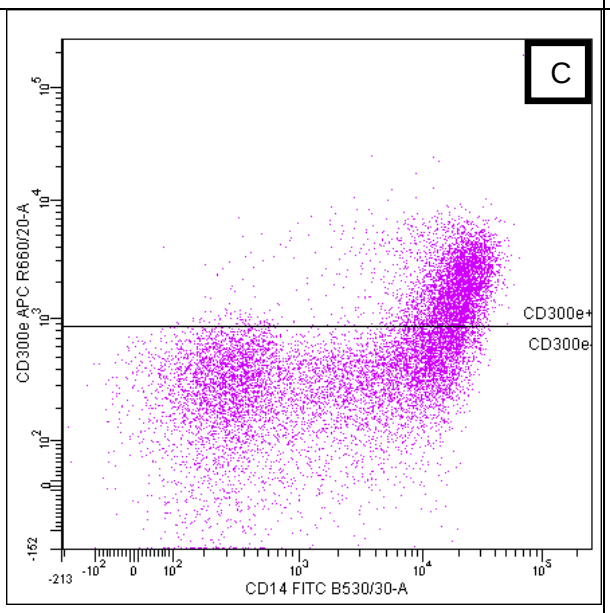


Figure 2.5. Gating strategy and identification of plasmacytoid dendritic cells, basophils, and mast cells.

Plasmacytoid dendritic cells (pDC's)and basophils were identified on the basis of strong CD123 expression and low SSC (A). The basophils can be identified by strong CD38 expression and absence of HLA-DR, whilst the pDC's express HLA-DR and show weaker CD38 expression (B). The is also differential CD45 expression between these two populations with the pDC's showing higher CD45 expression (C). Mast cells are identified on the basis of very strong CD117 expression (D).







Monocytic cells were identified on the basis of strong CD64 expression and intermediate SSC (A). CD14 expression on this population was determined using granulocytes as a negative internal control (B). The expression of CD300e was determined using CD14$^-$ monocytes as a negative internal control as shown in (C).

Figure 2.6. Gating strategy for identification and enumeration of the monocytic populations

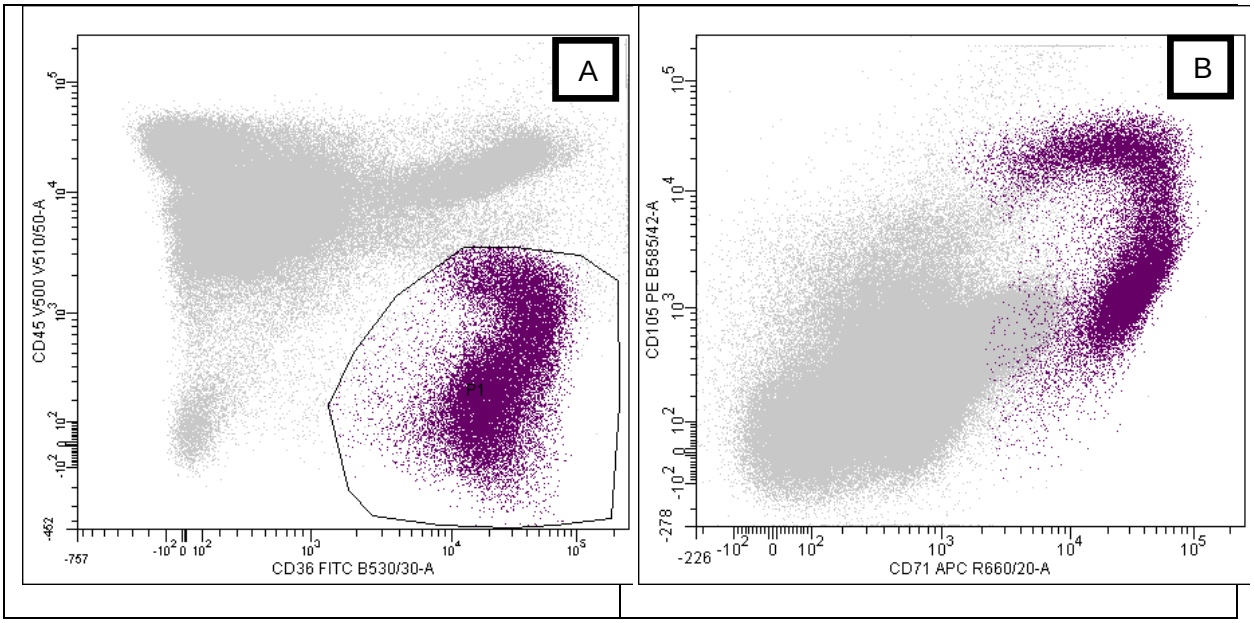


Figure 2.7. Gating strategy for assessing erythroid dysplasia

(A) The absence of CD45 and expression of CD36 was used to identify erythroid cells. All erythroid events expressed CD71 with variable CD105 expression (B).

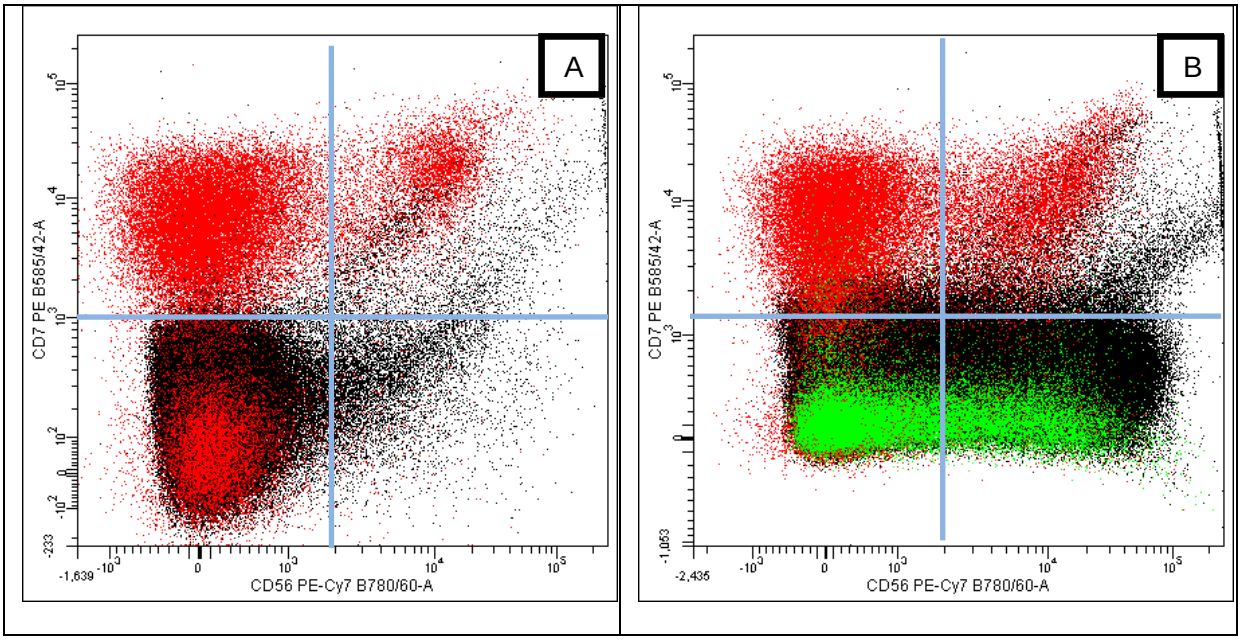


Figure 2.8. Identification of lineage infidelity antigen expression.

Positive expression of lymphocyte-related antigens by lymphoid cells (shown in red) was used as an internal control for the inappropriate expression of CD5, CD7, CD19 and CD56 by myeloid progenitor cells, granulocytes, or monocytes. (A) shows normal lymphoid and myeloid expression of CD5 and CD7, whilst (B) shows aberrant expression by CD34^{+} cells shown in green. Expression by >20% of the myeloid cell population for a lymphoid-related antigen was considered positive expression.

2.4.8 Data analysis post gating

For each gated population, the median fluorescent intensity (MFI) and the robust coefficient of variation (CV) of each antigen, whether expressed or not, were exported within a .csv file. For cases where there were less than 300 events for a specific haematopoietic population, the MFI and CV could not be used due to concerns regarding the validity of the result with this limited number of events.

This cut-off did not affect numerical population percentage results which could still be calculated for these cases. However, a standardised approach for enumerating cells in CLL cases has reported a lower limit of detection and lower limit of quantification as 20 and 50 events, respectively (Rawstron et al., 2007; Rawstron et al., 2013). Therefore, if the analysed population size did not reach 50 events, the number of events was set to the pseudovalue of 49, and a maximum population percentage was calculated using this value.

2.4.9 Antigen exclusion criteria

A median fluorescent intensity of 10^3 on the log scale of dot plots is often used as a cut-off for distinguishing positive and negative antigen expression. This can be seen with respect to CD7 expression by lymphocytes in plot (A) of Figure 2.8 above. In Chapter 4, when evaluating antigens for further assessment, it was undesirable to miss any potential significant differences in weakly expressed antigens between the control and MDS group. Therefore, a cut-off MFI of 500 was used and antigens where the mean group MFI was below 500 were not considered to be too weakly expressed for diagnostic purposes and were excluded from further analysis.

2.4.10 Immunophenotypic analysis for the flow scoring schemes

For evaluation of the Ogata FCM scoring scheme, all analysis and use of attribute cut-off values was performed in accordance with the published analytic methods (Ogata et al., 2009; Della Porta et al., 2012). For the FCSS scoring scheme, a visual approach to deviation from normal patterns of expression is used for the majority of the attributes (Wells et al., 2003) . Therefore, to standardise the approach and remove observer variability, any result greater or less than 2 standard deviations from the mean control group MFI was deemed abnormal. This approach could not, however, be applied to the visual assessment of the CD16/CD13 and CD11b/HLA-DR differentiation patterns. Finally, ELN guidelines recommend a “shift towards immature” assessment of monocytes (Westers et al., 2012). For this evaluation, this attribute was deemed abnormal if the percentage of CD14 expressing monocytes was greater than 2 standard deviations lower than the normal control mean value.

2.5 Statistical analysis and data normalisation

2.5.1 Use of R

2.5.1.1 Statistical analysis and logistic regression modelling

All formal statistical analysis and the construction of the logistic regression model from the Progenitor cell screen data and demographic data was performed using the R package (R Core Team, 2015). As well as the standard, pre-installed, Base Package, the following libraries were installed and used to prepare, interrogate, compare, analyse, and plot data: *aod; dpylr; e1071; ggplot2; glmnet; Hmisc; moments; nortest; OptimalCutpoints; PerformanceAnalytics; psych; RColorBrewer; reshape 2; rms; ROCR; scales; and xtable*.

For statistical analysis, χ^2 was performed for comparison between groups and Wilcoxon signed ranks for continuous variables. All tests used were two-sided. *P* values of <0.05 were considered significant. For between group statistical comparisons involving multiple attributes the Bonferroni correction was used to control for the familywise error rate and the Benjamini-Hochberg procedure was applied to control for the false discovery rate.

2.5.1.2 Feature scaling for use in classifier training and testing in Weka, and in dChip

Feature scaling of attributes allows all attributes to feature equally in a classifier or in hierarchical clustering. Feature scaling of attributes using standardisation was performed for all attributes in the training and test datasets, and also for attributes used in hierarchical clustering. Preparation of the data was done in R using the *scale* command. This command calculated the mean and standard deviation for each attribute. The attribute mean was then subtracted from each numerical value within that attribute, and divided by the attribute standard deviation. This value is also known as the Z-score or standard-score.

The mean and standard deviation for each attribute in the training set was then applied to the corresponding attribute values in the test set in order to normalize the test set attributes to the same scale. The exception to this was CD64 which was used as a FITC-conjugated antibody in the training set and a PE-conjugated antibody in the test set. In this case, feature scaling using standardisation was applied individually to CD64-FITC in the training set and to CD64-PE in the test set.

2.5.2 Use of dChip

dChip was developed for the analysis of SNP-array and gene-expression array data (Li and Wong, 2001). It also performs unsupervised hierarchical clustering which, in this case, is a method for clustering cases into discrete groups on the basis of attribute similarity. It

performs unsupervised hierarchical clustering using an *agglomerative approach* which assumes each case is separate and successively merges cases closest to one another until all groups are merged into a single cluster. For all unsupervised hierarchical clustering the attributes were standardised as defined in 2.5.1.2. Euclidean distance was used as the distance metric and average linkage was the linkage method.

2.5.3 Use of Weka

Weka is an acronym for *Waikato Environment for Knowledge Analysis*. The Weka software was developed at the University of Waikato in New Zealand and contains a collection of machine learning classifiers and algorithms (Hall et al., 2009). Datasets can be imported in Weka and it can be used to assess the result of classifier performance on a dataset. For this study, a total of 36 classifiers were tested on the training set. Default classifier parameters were retained for each classifier and standard measures of classifier performance were evaluated.

2.6 Cytogenetic analysis

The cytogenetic results for cases within the test set were obtained from patient reports on the HILIS database. All cytogenetic analyses were performed at the Regional Genetics Laboratory in St James's University Hospital, Leeds in accordance with the International System for Cytogenetic Nomenclature (Shaffer et al., 2009; Shaffer et al., 2013).

2.7 Molecular analysis

DNA was extracted bone marrow nucleated cells using the QIAamp DNA mini kit (QIAGEN, Manchester, UK) according to the manufacturer's instructions. Targeted gene sequencing was performed on the MiSeq (Illumina, Chesterford, UK) using panels designed to target 26 genes as shown in Table 2.8. The D3™ Assay Design service (Fluidigm®, San Francisco, CA, USA) was used to design amplicons. DNA libraries were built using Fluidigm® technology and all samples were included in runs of forty-eight pooled, barcoded patient samples. Samples were subjected to 150bp paired-end sequencing. Library construction and sequencing were performed according to the manufacturer's instructions. Details of the forward and reverse primer sequences can be found published in Supplementary Table S4 of Cargo *et al.* (Cargo et al., 2015).

Gene	Targeted Region
ASXL1	exon 12
BCOR	exon 2-15
CALR	exon 9
CBL	exon 8 & 9
c-KIT	exon 8 & 17
CSF3R	exon 14 & 17
DNMT3A	exon 2-23
EZH2	exon 2-20
FLT3	exon 20
IDH1	exon 4
IDH2	exon 4 & 5
JAK2	exon 12 & 14
KRAS	exon 2 & 3
MPL	exon 10
NPM1	exon 12
NRAS	exon 2 & 3
RUNX1	exon 4-8
SETBP1	exon 4
SF3B1	exon 12-16
SRSF2	exon 1
STAG2	exon 3-35
TET2	exon 3-11
TP53	exon 5-9
U2AF1	exon 2 & 6
WT1	exon 7 & 9
ZRSR2	exon 2-11

Table 2.8. Details of the genes and targeted regions which were investigated for somatic mutations in patients in the test set.

3 Insights into the diagnosis of MDS in a routine laboratory setting

3.1 Rationale and overview

MDS poses significant diagnostic problems both in the accurate morphological distinction from other malignant and non-malignant cytopenic conditions, and in the subclassification of MDS according to the WHO classification scheme (Parmentier et al., 2012; Font et al., 2013; Font et al., 2015). In this chapter, the extent of this problem in a diagnostic laboratory setting was assessed by use of database searches using the HMDS Laboratory Information System database (HILIS).

To investigate the difficulty in diagnosing MDS in a routine setting, HILIS was interrogated to determine what proportion of bone marrow samples referred for the investigation of cytopenia and/or monocytosis were diagnosed with MDS. A further search was performed to ascertain how many patients diagnosed with MDS in a given year had been previously referred for the investigation of cytopenia and had a non-diagnostic bone marrow.

HILIS was further interrogated for demographic data and flow cytometric results for the composition of CD34 positive progenitor cells for both MDS patients and Reactive patients. This could used to determine whether there were significant inter- and intra-subgroup differences and whether these differences could be exploited to aid in accurate MDS diagnosis. The resulting data could also be further investigated to determine whether simple demographic and biological attributes could distinguish MDS from other cytopenic conditions.

3.2 What percentage of patients referred for the investigation of cytopenia are diagnosed with MDS?

The proportion of patients who are referred for the investigation of cytopenia and are diagnosed with MDS is unknown. Beloosesky *et al.* investigated over 3000 patients admitted to a geriatric ward and reported that 7.5% had either cytopenia or macrocytosis, or a monocytosis, and that 15% of these 7.5% were diagnosed with MDS (Beloosesky et al., 2000). More recently, Kwok *et al.* reported an 8% MDS diagnostic rate for patients referred for the investigation of cytopenia, with 65% of patients having a non-diagnostic bone marrow (Kwok et al., 2015). The proportions of MDS and non-diagnostic patients referred to HMDS for investigation of cytopenia was unknown. Therefore, a HILIS database search of all samples received during a representative timeframe of one calendar month (January 2010) was performed to allow an insight into the number of cases referred and, also, whether the proportions tallied with those previously reported.

Bone marrow aspirate samples (with or without trephine biopsies) from 507 patients were referred to HMDS for the investigation of any haematological malignancy. 389 patients were excluded from further analysis on the basis of the following: referral for staging purposes or follow-up of previously diagnosed haematological malignancy, or for the investigation of the following conditions: chronic myeloid leukaemia, chronic myeloproliferative disorder, myeloma or monoclonal gammopathy of undetermined significance, bone marrow infiltration by carcinoma, or storage disorders.

118 bone marrow aspirate samples were referred for the investigation of cytopenia or, explicitly, of MDS or acute leukaemia. Of these 118 cases, 24 were diagnosed with non-MDS haematological malignancies (AML (n=11), B-ALL (n=1), bone marrow involvement by lymphoma (n=7), myeloma or MGUS (n=3), and PNH (n=2)).

61 cases were reported as non-diagnostic, or *reactive*, with no evidence to indicate that the cytopenia was due to a primary bone marrow disorder. 15 cases were classified as either "*suspicious of malignancy but not diagnostic*" or as "*see comments*". In the context of this cohort of patients, these two latter diagnostic terms were used for *non-diagnostic* specimens where a primary bone marrow disorder could not be excluded but there was insufficient evidence to definitively diagnose malignancy.

15% of cases were diagnosed as MDS (18 cases in total: 8 RAEB, 9 RCMD, and 1 RARS). A further case was suspicious of MDS but not diagnostic and a follow up sample was not received. Figure 3.1. shows the breakdown of the initial 507 patients into the different diagnostic subgroups.

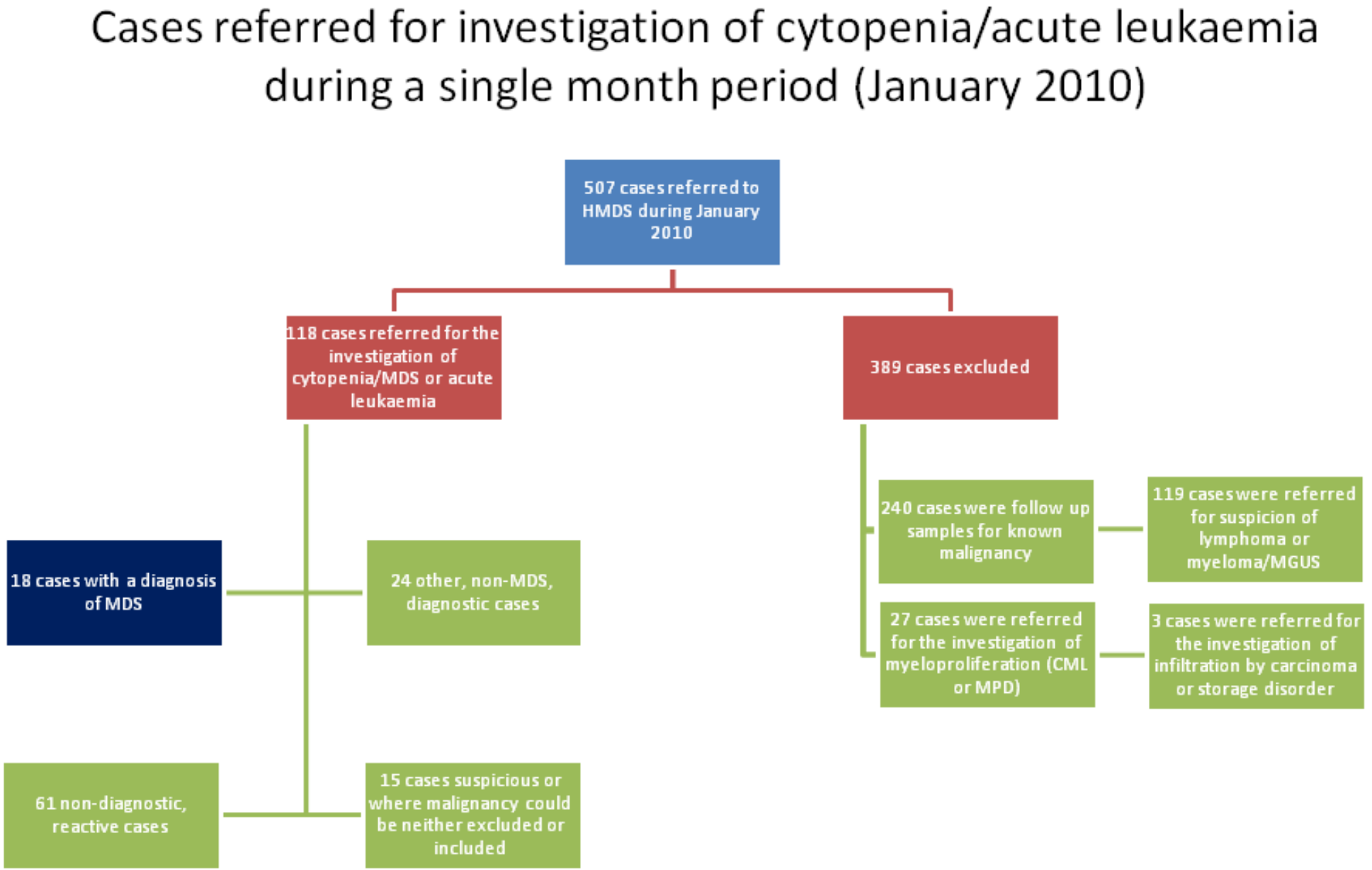


Figure 3.1. Breakdown of the cases referred for investigation of cytopenia/acute leukaemia during a single month (January 2010)

3.2.1 Follow up of non-diagnostic cytopenic patients

Due to the diagnostic difficulties associated with MDS combined with the finding that only 15% of cases referred with cytopenia were diagnosed with MDS, a follow-up database search was performed in January 2015, on all 76 patients who were classified as “reactive”, “see comments”, or “suspicious of malignancy but not diagnostic” to determine whether any had subsequently presented with haematological malignancy. This represented a follow-up period of 60 months. During this timeframe, two patients were diagnosed with a haematological malignancy: one patient who was initially classified as “reactive changes only” was diagnosed with AML (6 months after the initial “reactive” diagnosis). Another patient with, “see comments”, was subsequently diagnosed with RCMD, albeit 38 months after the initial “see comments” report.

The finding that 2 out of 76 patients progressed from a non-diagnostic classification to a diagnosis of myeloid neoplasm emphasised not only the diagnostic difficulties involved in the investigation of cytopenia, but brought to light the question of what proportion of patients presenting with MDS had been previously referred for investigation and had been reported as non-diagnostic.

3.3 What proportion of MDS patients are previously investigated for cytopenia and reported as non-diagnostic?

It was hypothesized that there was a proportion of patients, referred for the investigation of cytopenia, who were classified with a non-diagnostic bone marrow and who may be either en-route to developing MDS or misdiagnosed as non-MDS. There may be various reasons for this suggestion. Firstly, there are the well-reported difficulties in the accurate diagnosis of MDS. Secondly, the above discovery that only 15% of cases referred for the investigation of cytopenia in a single month were diagnosed with MDS and nearly two-thirds were given a non-diagnostic classification. Finally, the finding that, in a random month, 2 patients reported as non-diagnostic subsequently developed a myeloid disorder. It was unclear whether this latter finding was representative as the results were based on a single month’s data. Especially as patients with non-diagnostic bone marrow samples are not currently subjected to active, long-term monitoring.

To establish the true extent of the phenomenon, the database was searched to identify all patients who presented in 2014 with RARS, RCMD, RAEB, or CMML – the four largest MDS diagnostic subgroups. These patients were further investigated as to whether a bone marrow sample had previously been referred for the investigation of cytopenia and classified as non-

diagnostic. Whilst this approach may not be applicable in specialized care centres which may not have access to bone marrow results from secondary care centres, as HMDS is a regional referral centre it holds data on patients who have undergone investigation in secondary care centres within the region, before being transferred to a specialized care centre. As the HILIS database began in 2004, this allowed for at least 120 months of referrals to be examined. The results of this are shown in Table 3.1.

MDS Subgroup	**No. of patients presenting with MDS**	**No. of patients previously investigated**	**No of months between non-diagnostic and diagnostic bone marrow samples**		
			Mean	**Median**	**Range**
RARS	30	2 (7%)	13.5	13.5	1-26
RCMD	106	23 (22%)	28.4	16.0	1-109
RAEB	100	4 (4%)	19.3	15.5	6-28
CMML	49	3 (6%)	52.7	34.0	29-95
All	**285**	**32 (11%)**	**27.7**	**19.8**	**1-109**

Table 3.1. Patients diagnosed with MDS in 2014 who were previously referred for the investigation of cytopenia and reported as non-diagnostic.

Overall, 11% of MDS cases in 2014 had been previously referred for the investigation of cytopenia and found to be non-diagnostic. This percentage varied between the diagnostic subgroups. Patients diagnosed with RCMD showed the highest percentage of previously investigated cases with 23%. Whilst those patients diagnosed with RAEB, RARS, or CMML all demonstrated less than 10% of cases as being previously investigated, with those patients presenting with RAEB showing the lowest percentage overall (4%).

The median length of time between the initial, non-diagnostic referral and actual MDS diagnosis for all subgroups was just under 20 months (19.75 months). For individual MDS subgroups, the time scale varied from 1 month to just over 9 years (109 months). Both the RCMD and CMML subgroups had patients with a prolonged interval between initial referral

and diagnosis (109 and 95 months, respectively), whilst for the RAEB and RARS patients, the longest interval was shorter (28 and 26 months, respectively).

3.4 Do MDS and non-malignant cytopenic patients share similar demographic and biological features?

Based on data provided by HMDS, the median age and age specific incidence for patients with a confirmed diagnosis of MDS has been reported for the UK (HMRN., 2016.). However, it is unknown whether these ages differ from patients referred for investigation of cytopenia or whether the skewing of the male:female ratio, as found in MDS, is a phenomenon found in cytopenic patients. Furthermore, although there are reported differences in the percentage and composition of bone marrow progenitor cells between MDS patients and Reactive patients, it is also unknown whether these findings are independent of age or sex.

From January 2007, when the flow cytometry progenitor cell screening tube (SCS) was introduced, until September 2010, 4756 bone marrow aspirate samples from a range of diagnostic and non-diagnostic categories were identified by a HILIS database search as having had this screening tube performed. This flow cytometry screening tube enumerates the percentage of CD34 positive progenitor cells and denotes whether the B-progenitors within the CD34 positive compartment are greater than 5%. Patients with greater than 5% B-progenitors were classed as having “B-progenitors present”, whilst those with less than 5% were classed as having “decreased B-progenitors”.

This data was used in a threefold manner. Firstly, to determine whether there were differences between MDS and non-malignant cytopenic conditions with respect to age, sex, and progenitor cell composition. Secondly, whether any differences could be exploited to distinguish between the two using a logistic regression model. Thirdly, to discover the extent of biological differences between the different MDS subgroups.

The development of a logistic regression model required the creation of a training set containing two classes of patients: (i) patients with a confirmed diagnosis of MDS and (ii) patients who were referred for the investigation of cytopenia but were given a non-malignant diagnostic classification. Henceforth, these patients were given the broad classification of “Reactive”, although these patients can also be referred to as *pathological controls* (Malcovati et al., 2005).

As this was a training set, and due to the aforementioned phenomena described in 3.2.1, all patients with a non-diagnostic bone marrow in whom, by June 2015, there was a subsequent diagnosis of MDS or AML were subject to secondary exclusion. 36 patients were identified

as fulfilling this criteria and the diagnostic breakdown of these cases was: AML (n=12), MDS/MPN-U (n=1), RAEB (n=7), RARS (n=1), and RCMD (n=15). The results for these patients were retained for later analysis to evaluate whether the logistic regression model would have predicted their malignancy.

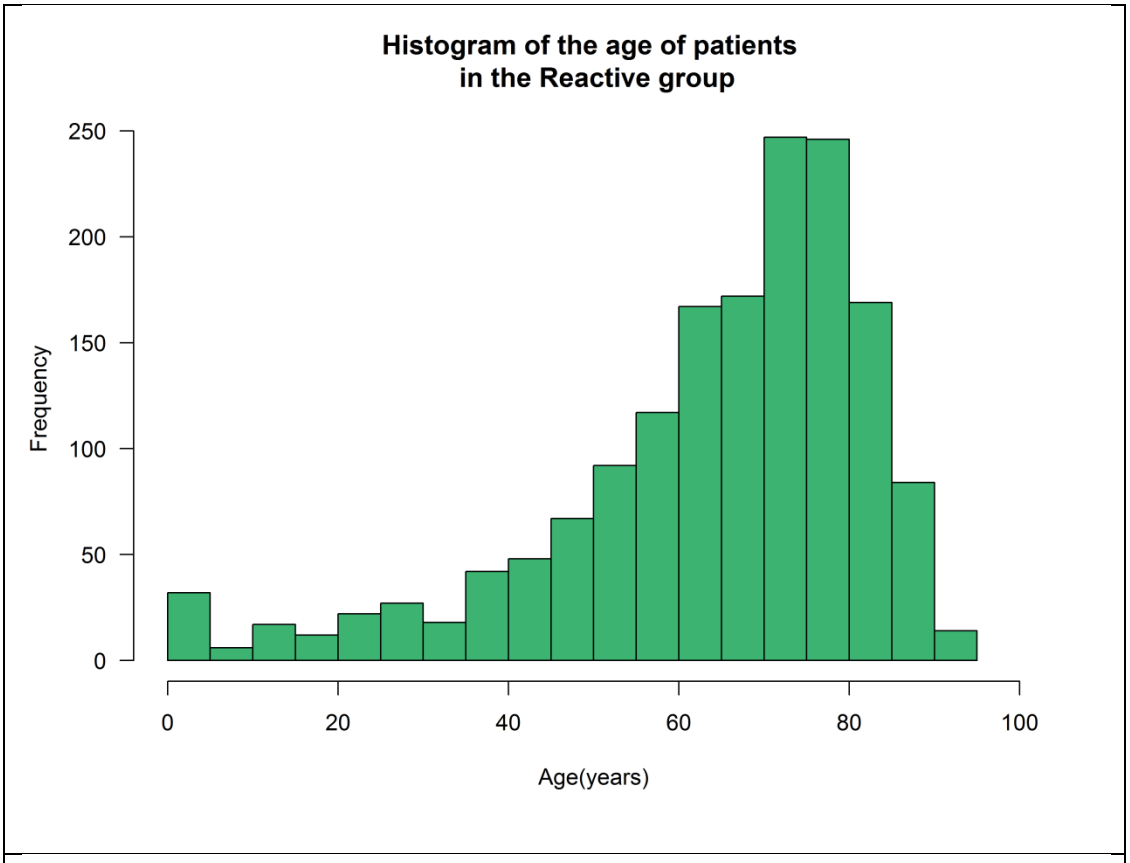
In total, 2710 cases were subject to primary exclusion and 36 to secondary exclusion. 2011 cases remained: 412 cases were diagnosed with MDS and 1599 cases were classified as the reactive group. The descriptive statistics for both the MDS and the reactive subgroups are shown in

	MDS	**Reactive**	***P* value**
Number of cases	412	1599	NA
Median Age	75.5	70.0	<0.001*
Number of females	136	743	NA
Median Age (female)	75.0	69.0	<0.001*
Number of males	276	845	NA
Median Age (male)	76.0	70.0	<0.001*
Male:Female ratio	2.03	1.14	<0.001**
Sex unknown (Median Age)	0	11 (63.0)	NA

Table 3.2. Descriptive statistics for age and sex of the MDS and Reactive groups. **p* value obtained from Wilcoxon signed ranks test. ***p* value obtained from χ^2 test of number of males and females in the MDS and reactive groups.

Patients with a Reactive diagnosis had a lower age. This was true both overall and for the specific sexes. There was no skew in the sex ratio, which is found in MDS. There was no difference between the intra-group ages of the males and females in the either the Reactive group or the MDS group (p = 0.190 and 0.935, respectively).

As can be seen from the histogram plots in Figure 3.2, and as indicated by the difference in median age between the groups, there was a skew towards younger cases in the Reactive group but not in the MDS group. In the MDS group, there was only one paediatric patient (male, 10 years old). The next youngest MDS patient was a 29 year old female, whilst in the reactive group, 89 patients were 25 years old or under.



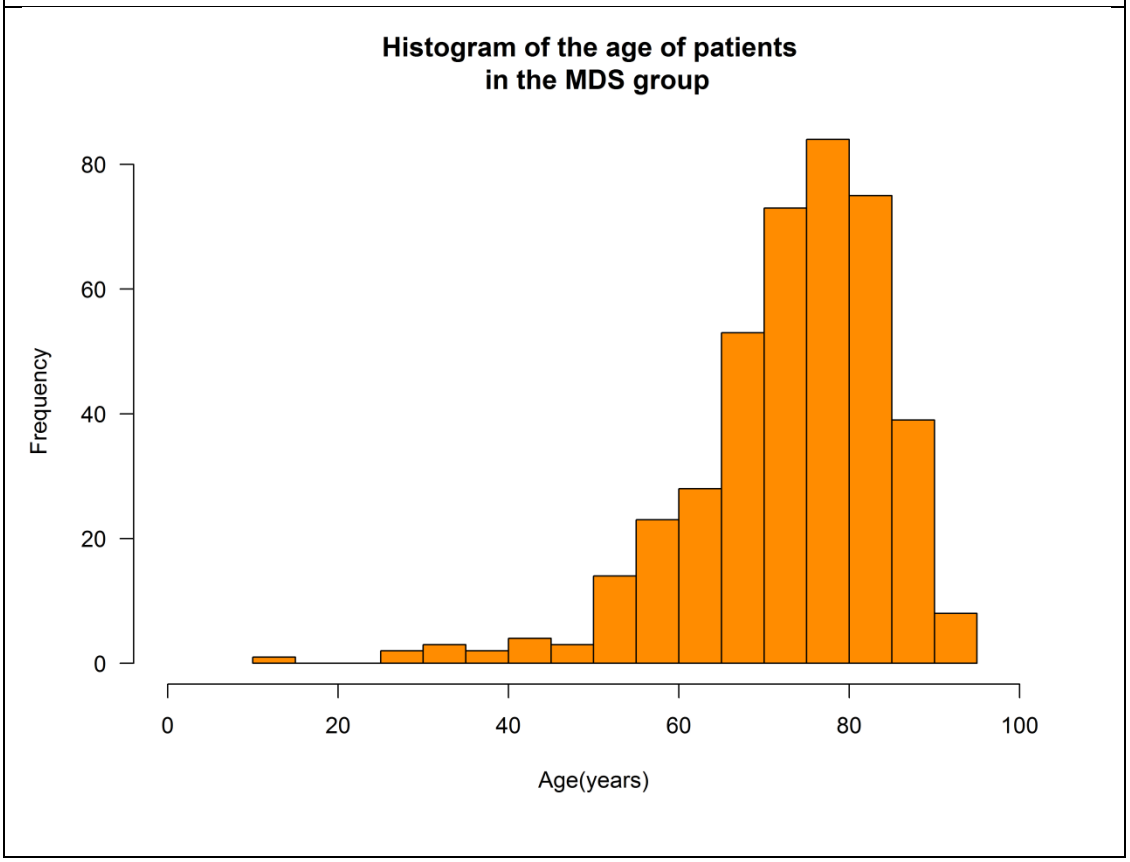


Figure 3.2. Histograms showing the age distribution for the Reactive and MDS groups

Paediatric MDS is a rare entity, with a reported incidence in under 15 year olds in England, Scotland and Wales of 1.35 per million (age standardised rate) (Passmore et al., 2003). Furthermore, a study has shown that nearly half of reviewable MDS cases in this age range were diagnosed with Juvenile CMML, which has easily distinguishable characteristics (Niemeyer et al., 1997; Emanuel, 2008). Therefore, there was a concern that the presence of this age range within the Reactive category may have an effect on the generation of any logistic regression model.

To determine whether the presence of paediatric cases could skew the percentage of CD34 positive cells in a logistic regression model, a scatter plot of CD34 percentage versus age was produced for the Reactive group Figure 3.3. It was noted that patients with a Reactive diagnosis under the age of 10 years had a significantly higher percentage of CD34 positive cells compared to those over the age of 10 years (p = <0.001). This difference can be seen in Figure 3.4.

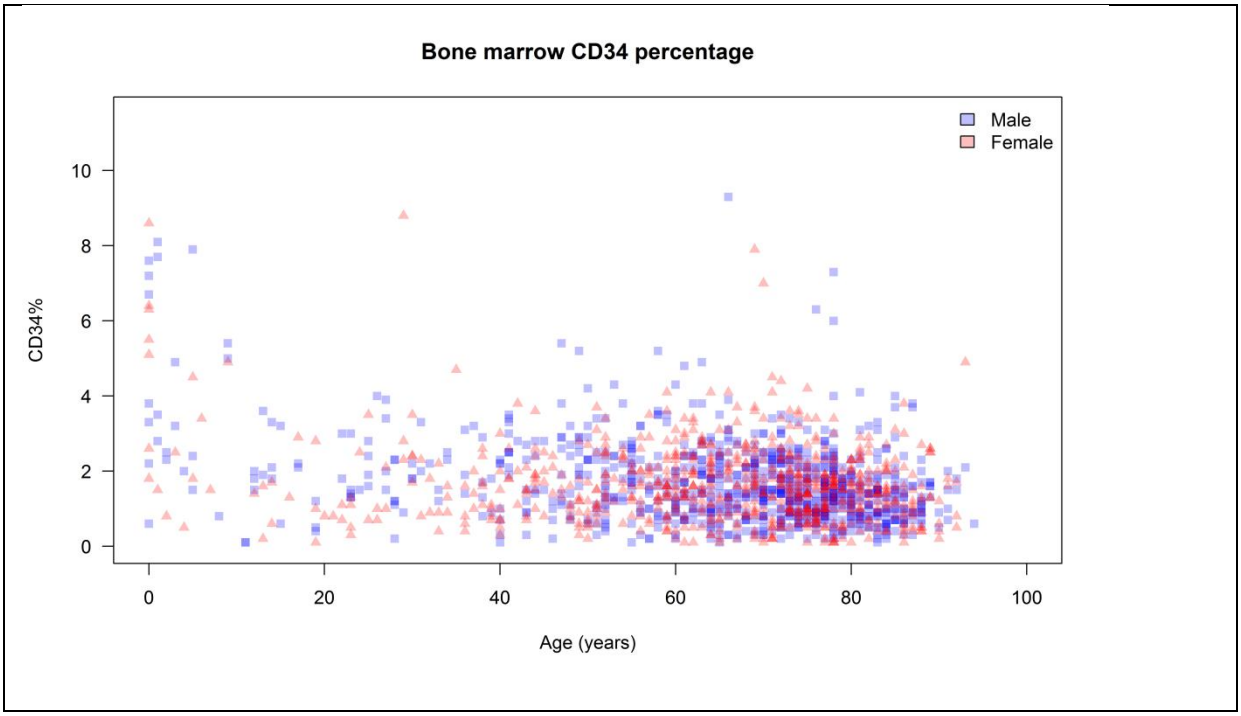


Figure 3.3. Scatter plot of age versus percentage of CD34 positive cells for the Reactive group and categorised by gender

Due to these findings, 38 patients under the age of 10 years old were excluded from further analysis. All patients were from the Reactive group. However, it is recognised that this cut-off of 10 years old is arbitrary and specific for this cohort. This removal of younger patients did not alter any significant difference between the ages of the Reactive and MDS groups as shown in the updated table of descriptive statistics (Table 3.3).

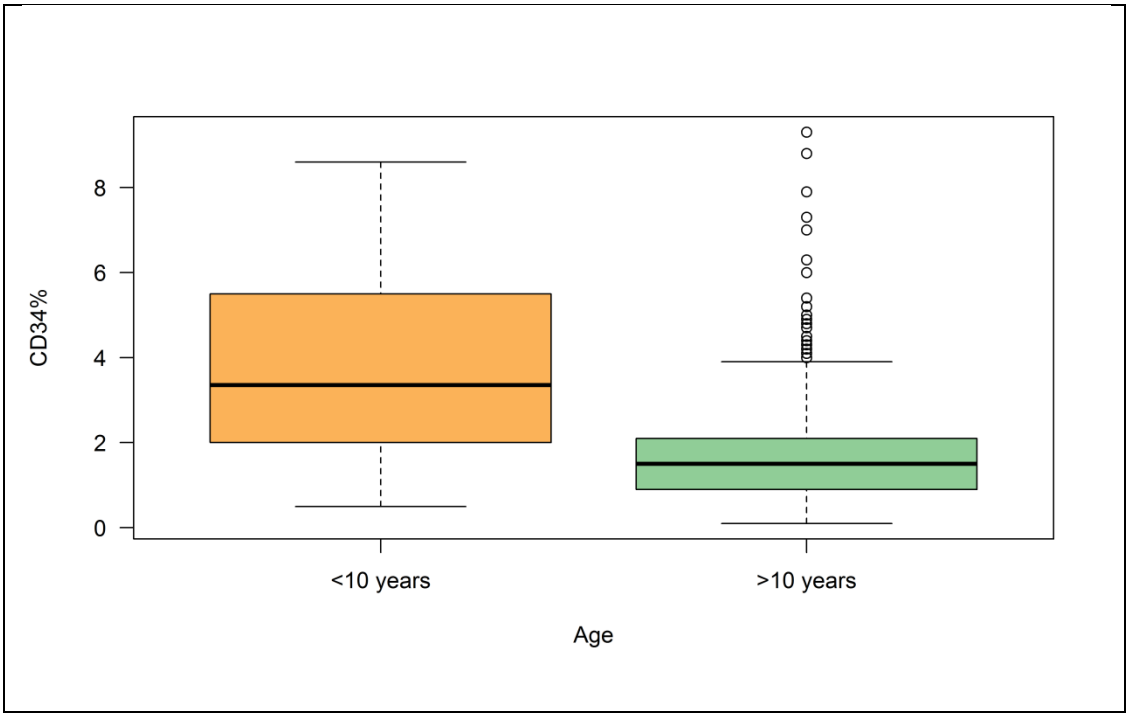


Figure 3.4. A box and whisker plot showing the percentage of CD34 positive cells for under- and over-10 year olds in the Reactive group.

	MDS	Reactive	*P* value
Number of cases	412	1561	NA
Median Age	75.5	70.0 (11-94)	<0.001*
Number of females	136	727	NA
Median Age (female)	75.0	69.0 (12-93)	<0.001*
Number of males	276	823	NA
Median Age (male)	76.0	71.0 (11-94)	<0.001*
Male:Female ratio	2.03	1.13	<0.001**
Sex unknown (Median Age)	0	11 (63.0)	NA

Table 3.3. Descriptive statistics for age and sex of the MDS and Reactive groups form patients over the age of 10 years.

*** *p* value obtained from Wilcoxon signed ranks test. ** *p* value obtained from χ^2 test of number of males and females in the MDS and reactive groups.**

3.5 Identifying biological differences between and within the MDS and Reactive Groups

Previous studies have noted an increase in the percentage of CD34 positive progenitors and a reduction in the B-progenitor population within the CD34 population in MDS patients in comparison to patients with a Reactive diagnosis (Sternberg et al., 2005; Ogata et al., 2006). These studies had smaller cohorts in comparison to this study and it was unknown whether differences would still be present in a larger dataset of unselected patients or whether differences were sex specific.

Descriptive statistics for CD34 positive cell attributes for the Reactive and the MDS groups are shown in Table 3.4. There was a difference between the Reactive and the MDS group for the percentage of CD34 positive progenitors (p=<0.001). This difference was found when inter-group male-to-male and female-to-female comparisons were performed (p=<0.001 for both).

The MDS group showed a significant higher percentage of cases with decreased B-progenitors compared to the Reactive group (p<0.001). This difference was not due to the skewed male to female ratio in the MDS subgroup as there was no difference between the proportions of males and of females who had decreased B-progenitors present in the MDS subgroup (p= 0.302). However, more males had decreased B-progenitors than females (p=0.030) within the Reactive group.

Within the Reactive group, the females had a lower median age (69 years versus 71 years for males), although this was not significant (p=0.190). Also, within the Reactive group, patients with decreased B progenitors were found to be significantly older than patients with B-progenitors present (p=<0.001).

3.6 Are there intra-subgroup differences within the Reactive group?

In the literature, the different Reactive subgroups are collected into one main umbrella group (Ogata et al., 2006; Chu et al., 2011; Della Porta et al., 2012). For example, the Ogata *et al.* used a broad range of control patients with the largest single subgroup being Idiopathic Thrombocytopenia Purpura (ITP) patients (Ogata et al., 2006). However, it was unknown whether there were differences in the male to female rate, age, and CD34 positive percentage and composition between different non-malignant cytopenic conditions. If so, the

Diagnosis	CD34%					B-progenitors Present		Decreased B-progenitors	
	Median	Mean	Min	Max	SD	Number of cases	Median Age (years)	Number of cases	Median Age (years)
MDS all	2.20	4.65	0.1	34	5.51	107	75.0	305	76.0
MDS ♂	2.30	4.36	0.1	29	5.02	76	75.0	200	76.0
MDS ♀	2.05	5.23	0.1	34	6.37	31	74.0	105	76.0
Reactive all	1.50	1.61	0.1	9.3	0.99	1079	67.0	482	74.0
Reactive ♂	1.50	1.59	0.1	9.3	1.00	550	69.0	273	74.0
Reactive ♀	1.50	1.62	0.1	8.8	0.99	523	66.0	204	75.0
Reactive (U)	1.50	1.95	0.7	5.0	1.26	6	54.0	5	76.0

Table 3.4. Percentage CD34 positive progenitors and B-progenitor cell status in the MDS and Reactive groups as determined by sex.

composite make-up of a Reactive control group could affect any differences between this group and the MDS group.

In this dataset, two large subgroups existed which could be evaluated: an Anaemia of Chronic Disease (ACD) group (n=343) and an ITP group (n=102). The descriptive statistics for these two subgroups are shown in Table 3.5 and Table 3.6. The ACD group was found to be significantly older (p=<0.001) and had a significantly lower male to female ratio (p=0.032). Despite these differences, there were no significant differences for the percentage of CD34 positive progenitors (p=0.162) or for the proportion with presence of B-progenitors (p=0.134) between the ACD and ITP groups.

3.7 Does the percentage of CD34 positive cells show age related changes in the Reactive and MDS patient groups?

Age-related changes are reported to occur in the haematopoietic stem cell (HSC) compartment in humans, including an increase in HSC's but a decrease in function (Geiger et al., 2013). Despite the CD34 positive compartment containing HSC's, a slight decrease in the percentage of CD34 positive cells across four age groups from 0 to 80 years has been reported in a series of 332 spinal cord injury patients (Dedeepiya et al., 2012). It is unknown whether these characteristics are present in the Reactive group and in MDS patients across the different age groups. To evaluate this, the percentage of CD34 positive progenitor cells for 10 year age ranges were examined in both groups.

Figure 3.5 shows the box and whisker plots for the percentages of CD34 positive cells across the different age ranges in the Reactive and the MDS groups. In both groups, there is a slight decrease in the percentage of CD34 positive progenitors with age. In the Reactive group this reduction appears to occur continuously from the 41-50 age range until the 81-90 age range. For the MDS group, due to the limited number of patients there can be no comment regarding patients below 50 years of age. However, there does not appear to be a downward trend. As it was feasible that the presence of RAEB cases may skew the percentage of CD34 positive cells, these patients were further excluded and the box and whisker plots were re-plotted as shown in **Figure 3.6**. However, even with this subgroup excluded, there does not appear to be an upward or downward trend for MDS patients with age.

Diagnosis	Age					B-progenitors Present		Decreased B-progenitors	
	Median	Mean	Min	Max	SD	Number of cases	Median Age (years)	Number of cases	Median Age (years)
ACD all	76.0	73.9	33.0	94.0	10.1	225	76.0	118	76.5
ACD ♂	77.0	75.0	41.0	94.0	9.0	100	77.0	66	74.5
ACD ♀	75.0	73.0	33.0	93.0	11.0	124	74.0	51	78.0
ACD (U)	69.5	69.5	63.0	76.0	9.2	1	63.0	1	76.0
ITP all	69.5	64.4	12.0	91.0	17.3	75	67.0	27	75.0
ITP ♂	72.0	65.9	12.0	91.0	18.3	45	70.0	17	77.0
ITP ♀	64.0	62.0	19.0	87.0	15.6	30	64.0	10	63.5
ITP (U)	NA	NA	NA	NA	NA	0	NA	0	NA

Table 3.5. Descriptive statistics for age for the ACD and ITP subgroups within the Reactive group.

Diagnosis	CD34%					B-progenitors Present		Decreased B-progenitors	
	Median	Mean	Min	Max	SD	Number of cases	CD34% median	Number of cases	CD34% median
ACD all	1.50	1.54	0.10	4.90	0.73	225	1.60	118	1.30
ACD ♂	1.40	1.45	0.10	4.00	0.66	100	1.50	66	1.30
ACD ♀	1.50	1.63	0.10	4.90	0.78	124	1.70	51	1.20
ACD (U)	1.75	1.75	1.50	2.00	0.35	1	1.50	1	2.00
ITP all	1.65	1.67	0.30	3.80	0.81	75	1.90	27	1.10
ITP ♂	1.80	1.71	0.30	3.80	0.84	45	1.90	17	1.10
ITP ♀	1.60	1.61	0.30	3.00	0.77	30	1.70	10	1.30
ITP (U)	NA	NA	NA	NA	NA	0	NA	0	NA

Table 3.6. Descriptive statistics for percentage of CD34 positive cells for ACD and ITP subgroups within the Reactive group.

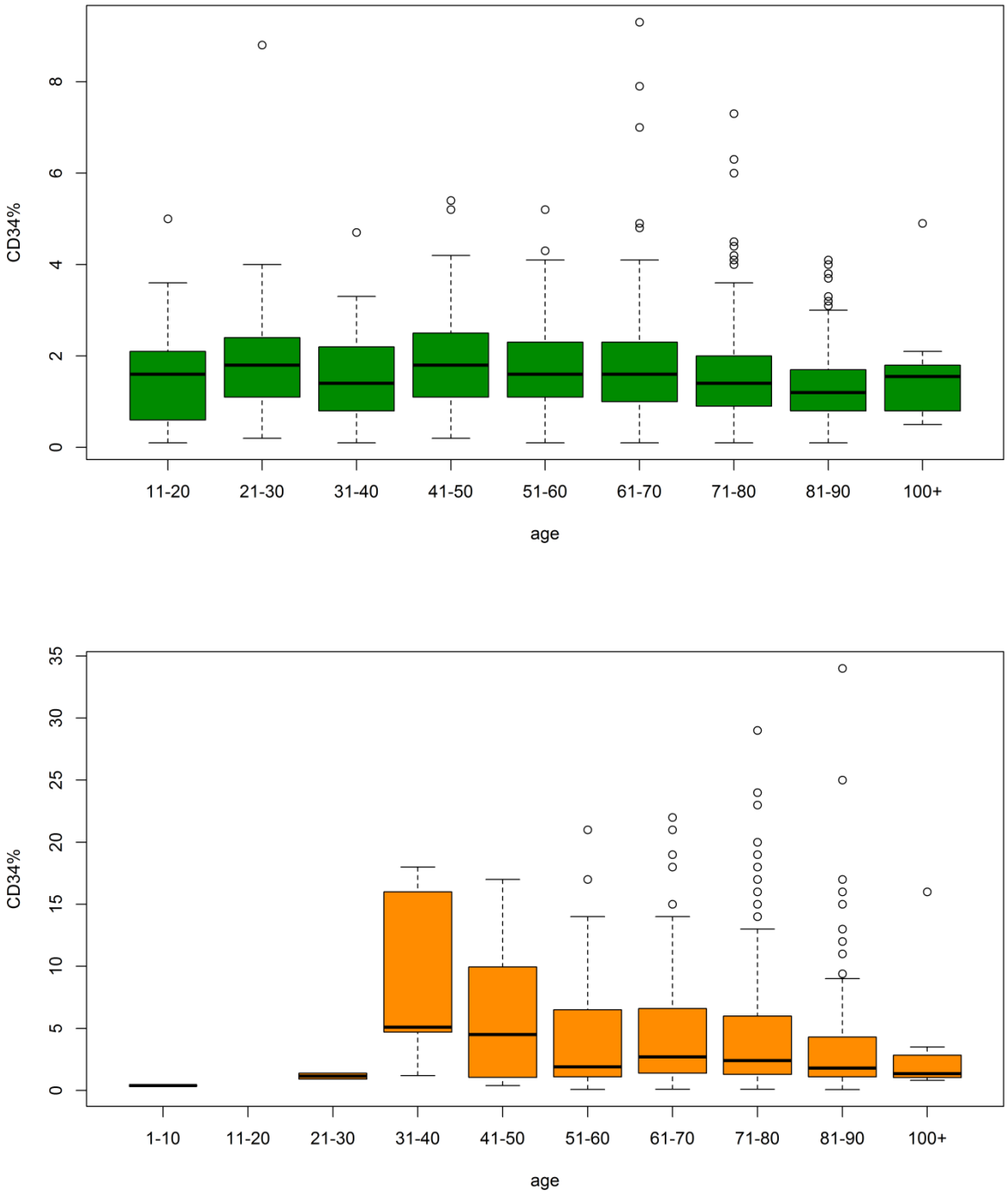


Figure 3.5. Box and whisker plots showing the percentage of CD34 positive cells per 10 year age range for the Reactive and the MDS groups.

The top plot with the green box and whisker plots shows the Reactive group whilst the bottom, orange, box and whisker plots shows the MDS group.

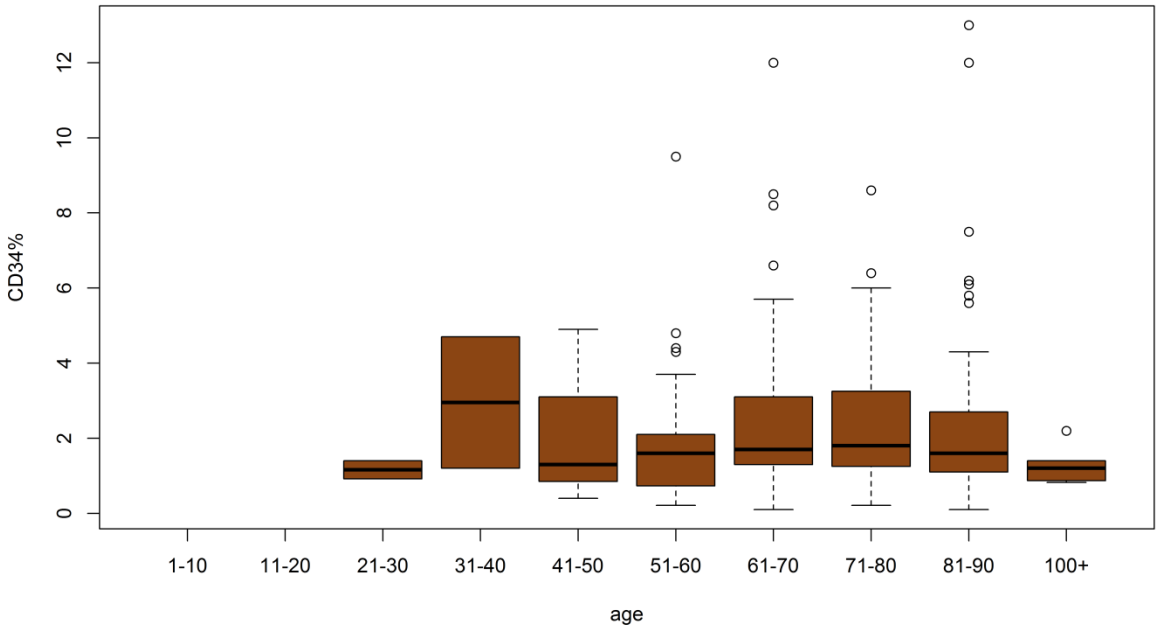


Figure 3.6. Box and whisker plots showing the percentage of CD34 positive cells per 10 year age range for the MDS group following exclusion of RAEB cases.

3.8 Is the decreased B-progenitor phenomenon a consequence of age-related changes?

The different age range did not affect the percentage of CD34 positive progenitors between the ACD and ITP subgroups, nor did it affect the incidence of decreased B-progenitors. For the Reactive group as a whole, there was a difference between the ages of patients with B-progenitors present and those with decreased B-progenitors. This difference was not found within the MDS group.

Studies have shown that a decrease in B-progenitors occurs with age and this may be as consequence of alterations in frequency of populations within the stem cell compartment (McKenna et al., 2001; Kuranda et al., 2011). As patients within the MDS group had an older median age, it was unclear whether the increased number of patients with decreased B-progenitors within the MDS group was a result of aging or the underlying biology of the disease.

To investigate the possibility that the decrease in B-progenitors was an age-related phenomenon, patients in both the Reactive group and in the MDS group, were grouped into

10 year age bins and the percentage of patients with decreased B-progenitors was evaluated for each age bin.

In the Reactive group there was an increase of percentage of patients with decreased B-progenitors as age increased. This trend is shown in the Figure 3.7 and Table 3.7. This trend was not seen for the MDS group, with the majority of patients with MDS having decreased B-progenitors throughout all age ranges. Taken together, these data suggest that, although decreased B-progenitors do appear to be a feature of aging, the decreased B-progenitor phenomenon in MDS is not an age-related consequence.

Age Range	B-progenitors Present (no. of cases)		Decreased B-progenitors (no. of cases)		% of cases with Decreased B-progenitors	
	Reactive	MDS	Reactive	MDS	Reactive	MDS
10-19	27	0	1	1	3.6	100.0
20-29	39	0	4	1	9.3	100.0
30-39	42	2	10	4	19.2	66.7
40-49	90	1	23	5	20.4	83.3
50-59	152	13	41	19	21.2	59.4
60-69	243	18	89	57	26.8	76.0
70-79	325	38	168	116	34.1	75.3
80-89	149	34	138	92	48.1	73.0
90-99	12	1	8	10	40.0	90.9
Total	**1079**	**107**	**482**	**305**	**30.9**	**74.0**

Table 3.7. Age distribution and proportion of cases with present or decreased B-progenitors in the Reactive and the MDS groups.

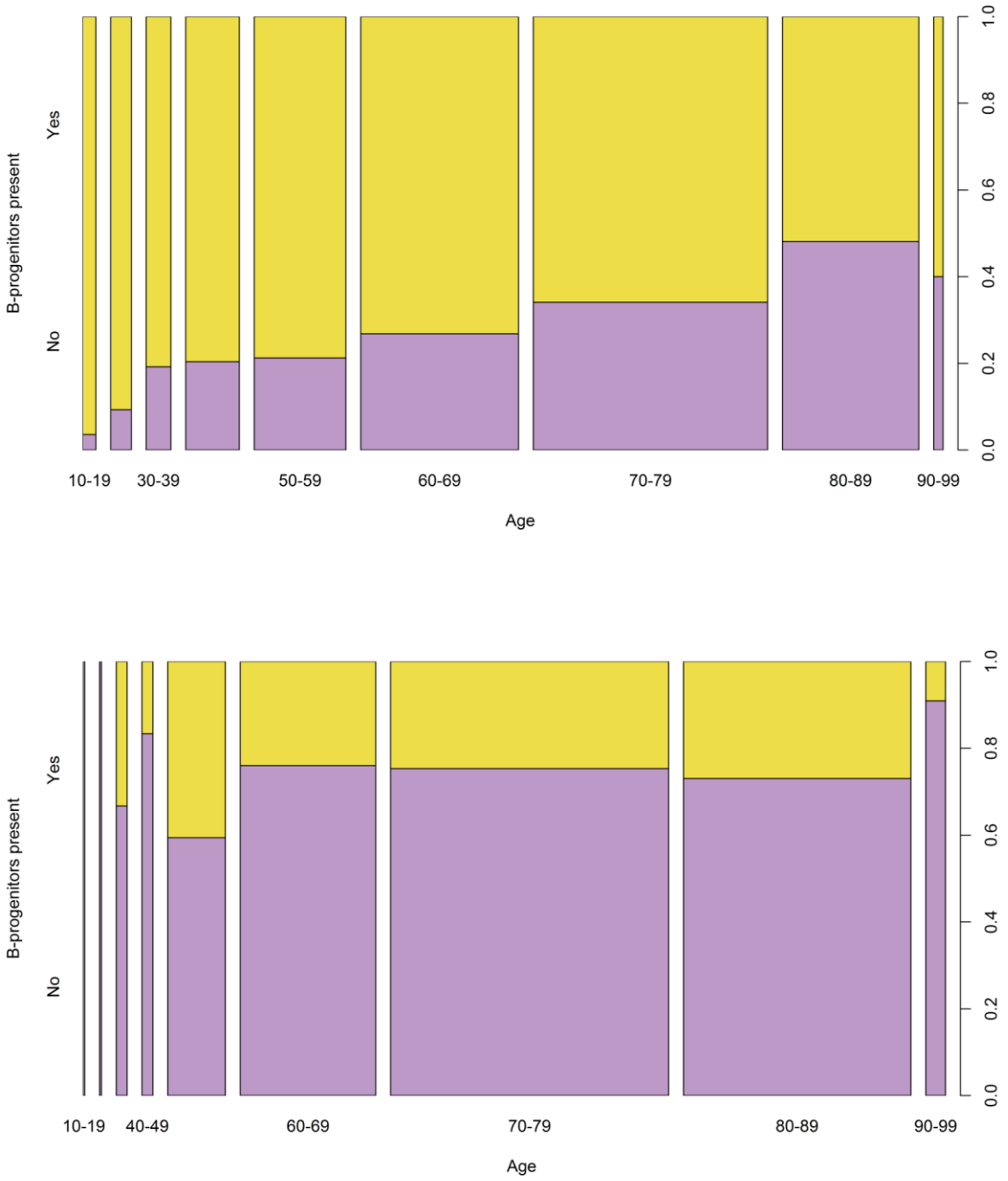


Figure 3.7. Spineplots showing the distribution and proportion of cases with present or decreased B-progenitors with age.

The Reactive group is depicted in the top plot and the MDS group in the bottom plot. Yellow indicates the proportion (on a scale of 0 to 1) of cases within that age bin which have B-progenitors present whilst purple indicates the inverse proportion with decreased B-progenitors. The width of the age bins is proportional to the number of patients within that bin.

3.9 Is the decreased B-progenitor proportion a consequence of an increased CD34 percentage?

To determine whether the proportion of patients with decreased B-progenitors within the CD34 positive compartment decreased with increasing CD34 percentage, and whether this was constant for both the Reactive group and the MDS group, patients were grouped into increasing one percent CD34 positive bins and the proportion of cases with decreased B-progenitors was evaluated for each bin.

In the Reactive group, there was an increase in the proportion of patients with B-progenitors present, as seen in Table 3.8. This feature occurred for increasing percentages of CD34 positive cells up to 4% and implies that there is a linear relationship with an increase of B-progenitors with increasing CD34 positive cells. This trend did not, however, continue for the patients with >4% CD34 positive cells, although this may have been a consequence of the small sample size with only 11 cases in this group. Statistically, there was a significant difference between the median value for CD34 positive percentage between the group with B-progenitors present and the group with decreased B-progenitors (1.6% versus 1.2%, p=<0.001).

The MDS group showed an opposite trend to the Reactive group, as shown in Table 3.9 and Figure 3.8. For this group, each CD34 positive percentage range contained at least 60% of patients with decreased B-progenitors and this percentage increased with increasing CD34 percentage. In contrast to the Reactive group, the median CD34 positive percentage for cases with B-progenitors present was significantly lower compared to cases with decreased B-progenitors (1.70% versus 2.70%, p=<0.001).

CD34+ Range (%)	B-progenitors Present (no. of cases)	Decreased B-progenitors (no. of cases)	% of cases with Decreased B-progenitors
0.1-1.0	471	208	30.6
1.1-2.0	284	71	20
2.1-3.0	74	16	17.8
3.1-4.0	15	3	16.7
4.1+	6	5	45.5

Table 3.8. B-progenitor status for the different percentage CD34 positive cell bins for Reactive patients

CD34+ Range (%)	B-progenitors Present (no. of cases)	Decreased B-progenitors (no of cases)	% of cases with Decreased B-progenitors
0.1-1.0	38	73	65.8
1.1-2.0	18	30	62.5
2.1-3.0	8	25	75.8
3.1-4.0	6	17	73.9
4.1-5.0	1	17	94.4
5.1-6.0	4	16	80
6.1-7.0	1	8	88.9
7.1-8.0	0	7	100
8.1-9.0	1	5	83.3
9.1-10	0	3	100
10+	6	51	89.5

Table 3.9. B-progenitor status for the different percentage CD34 positive cell bins for MDS patients

Inter-group comparison showed that there was a significant difference between the median CD34 positive percentage between the Reactive group with decreased B-progenitors and the MDS group with decreased B-progenitors (p=<0.001). However, no difference was seen between the Reactive group with B-progenitors present and the MDS group with B-progenitors present (p=<0.087) which may have implications for scoring schemes based on these two attributes.

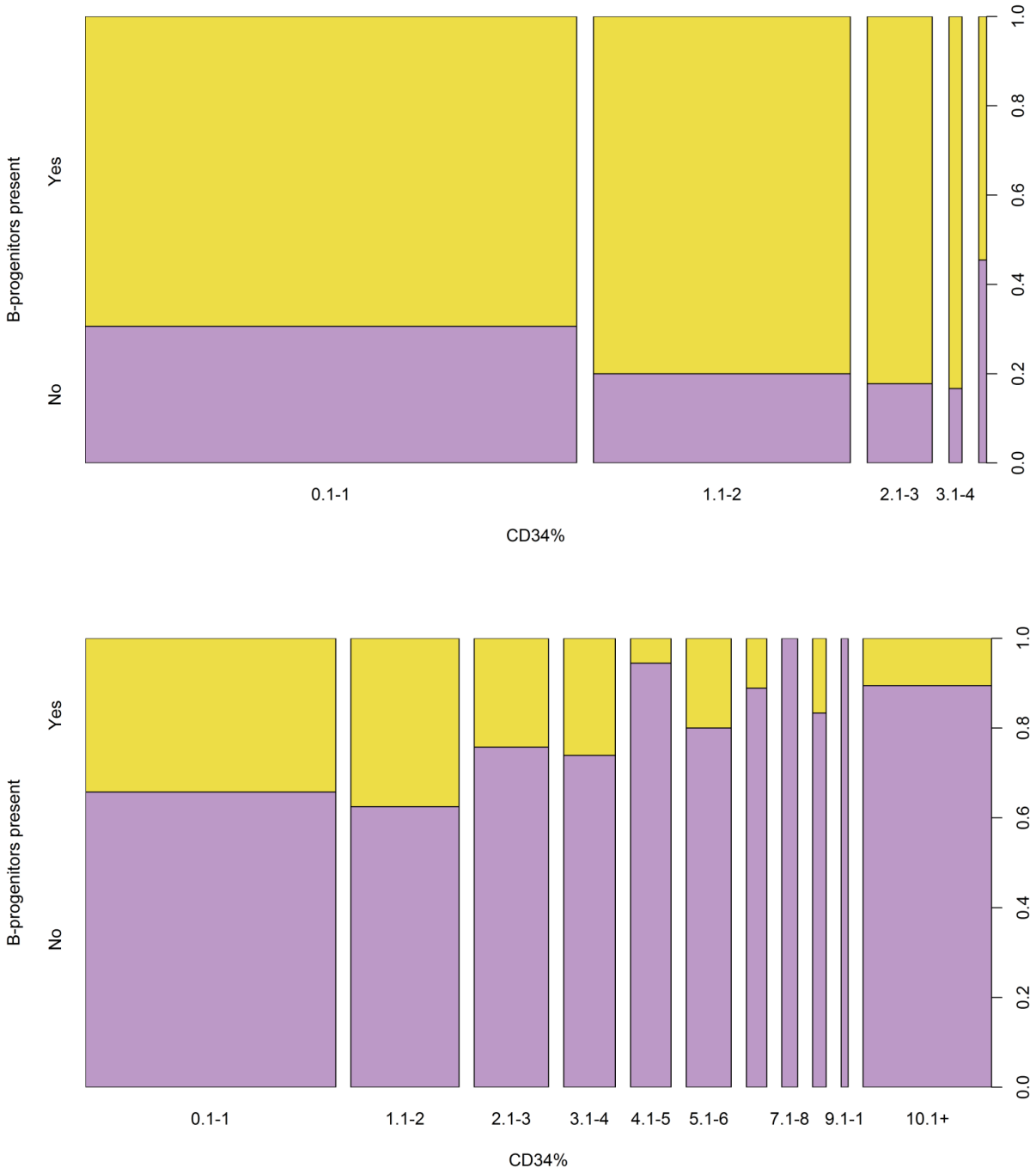


Figure 3.8. Spineplots showing the distribution and proportion of cases with present or decreased B-progenitors with percentage of CD34 positive cells.

The Reactive group is depicted in the top plot and the MDS group in the bottom plot. Yellow indicates the proportion (on a scale of 0 to 1) of cases within that age bin which have B-progenitors present whilst purple indicates the inverse proportion with decreased B-progenitors. The width of the age bins is proportional to the number of patients within that bin.

3.10 Creation of a Logistic Regression Model

As all the attributes age, sex, percentage of CD34 positive cells and B-progenitors status all differed between the MDS and the Reactive group, and as age and percentage of CD34 positive cells are continuous variables, a logistic regression model was used to produce a probability model based on all these attributes.

This data series was used as a training set to produce the model. To simplify the model, the cases in whom the gender could not be determined (n=11) were excluded from analysis. The model was therefore constructed using 1550 Reactive patients and 412 MDS patients. The baseline accuracy was calculated on the basis of every case belonging to the largest class which, in the training set, was the Reactive group and was 0.7900. The performance metrics (accuracy, Kappa statistic, sensitivity, specificity, positive predictive value, negative predictive value) were all reported using the MDS group as the “Positive” Class. Class prediction was on the basis of having a probability of >0.5 for class membership for class membership of the MDS group and <0.5 for the Reactive group.

Table 3.10 shows the confusion matrix for predicted class versus actual class and Table 3.11 shows the model results for all attributes. All 4 attributes were statistically significant (all p<0.001) with a decrease in B-progenitors showing the largest odds ratio.

		Predicted Class	
		MDS	**Reactive**
Actual Class	**MDS**	160	252
	Reactive	24	1526

Table 3.10. Confusion matrix showing the outcomes of the logistic regression model.

The variance inflation factor (VIF) for age, sex, percentage of CD34 positive cells and B-progenitor status was 1.055, 1.000, 1.037, and 1.033 respectively. The square root of the VIF (sqrtVIF) was 1.027, 1.000, 1.018, and 1.017 respectively. The VIF indicates evidence of multicollinearity and if the value is not unusually larger than 1.0, as found here, then multicollinearity does not pose a problem (Mansfield and Helms, 1982). The sqrtVIF indicates the standard error for the attribute, for example, the largest sqrtVIF is for age and is 1.027, indicating that the standard error for this attribute is 2.7% higher were it uncorrelated with any other attribute.

			Confidence intervals for parameter estimate			Confidence intervals for Standard error	
	Parameter Estimate	Standard Error	2.5%	97.5%	Odds Ratio	2.5%	97.5%
Intercept	-6.168451	0.452953	-7.08564889	-5.3091744	0.002094478	0.0008370315	0.004946009
Age	0.036263	0.005707	0.02534317	0.0477265	1.036929041	1.0256670346	1.048883747
Sex (Male)	0.541354	0.141315	0.26663068	0.8210754	1.718383481	1.3055581876	2.272942838
CD34	0.522281	0.046539	0.43470005	0.6170333	1.685868636	1.5444997123	1.853421385
B-progenitors (decreased)	1.511978	0.140263	1.23985912	1.7901585	4.535691971	3.4551266670	5.990401837

Table 3.11. Results of the contributions of the individual attributes to the logistic regression model.

The *c* index, also known as the area under the ROC curve (AUROC) was 0.824 indicating the predictive accuracy of the model (Austin and Steyerberg, 2012). The accuracy itself of the model was 0.8593, compared to the baseline of 0.7900. The Kappa statistic, which takes into account a correct prediction by chance, was 0.4679. Sensitivity was 0.3883, specificity was 0.9850, the positive predictive value was 0.8696, and the negative predictive value was 0.8621.

As MDS is a heterogeneous group comprising multiple diagnostic categories, the model prediction outcome for actual cases within each subgroup of MDS was determined. This would ascertain whether the model predicted specific subgroups better than others. The results of this are shown in Table 3.12. With the exception of the RAEB and RAEB-F WHO subgroups, the model fails to correctly predict more than 30% of cases within any other WHO subgroup.

	5q-	CMML	MDS/MPN-U	RAEB	RAEB-F	RARS	RARS-T	RCMD	RCUD
Correct Prediction (as MDS)	1	3	2	96	4	6	0	48	0
Incorrect Prediction (as Reactive)	6	30	6	19	0	68	4	117	2
Predicted correctly (%)	14	9	25	83	100	8	0	29	0

Table 3.12. Model prediction accuracy for the different MDS subgroups contained within the logistic regression training set.

3.11 Testing the performance of the logistic regression model on an independent dataset

The logistic regression model produced a probability of an individual patient having MDS on the basis of age, gender, CD34% and presence of B-progenitors >5%. This model could be then used to give a probability value for MDS on unseen, individual patient results via the input of results into the model. For example, an 86 year old male patient with a CD34 of 4.2% and decreased B-progenitors would have a probability of 0.753 (75.3%) of belonging to the MDS class. Likewise, a 35 year old female with a CD34% of 1.5% and B-progenitors >5% would have a probability of 0.016 (1.6%) of belonging to the MDS class. However, to determine the actual performance of the model, an independent patient cohort was required

and a dataset comprising of patients whose samples had been subject to the SCS for the investigation of cytopenia between September 2010 and April 2013 was used.

The same exclusion criteria as those applied to the training set were applied to the test set. 1753 cases were initially identifying as either Reactive (n=1606) or MDS (n=147). 26 patients from the Reactive group subsequently presented with either MDS or AML by June 2015 and were excluded from further analysis, but were retained to be added to the original transformed cases. 57 cases were excluded due to age being less than 10 years old, all from the Reactive group. 11 patients were of unknown gender and were excluded, all from the Reactive group. The descriptive statistics for this test set are shown in Table 3.13.

	MDS	**Reactive**	***P* value**
Number of cases	147	1512	NA
Median Age	76.0	70.0	<0.001*
Number of females	61	696	NA
Median Age (female)	76.0	68.0	<0.001*
Number of males	86	816	NA
Median Age (male)	76.0	71.0	<0.001*
Male:Female ratio	1.41	1.18	0.2912**

Table 3.13. Descriptive statistics for age and sex of the MDS and Reactive groups of the independent test set.

***p* value obtained from Wilcoxon signed ranks test. ****p* value obtained from χ^2 test of number of males and females in the MDS and Reactive groups.**

Like the training set, the test set was skewed towards the inclusion of Reactive cases and this resulted in a baseline accuracy of 0.9114. Unlike both the training set and published data, in this set there was no significant difference in the male-to-female rate ratio in the MDS group.

Application of the model derived from the training set on the test set resulted in a probability that the individual case belonged to the MDS class. The performance metrics (accuracy, Kappa statistic, sensitivity, specificity, positive predictive value, negative predictive value) were all reported using the MDS group as the “Positive” Class. The logistic regression model calculates a probability for each patient. Therefore, although the usual probability for class membership is 0.5, the performance metrics could be determined using a probability range from 0.1 to 0.9 in incremental cut-offs of 0.1 for class membership of the MDS class. The results for the range of different probabilities are shown in Table 3.14.

Probability of MDS	Reactive classed as Reactive (no. of cases)	Reactive classed as MDS (no. of cases)	MDS classed as Reactive (no. of cases)	MDS classed as MDS (no. of cases)	Accuracy	Kappa statistic	Sensitivity	Specificity	Positive predictive value	Negative predictive value
0.1	765	747	22	125	0.5365	0.1104	0.8503	0.5060	0.1433	0.9720
0.2	1120	392	47	100	0.7354	0.2044	0.6803	0.7407	0.2033	0.9597
0.3	1309	203	75	72	0.8324	0.2552	0.4898	0.8657	0.2618	0.9458
0.4	1432	80	100	47	0.8915	0.2843	0.3197	0.9471	0.3701	0.9347
0.5	1486	26	116	31	0.9144	0.2677	0.2109	0.9828	0.5439	0.9276
0.6	1500	12	127	20	0.9162	0.1981	0.1361	0.9921	0.6250	0.9219
0.7	1506	6	133	14	0.9162	0.1496	0.0952	0.9960	0.7000	0.9189
0.8	1510	2	137	10	0.9162	0.1139	0.0680	0.9987	0.8333	0.9168
0.9	1511	1	140	7	0.9150	0.0819	0.0476	0.9993	0.8750	0.9152

Table 3.14. Performance metrics for the different probabilities of class membership for the MDS class for the test set.

Using the 0.5 probability for class membership resulted in the correct classification for 98.3% Reactive cases. However, only 21.1% of MDS cases were correctly classified. If the cut-off for class membership of the MDS class was set low at 0.1, then 85.0% of MDS cases would be correctly classified as MDS. However, at this threshold only 50.6% of Reactive cases were correctly classified (specificity = 0.5060). Conversely, if the probability for class membership of the MDS class was set high at 0.9, only 4.8% of MDS cases would be correctly classified as MDS. However, this threshold would result in the correct classification of 99.9% of Reactive cases, with only one case misclassified. This single case was a 86 year old male with metastatic carcinoma, a CD34 of 6.3% and <5% B-progenitors whose probability of MDS was 0.9084.

3.12 Testing the performance of the logistic regression model on patients who developed myeloid malignancy

Patients from both the training set and the test set were excluded on the basis of the subsequent development of a myeloid malignancy, MDS or otherwise. To determine whether these patients could have been identified as being at risk of development, the logistic regression model was applied to this cohort.

Overall, 62 patients (36 patients from the training set and 26 patients from the test set) were excluded from initial training or test set analysis and available for analysis as a separate cohort. In this cohort, there were 26 females and 36 males, the median age was 71.0 years (range 38.0-87.0), the median percentage of CD34 positive cells was 1.70% (range 0.2-8.1%), and 40 out of the 62 patients had reduced B-progenitors. The diagnostic breakdown of this group was as follows: 2 patients with MDS with 5q- as a sole abnormality, 19 patients with AML, 1 patient with blastic plasmacytoid dendritic cell neoplasm, 1 patient with chronic myeloproliferative neoplasma with myelofibrosis, 1 patient with MDS/MPN-U, 13 patients with RAEB, 1 patient with RARS, and 24 patients with RCMD.

The classification of these patients according to different probabilities of MDS class membership is shown in Table 3.15. Using a probability of 0.5 for inclusion into the MDS class resulted in only 9.7% of patients being classified as MDS in their non-diagnostic sample. However, with the exception of the very high probabilities for membership of the MDS class (0.8 and 0.9), there appeared to be a greater percentage classified as MDS at every probability level than the percentage of Reactive cases misclassified as MDS in the test set.

Probability of MDS	No of cases classified as Reactive	No of cases classified as MDS	% classified as MDS	% of Reactive cases classified as MDS in the test set
0.1	11	51	82.3%	49.4%
0.2	29	33	53.2%	25.9%
0.3	39	23	37.1%	13.4%
0.4	48	14	22.5%	5.3%
0.5	56	6	9.7%	1.7%
0.6	59	3	4.8%	0.8%
0.7	61	1	1.6%	0.4%
0.8	62	0	0%	0.1%
0.9	62	0	0%	0.1%

Table 3.15. Percentage of cases classified as MDS per probability cut-off according to the logistic regression model in the cohort of Reactive diagnosis patients who developed a myeloid malignancy

3.13 Evaluation of demographic and biological features on the combined cohort of training and test set MDS patients

As the predictive model showed better prediction for certain subgroups of MDS (Table 3.12 above), MDS patients from the training (412) and test (147) sets were combined to form one cohort which could be examined for biological and demographic differences between the MDS subgroups. Box and whisker plots showing the age distribution per MDS subgroup for all patients is shown in Figure 3.9 and sex-specific distribution is shown in Figure 3.10. A tabulated version for age and gender of patients per MDS diagnostic group is shown in Table 3.16.

3.13.1 Are there differences in age between the MDS subgroups?

To determine whether there were differences between ages for the different MDS subgroups, a pairwise Wilcoxon rank sum test with Bonferroni correction applied to account for multiple comparisons was performed. The only difference was between the RCMD and RAEB groups with RAEB patients having a lower age (Table 3.17).

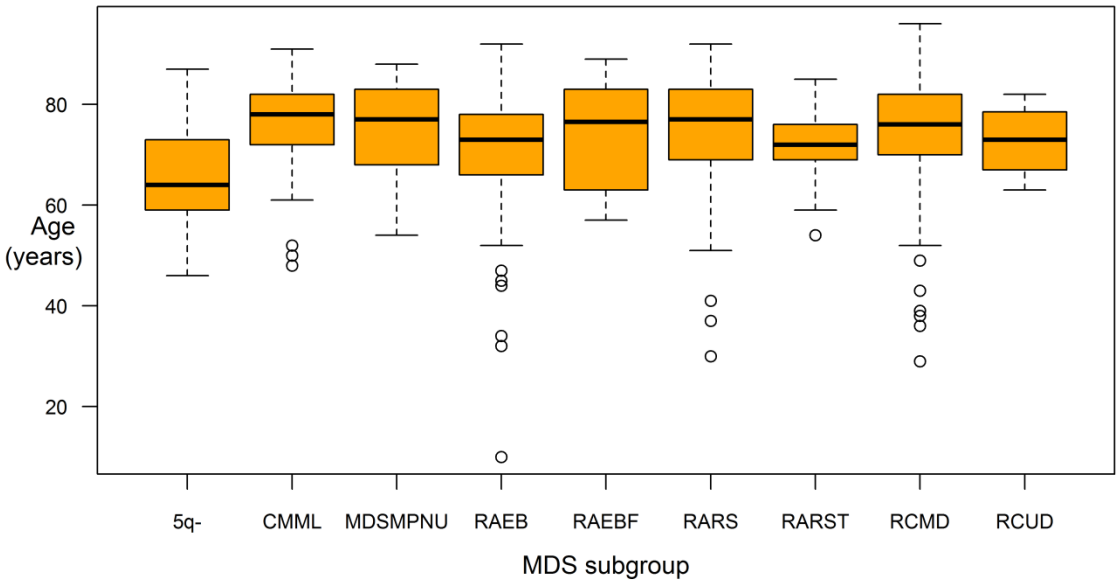


Figure 3.9. Box and whisker plots showing the age distributions within the different MDS subgroups

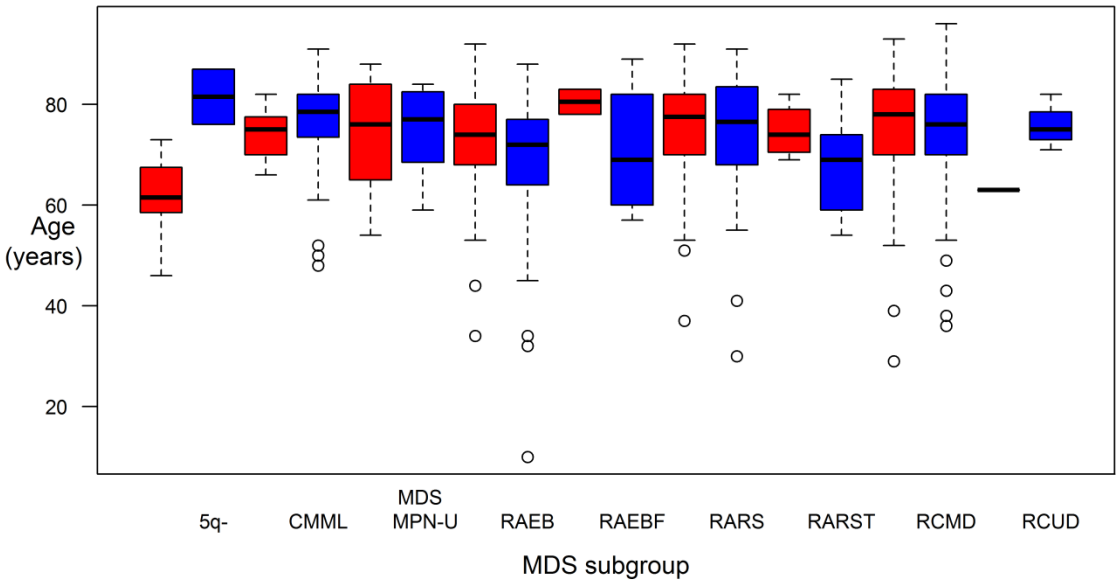


Figure 3.10. Box and whisker plots showing the age distributions by sex within the different MDS subgroups.

Males are shown in blue and females are shown red.

Subgroup	No of patients	Median Age (range)	No of Females	Median Age F (range)	No of Males	Median Age M (range)	M:F Ratio
5q-	10	64 (46-87)	8	61.5 (46-73)	2	81.5 (76-73)	0.25:1
CMML	36	78 (48-91)	8	75 (66-82)	28	78.5 (48-91)	3.5:1
MDSMPNU	21	77 (54-88)	9	76 (54-88)	12	77 (59-84)	1.33:1
RAEB	127	73 (10-92)	49	74 (34-92)	78	72 (10-88)	1.59:1
RAEBF	6	76.5 (57-89)	2	80.5 (78-83)	4	71 (57-89)	2:1
RARS	106	77(30-92)	42	77.5 (37-92)	64	76.5 (30-91)	1.52:1
RARST	9	72(54-85)	4	74 (69-82)	5	69 (54-85)	1.25:1
RCMD	240	76 (29-96)	74	78 (29-93)	166	76 (36-96)	2.24:1
RCUD	4	73 (63-82)	1	63	3	75 (71-82)	3:1
All cases	**559**	**76 (10-96)**	**197**	**75(29-93)**	**359**	**76(10-96)**	**1.82:1**

Table 3.16. Demographic results for MDS patients per diagnostic subgroup from the combined training and test set data

	5q-	CMML	MDS MPN-U	RAEB	RAEBF	RARS	RARST	RCMD
CMML	0.298							
MDS MPN-U	1.000	1.000						
RAEB	1.000	0.274	1.000					
RAEBF	1.000	1.000	1.000	1.000				
RARS	0.388	1.000	1.000	0.294	1.000			
RARST	1.000	1.000	1.000	1.000	1.000	1.000		
RCMD	0.260	1.000	1.000	**0.012**	1.000	1.000	1.000	
RCUD	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table 3.17. *P* values for pairwise comparisons for evaluation of differences in age per MDS subgroup.

Wilcoxon rank sum test with Bonferroni correction for multiple comparisons was performed. Significant differences are highlighted in red

3.13.2 Evaluation of CD34 positive cells in the different MDS subgroups

In the classification and prognostication of MDS, morphological blast cell percentage cut-offs are used to denote membership of the different WHO MDS subgroups, and are used as a component of the IPSS-R. As some of these cut-offs appeared arbitrarily defined, it was unclear whether there was have an underlying biological basis with respect to the percentage of progenitors. It was, therefore, of interest to determine whether the percentage of CD34 positive cells in MDS was normally distributed with overlaps between MDS subgroups, or whether distinct cut-offs occurred which correlated with diagnostic subgroups.

To determine whether the percentage of CD34 positive cells for all cases of MDS showed a normal distribution, a Quantile-Quantile plot (Q-Q plot) was produced which shows a non-normal distribution (Figure 3.11). The skew in distribution can also be seen in the male and female histogram plots in Figure 3.12. These histograms also show that there are no obvious cut-offs at or around the 2% and the 5% levels which would correspond to the identification of different IPSS-R prognostic entities or WHO diagnostic subgroups.

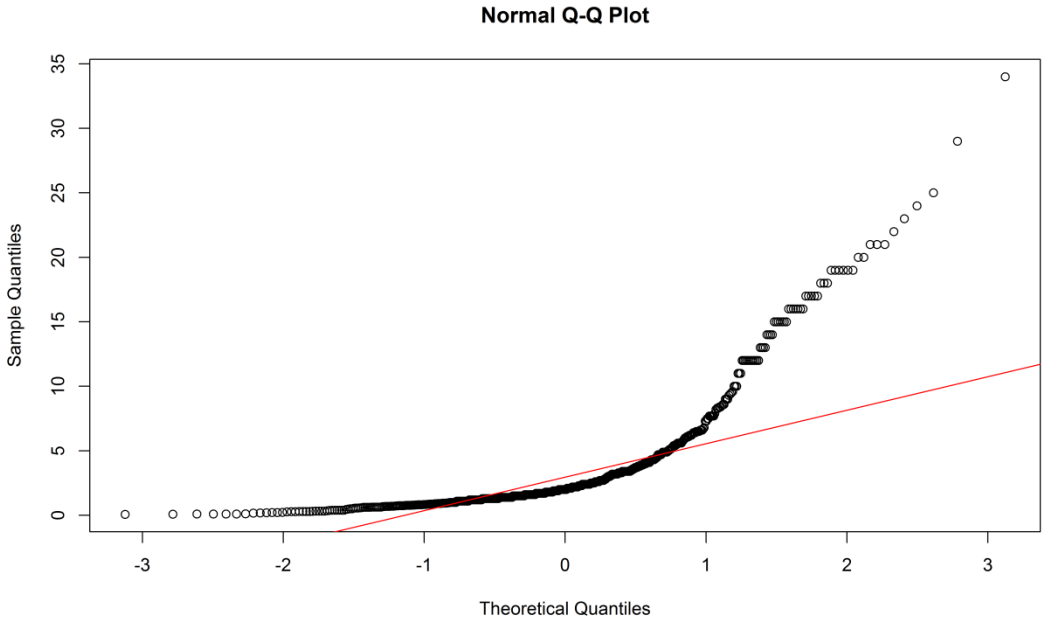


Figure 3.11. Q-Q plot to show the distribution of the percentage of CD34 positive cells for all MDS cases in the training and test set

The red line is a theoretical normal distribution line which passes through the 1st and 3rd quantiles. In this plot, the data does not follow the theoretical line and is non-linear and, therefore, non-normally distributed.

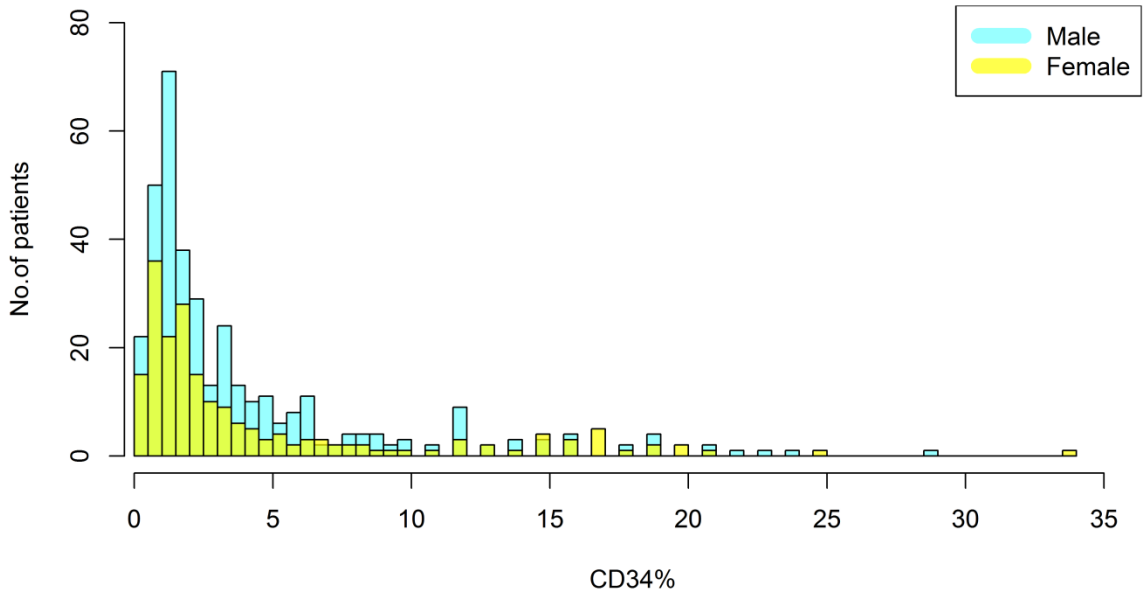


Figure 3.12. Histogram of percentage CD34 positive cells for males and females.

Each bin represents a 0.5% CD34 positive increment.

Unsurprisingly, the RAEB and RAEB-F subgroups had the highest median percentage of CD34 positive progenitors as shown in Table 3.18. However, box and whisker plots in Figure 1.1 showed that there was overlap between the different MDS diagnostic subgroups although, statistically, the different MDS subgroups did show inter-group differences for the percentage of CD34 positive cells (Table 3.19).

Subgroup	No of patients	Median CD34% All cases	CD34% Range All cases	Median CD34% Female (range)	Median CD34% Male (range)
5q-	10	2.10	0.20-6.60	2.40 (0.20-6.60)	1.35 (0.40-2.30)
CMML	36	1.05	0.10-19.00	1.00 (0.30-2.60)	1.05 (0.10-19.00)
MDSMPNU	21	1.80	0.20-8.40	1.70 (0.20-3.40)	2.20 (0.20-8.40)
RAEB	127	9.00	0.10-34.00	12.00 (0.10-34.0)	7.70 (0.40-29.00)
RAEBF	6	7.55	2.00-19.00	4.25 (2.00-6.50)	10.30 (6.50-19.00)
RARS	106	1.50	0.100-8.20	1.65 (0.10-5.40)	1.45 (0.40-8.20)
RARST	9	1.30	0.30-2.60	1.85 (0.30-2.60)	0.90 (0.30-1.80)
RCMD	240	1.80	0.10-13.00	1.60 (0.10-13.0)	1.90 (0.10-12.00)
RCUD	4	1.45	0.70-1.70	1.90 (1.30-1.70)	1.60 (0.70-1.70)
All cases	**559**	**2.00**	**0.10-34.00**	**2.00 (0.10-34.00)**	**2.05 (0.10-29.00)**

Table 3.18. Percentage of CD34 positive progenitors per MDS diagnostic subgroup for male and female cases

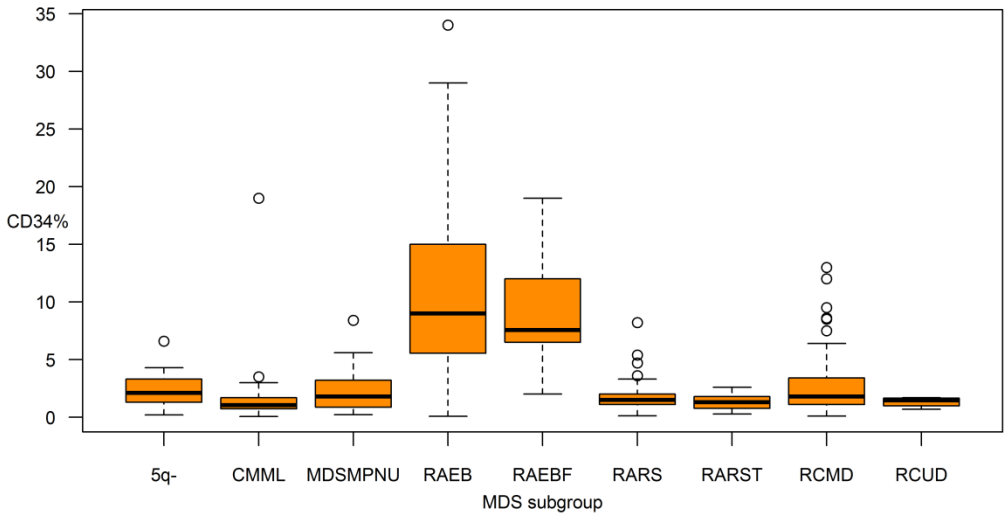


Figure 3.13. Box and whisker plots showing the percentage of CD34 positive progenitor cells for each MDS diagnostic subgroup

	5q-	CMML	MDS MPN-U	RAEB	RAEBF	RARS	RARST	RCMD
CMML	1.0000							
MDS MPN-U	1.0000	1.0000						
RAEB	**0.0015**	**<0.0001**	**<0.0001**					
RAEBF	0.5228	**0.0213**	0.0870	1.0000				
RARS	1.0000	0.4914	1.0000	**<0.0001**	**0.0065**			
RARST	1.0000	1.0000	1.0000	**0.0004**	0.1387	1.0000		
RCMD	1.0000	**0.0067**	1.0000	**<0.0001**	**0.0231**	0.0717	1.0000	
RCUD	1.0000	1.0000	1.0000	0.1068	0.5012	1.0000	1.0000	1.0000

Table 3.19. *P* values for pairwise comparisons for evaluation of differences in percentage of CD34 positive cells per MDS subgroup.

Wilcoxon rank sum test with Bonferroni correction for multiple comparisons was performed. Significant differences are highlighted in red

3.14. Assessment of B-progenitors in the different MDS subgroups

Although a decrease in B-progenitors is a consistent feature in MDS, it was unknown if the MDS subgroups differed in the proportion of cases in which there were decreased B-progenitors. Overall, 75% of all MDS cases showed decreased B-progenitors. However, there was variability across the MDS subgroups (Figure 3.14) with the lower grade MDS subgroups like RARS and RARS-T having a lower percentage of patients with decreased B-progenitors than the higher grade MDS groups like RAEB and RAEB-F. Table 3.20 shows the differences between the MDS subgroups for age and percentage CD34 positive cells for with respect to sex and B-progenitor cell status.

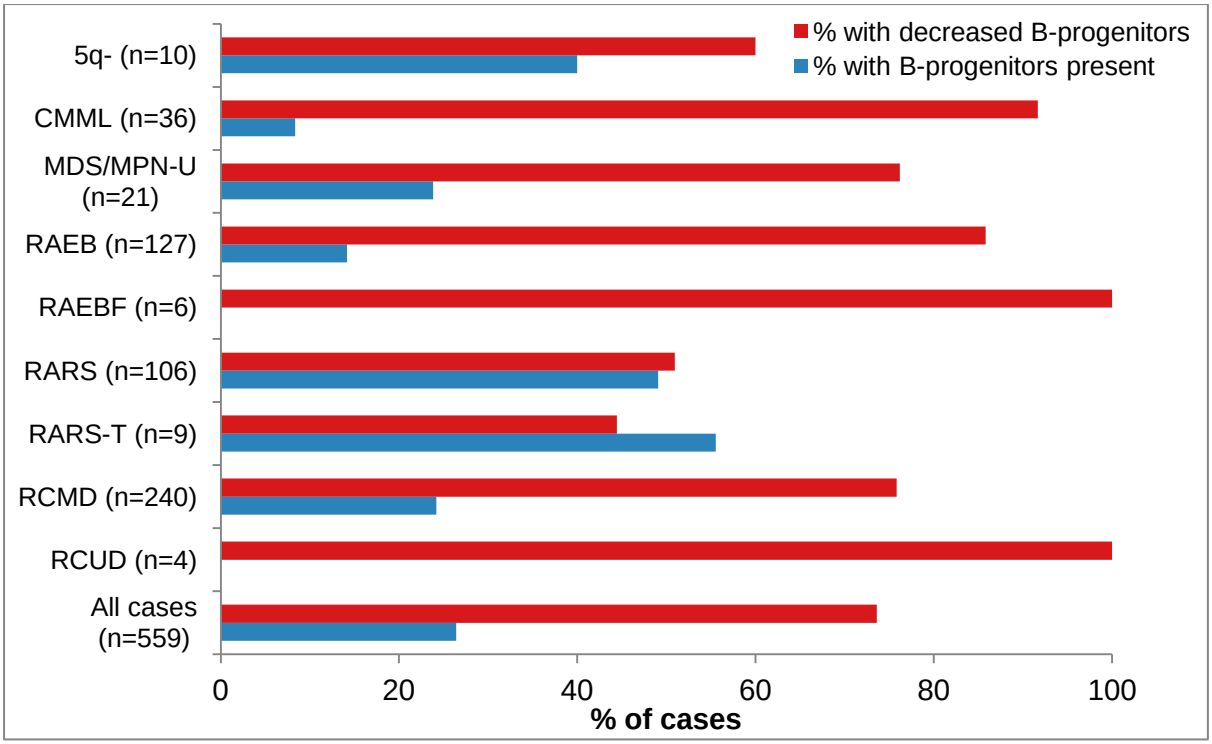


Figure 3.14. Percentage of MDS cases which show present and decreased B-progenitor cells per diagnostic subgroup.

The number of patients in each subgroup is denoted in parentheses after the subgroup name.

Subgroup	B-progenitors decreased						B-progenitors present					
	Female			Male			Female			Male		
	Number of patients	Median Age (range)	%CD34 (range)	Number of patients	Median Age (range)	%CD34 (range)	Number of patients	Median Age (range)	%CD34 (range)	Number of patients	Median Age (range)	%CD34 (range)
5q-	6	61.5 (46-73)	2.60 (0.20-6.60)	0	NA	NA	2	63.5 (58-69)	2.10 (1.30-2.90)	2	81.5 (76-87)	1.35 (0.40-2.30)
CMML	8	75.0 (66-82)	1.01 (0.32-2.60)	25	78.0 (48-91)	1.20 (0.59-19.00)	0	NA	NA	3	81.0 (50-82)	0.30 (0.07-0.80)
MDSMPNU	7	76.0 (64-88)	1.89 (0.92-3.40)	9	77.0 (60-84)	1.90 (0.21-8.40)	2	68.0 (54-82)	0.58 (0.22-0.94)	3	77.0 (59-83)	2.50 (0.27-3.80)
RAEB	46	74.5 (34-92)	12.00 (0.10-34.00)	63	72.0 (10-88)	9.00 (0.35-29.00)	3	63.0 (56-73)	0.81 (0.09-11.00)	15	71.0 (52-86)	6.20 (1.30-15.00)
RAEBF	2	80.5 (78-83)	4.25 (2.00-6.50)	4	69.0 (57-89)	10.30 (6.50-19.00)	0	NA	NA	0	NA	NA
RARS	24	78.5 (65-92)	1.65 (0.50-5.40)	30	82.0 (55-91)	1.50 (0.86-8.20)	18	74.0 (37-88)	1.60 (0.12-2.70)	34	74.5 (30-87)	1.40 (0.40-3.20)
RARST	1	69.0 (NA)	0.30	3	69.0 (54-74)	0.91 (0.28-1.40)	3	76.0 (72-82)	2.40 (1.30-2.60)	2	72.0 (59-85)	1.29 (0.77-1.80)
RCMD	54	78.0 (29-93)	1.60 (0.10-8.50)	128	76.0 (36-96)	2.00 (0.10-12.00)	20	76.5 (39-88)	1.95 (0.19-13.00)	38	77.5 (54-95)	1.70 (0.17-9.50)
RCUD	1	63.0 (NA)	1.30	3	75.0 (71-82)	1.60 (0.69-1.70)	0	NA	NA	0	NA	NA
All cases	149	76.0 (29-93)	2.40 (0.10-34.00)	265	76.0 (10-96)	2.30 (0.10-29.00)	48	73.5 (37-88)	1.50 (0.09-13.00)	97	75.0 (30-95)	1.70 (0.07-15.00)

Table 3.20. Age and percentage of CD34 positive cells by MDS subgroup, sex, and B-progenitor status

3.15 Discussion

3.15.1 MDS in the context of the investigation of cytopenia

At the time of investigation in 2010, it was unclear how many patients, referred for the investigation of cytopenia, were returned with a diagnosis of MDS. The largest study was on an unselected cohort of elderly hospitalised patients in whom MDS was reported in 15% of cytopenic patients (Beloosesky et al., 2000). This prompted the approach adopted here to assess the features of a more diverse cohort of patients referred for the investigation of cytopenia. More recently, Kwok *et al.* reported that 8% of cases referred for the investigation of cytopenia were diagnosed with MDS (Kwok et al., 2015). Therefore, the finding that in a single calendar month, 15% of patients referred to HMDS were diagnosed with MDS was in keeping with previous reports.

Specific guidelines regarding the referral of patients for the investigation of cytopenia do not exist, although there are British Society of Haematology MDS guidelines which state that MDS should be suspected in unexplained cytopenic patients (Killick et al., 2014). Therefore, although this figure of 15% appears to be low for a MDS diagnostic hit rate, the value in referring a sample for the investigation of cytopenia is not solely about the diagnosis of MDS. Indeed, in this study, 20% of referred patients had a non-MDS haematological malignancy. Furthermore, although the majority of referred patients had a non-diagnostic sample, this result in itself can be equally as informative as its diagnostic counterpart when considered as a component of an investigatory workup.

However, it is the finding that a proportion of non-diagnostic, cytopenic cases (2 patients in this cohort) develop a myeloid malignancy that presents a challenge. Clinical monitoring of non-diagnostic, cytopenic patients could aid in the identification of those who are at risk of progression. The finding that, in 2014, 11 percent of patients diagnosed with MDS had been previously referred for bone marrow investigation gives credence to this approach. Indeed, this approach is recommended for those patients who are classified as ICUS and IDUS, into which many of these patients will undoubtedly fall (Valent et al., 2012). However, the progression rate of patients with ICUS has never been formally reported in a peer reviewed journal, only as a presentation at an international conference (Hanson et al., 2009).

Clinical monitoring would, though, pose a challenge. 76 non-diagnostic patients were identified in January 2010 alone and, assuming the same monthly rate, would equate to nearly 1000 extra patients requiring clinical monitoring per annum. Furthermore, the duration of follow-up could be indefinite as, although the median duration between the non-diagnostic and the diagnostic marrow for all MDS subgroups was 19.8 months and the maximum

latency between samples varied from 26 to 109 months depending upon subgroup. A community monitoring based approach whereby patients could send biannual or yearly samples for full blood count analysis may be one feasible option.

The actual reason for the differences in progression rate to, and the median and maximum latency between samples, in the different MDS subgroups is unclear. The aforementioned difficulty in the accurate diagnosis of MDS and inter-observer variability could result in an under diagnosis of MDS. It is, indeed, likely that the classical morphological findings of in RCMD are a relatively late-stage event in the natural history of MDS, following several genetic insults then development of cytopenia, before the advent of morphological dysplasia. Currently, the latency period post genetic insult in humans is unknown. In-house evidence has shown that a patient with a somatic mutation at MDS presentation had the same mutation over 6 years previously, implying that a second or multiple hits were involved in the transformation to MDS (Cargo et al., 2015).

The term Clonal hematopoiesis of interdeterminate potential (CHIP) has been proposed to define patients with somatic mutation but no evidence of dysplasia, and these patients are reported to have a low rate of progression (0.5-1% per annum) (Steensma et al., 2015). However, mutations in genes associated with MDS can be found in 10% of American people over the age of 70 years old with no evidence of haematological malignancy or cancer (Jaiswal et al., 2014). In contrast, the overall MDS incidence rate for Americans has been reported as 3.3 per 100,000, rising to 20.0 per 100,000 in patients in the 70-79 age range (Rollison et al., 2008). Therefore, as CHIP is more common than MDS, this suggests a requirement for a specific combination of genetic and epigenetic events in the pathogenesis of MDS.

It is clear that additional tools or screening tests could be useful adjuncts to genetic mutation studies and/or long-term clinical monitoring in cytopenic patients to identify those at risk of progression. Full blood count parameters may aid and it may be that the depth of cytopenia is important or that patients with mutlilineage cytopenia are more at risk of progression. Unfortunately, haematological parameters were unavailable on the majority of patients due to sole referral of a bone marrow from external sources. The department now recommends a peripheral blood sample to accompany any bone marrow sample referrals. This may help to clarify which patients are at risk of progression. The use of flow cytometric immunophenotyping may aid in a more accurate identification of patients with MDS or, alternatively, in identifying patients with cytopenia who may require closer monitoring. This approach in the use of flow cytometry in cytopenic patients and its potential utility will be the focus of the subsequent chapters of this thesis.

3.15.2 Biological and demographic features of MDS and Reactive patients

When filtering cases for inclusion in the training set, the finding that 36 non-diagnostic patients (2.3%) progressed to either MDS or AML echoes the findings of 3.15.1 above. As does the finding that Reactive patients outnumbered MDS patients, with initial ratio being 412 MDS patients (20.5%) and 1599 Reactive cases (79.5%).

Epidemiological studies report that the incidence rate of MDS increases with age and that there is a higher incidence in males (Rollison et al., 2008; National Cancer Institute. SEER Cancer Statistics Review 1975-2012.; HMRN., 2016.). The finding of an skewed male-to-female rate ratio in MDS is not unexpected as most haematological malignancies show this skew (HMRN., 2016.). The age of the MDS patients in this study was similar to that reported. The absence of younger patients indicates the rarity of patients under the age of 30 with this disorder. In contrast, those patients referred for the investigation of cytopenia who were non-diagnostic were slightly younger than those with MDS. This was true even after the removal of a cohort of paediatric cases and remained true when gender was taken into account. Furthermore, there was no skew of the male-to-female rate ratio in the Reactive group. This implies that the increased incidence in males in MDS is not simply because more males are referred for the investigation of cytopenia.

With respect to biological differences between the Reactive group and the MDS group, the MDS group showed a statistically increased percentage of CD34 positive progenitor cells. This was unsurprising given that this group contained RAEB patients, an MDS subgroup in which there are, by definition, increased myeloid progenitor cells. This increase was found when both male-to-male and female-to-female Reactive to MDS group comparisons were performed. This study further confirms the previously described tendency for MDS patients to have decreased B-progenitors (Sternberg et al., 2005). Decreased B-progenitors were not unique to the MDS group as they were found in some, but significantly fewer, patients in the Reactive category.

For this study, the composition of the Reactive control group did not contain a small series of cytopenic cases. Rather, an unselected group of patients referred for investigation of cytopenia to a routine, diagnostic service was used. This, thereby, represented a real patient cohort. Furthermore, to determine whether there was a group composition effect, a comparison of the ACD and ITP subgroups within the Reactive group was performed. Although there were significant differences with respect to median age and sex ratio for these two subgroups, there was no overall difference for the percentage of CD34 positive cells or proportion of cases with B-progenitors. This finding implies that the subgroup

composition of the Reactive group may not play a critical role in any biological differences found between the Reactive group and the MDS group.

As the Reactive group was found to have a lower percentage of CD34 positive cells and a lower proportion of cases with decreased B-progenitors, it was hypothesized that these attributes may be affected by the lower median age of this group. However, although the percentage of CD34 positive cells appeared to show a slight, progressive decrease between the ages of 40 and 90 in the Reactive group, the MDS group did not show this decrease.

Within the Reactive group, there was a relationship between age and decreased B-progenitors. Patients with decreased B-progenitors were older than patients with B-progenitors present. This is consistent with the findings that age affects B-lymphopoiesis (Min et al., 2006; Guerrettaz et al., 2008; Kuranda et al., 2011). This finding was not replicated in the MDS group as there was no difference between the ages of MDS patients with decreased B-progenitors and those with B-progenitors present. This implies that the decreased B-progenitor phenomenon found in MDS patients may not be solely due to age-related alterations, but may be a biological feature of the underlying disease whose mechanism is currently unknown. Furthermore, due to the differences in percentages of cases with B-progenitors present between MDS groups, it would be of interest to discover whether those patients with B-progenitors present have better clinical outcomes and whether the presence of B-progenitors indicates the retention of some normal haematopoiesis. Alternatively, due to high proportion of RAEB cases with a decrease in B-progenitors, it may be the case that a reduction in B-progenitors is associated with MDS progression.

When assessing the significance of any decrease in B-progenitors, there is the caveat of the 5% cut-off. From the HILIS database results used here, it was not possible to assess whether the percentage of B-progenitors is a continuous variable and/or whether the 5% cut-off was optimal for this dataset. There may be notable biological differences between those patients with a percentage of B-progenitors of 4.9% and those with B-progenitors below the limit of detection, despite both being classified the same. In addition, as this 5% cut-off depends upon the presence of myeloid progenitors, it is entirely possible that two patients with the same number of absolute B-progenitors can be classified differently dependent upon the numbers of myeloid progenitors present.

In relation to this caveat, it was noted that, in the Reactive group, with an increasing percentage of CD34 positive progenitors there was an increased proportion of patients with B-progenitors present. This implies that, in non-diagnostic cytopenic patients, there was a concomitant increase in myelopoiesis and B-lymphopoiesis. This feature was not found in

the MDS group, in whom the proportion of patients with decreased B-progenitors showed no discernible trend with an increasing percentage of CD34 positive progenitors.

Lastly, when examining the CD34 positive cells distribution for all subgroups of MDS, these were found not to show a normal distribution. This is unsurprising given that the RAEB cases with increased CD34 positive cell percentages would skew the distribution and the constitution of the distribution comprises of varying proportions of each MDS diagnostic subgroups. However, assessment of the histograms for this attribute also showed that there were no obvious breaks in the histogram distribution either. This is despite the use of myeloid progenitor percentages of 2%, and 5% and 10% in the IPSS-R and the WHO classification of MDS. This would imply that there is no biological basis behind these cut-offs.

3.15.3 Building a logistic regression model

As there were two classes, Reactive and MDS, and due to the continuous spectrum, non-Gaussian distribution and the overlap between Reactive and MDS groups for the percentage of CD34 progenitors, it was thought that using a logistic regression model might work better than a simple scoring scheme for distinguishing these two classes. The presence or decrease in B-progenitors could be provided as a binary class input whilst, unlike other flow cytometry scoring schemes, there was the benefit of being able to use age and sex, especially as MDS patients were older with a skewed male-to-female ratio. There were further benefits to using a logistic regression model over conventional flow cytometry scoring scheme. Firstly, the generation of weights for attributes instead of arbitrary assignment of points. Secondly, an individual patient probability would be produced and although, class membership would be reported using a probability cut-off of 0.5, these probabilities would indicate the degree of confidence of class membership and could be examined to identify cases where the model may perform either well or poorly.

The baseline accuracy for the training set was 0.7900 and was based on every case being classified into the majority class (also known as the Zero R classifier) which, in this case, was the Reactive group. The reason for this high figure was due to the class imbalance, with nearly four times as many Reactive cases as MDS cases. Although the logistic regression model improved on the baseline accuracy and the specificity was very high (0.9850), the sensitivity was poor (0.3883). The model showed good classification ability for RAEB and RAEB-F cases, but correctly predicted all other MDS subgroups less than 30% of the time. This was understandable due to 2 of the 4 attributes being CD34 positive cells and presence of B-progenitors.

Similar results were obtained for the test set. The model showed high specificity (0.9828) but low sensitivity (0.2109). Therefore, it would appear that this approach of using these four attributes as a method to accurately diagnose MDS is not a feasible option. The model may be improved by the addition of blood count parameters and use of depth of cytopenia, but these were unavailable on the majority of patients in the training set. Furthermore, despite a request to provide a peripheral blood sample with every referral, not every referral centre provides this.

However, although this model may not have utility in a diagnostic setting, a simple model such as this may have a use in a triage approach for further clinical monitoring or more comprehensive diagnostic testing of patients. As the logistic regression model produces a probability for each patient, different cut-off probabilities for class membership can be applied. For example, if a probability cut-off of 0.1 was applied to the test set to denote MDS class membership, 85% of MDS cases would be correctly classified. However, approximately 50% of the Reactive cases would be misclassified as MDS. Initially, this appears to be a poor cut-off. Although, if the 15% of misclassified MDS cases could be identified as MDS by morphological means, or by the additional of another simple attribute into the model, then the 50% of Reactive cases who were classified as Reactive could be excluded from further clinical monitoring or diagnostic testing.

This approach can be demonstrated on the cohort of non-diagnostic cytopenic cases who developed a myeloid malignancy. At a proposed MDS cut-off probability of 0.1, 51 out of 62 of these non-diagnostic cases were classified as MDS (82.30%). Therefore, the majority of these patients would have been further investigated. The challenge is to identify attributes which could be included in a simple model to accurately identify those 15-20% of MDS patients, or non-diagnostic patients who were at risk of progression, who were not classified as MDS by this approach

In summary, although a classification model based on simple parameters such as features of CD34 progenitor cells and age and gender works well for the higher grade subgroups, the biological features of the lower grade MDS groups such as RARS and RCMD overlap with Reactive cases to such an extent that a simple model is insufficient to identify these cases. Therefore, any flow cytometry classification approach will have to identify alternative attributes to aid in the accurate classification of these cases.

4 Immunophenotypic panel development for the identification MDS patients

4.1 Rationale and Overview

Initial flow cytometry immunophenotypic studies used approaches based primarily upon aberrant features of CD34 positive cells and, to a lesser, extent, granulocytes and monocytes (Stetler-Stevenson et al., 2001; Wells et al., 2003; Ogata et al., 2006). Further studies have concentrated on the immunophenotypic features of specific populations, for example, erythroid dysplasia or of monocytes (Malcovati et al., 2005; Xu et al., 2005; Della Porta et al., 2006). An extended analysis of the $CD34^+$ progenitor populations has also been performed (Matarraz et al., 2008).

However, at the time of experimentation, there was no reported approach looking at antigenic differences between MDS and normal patients using a systematic approach from stem cells through to the differentiated myeloid cells and it was unknown whether aberrancies might be present at all stages of differentiation.

The advent of routine 8-colour flow cytometry offered the potential of tracking phenotypic differentiation pathways from stem cells through erythroid, granulocytic and monocytic developmental pathways in both normal and MDS patients.

The aims of the work presented in this chapter were twofold: Firstly, to verify gating strategies for less commonly reported or less well-defined haematopoietic populations. Secondly, to analyse numerical and immunophenotypic attributes in MDS and normal individuals to identify features for inclusion in a smaller antibody panel which would be subsequently evaluated on a larger cohort of MDS and non-malignant cytopenic patients.

For the purposes of this chapter, it must be noted that, when referring to immunophenotypic studies, the use of the term CD34 expressing or $CD34^+$ cells refers solely to the myeloid/stem cell CD34 compartment and does not include the $CD34^+$ B-lymphoid progenitors, unless stated otherwise.

4.2 Patient cohort for panel testing

32 patients overall were selected for evaluation: 8 normal (lymphoma staging) patients and 24 patients from across the WHO diagnostic spectrum. The patient characteristics are shown in Table 4.1.

Group	Subgroup	Number of Patients	Male:Female Ratio	Median Age (years)(range)
Normal	Lymphoma Staging	8	5:3	52.5 (25-75)
MDS	*5q-*	*1*	*0:1*	*70*
	CMML	*3*	*1:2*	*78.0 (71-89)*
	MDS/MPN-U	*3*	*2:1*	*84.0 (71-89)*
	RARS-T	*1*	*1:0*	*77*
	RCMD	*10*	*7:3*	*69.0 (54-82)*
	RAEB	*6*	*3:3*	*75.0 (53-89)*
All MDS	N/A	24	14:10	74.0 (53-89)

Table 4.1. Patient demographics for the MDS and Normal control groups

4.3 Gating strategies for haematopoietic populations infrequently evaluated in MDS

Immunophenotypic gating strategies for most of the major bone marrow haematopoietic populations have previously been published and the populations assessed for differences between MDS and control samples. These include assessment of: $CD34^+$ progenitors; B-lymphoid progenitors, granulocytes, monocytes, erythroid cells, and platelets (Stetler-Stevenson et al., 2001; Wells et al., 2003; Sternberg et al., 2005; Xu et al., 2005; Ogata et al., 2006; Della Porta et al., 2006; Matarraz et al., 2008). However, some haematopoietic populations had not previously been reported as assessed in MDS and confirmation that these populations existed in MDS patients or that the immunophenotypic gating strategy was truly identifying the purported population was required.

4.3.1 Validation and comparison of $CD34^+CD38^-$ and $CD34^+CD38^+$ progenitors

It is now well established that both normal and MDS haematopoietic stem cells reside in the $CD34^+CD38^-$ compartment (Doulatov et al., 2010; Woll et al., 2014; Mian et al., 2015). However, although the expression of CD38 has been reported to be downregulated in MDS, it is unknown whether the $CD34^+CD38^-$ population differs immunophenotypically between normal and MDS patients (Goardon et al., 2009). To confirm and evaluate phenotypic differences $CD34^+CD38^-$ compartment between MDS and normal, an immunophenotypic analysis of normal $CD34^+CD38^-$ and $CD34^+CD38^+$ populations was performed to determine whether there were immunophenotypic differences between the two. Statistical analysis of these two populations can be seen in Table 4.2 whilst box and whisker plots can be seen in Figure 4.1.

	$CD34^+CD38^-$ MFI			$CD34^+CD38^+$ MFI				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	***P* value**	**Bonferroni correction**
CD34	1159	10766	5186	802	7414	3640	0.083122937	1
CD117	3597	8325	5443	1948	8306	5906	0.792895503	1
HLA-DR	766	3386	1946	2184	9691	5256	0.002761604	0.055232
CD45	1165	2039	1433	907	1386	1175	0.003849924	0.076998
CD13	1585	7560	2994	340	2425	1129	0.001947528	0.038951
CD71	550	1483	831.5	1047	4817	2324	0.010081694	0.201634
CD105	438	1048	694	533	1126	815.5	0.636502487	1
CD95	368	3270	690	775	1287	968	0.029662259	0.593245
CD33	121	1153	671	43	900	577	0.701478109	1
CD45RO	76	1923	1232	43	1005	451	0.052029618	1
CD43	2366	14949	8792	7762	27661	21962	0.040568856	0.811377
CD133	888	3589	1107	207	781	336	0.000939106	0.018782
CD62L	278	7931	1831	214	2625	689	0.372029339	1
CD123	157	1378	869.5	86	1404	461	0.066081916	1
CD59	6184	18635	13268	4939	15994	10114	0.201336485	1
CD84	389	1336	545	2363	5135	3072	0.002165029	0.043301
CD18	363	914	586	385	969	599	0.792895503	1
CD49d	810	1287	930	1847	2754	2466	0.002165029	0.043301
CD11a	135	1043	710	191	1323	559	0.798297847	1
CD81	3286	4524	4359	4622	7215	6202	0.01519361	0.303872

Table 4.2. Phenotypic comparison of the antigenic differences between the $CD34^+CD38^-$ and $CD34^+CD38^+$ cells in normal individuals.

Antigens showing significantly different expression between groups by both the Wilcoxon signed rank test and the Bonferroni correction are shown in red, whilst those significant by the Wilcoxon signed rank alone are shown in blue.

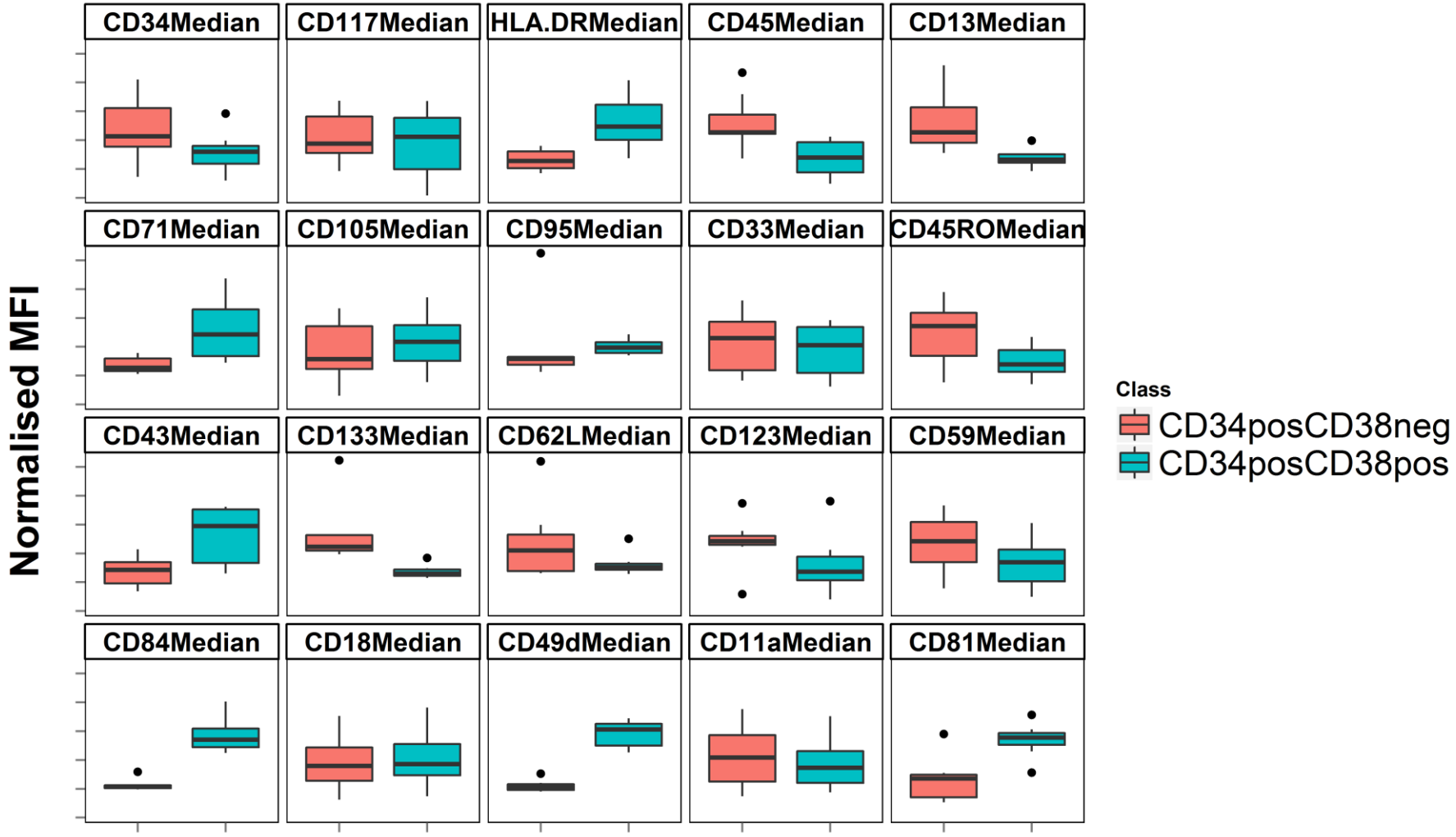


Figure 4.1. Box and whisker showing differential antigen expression for the $CD34^{+}CD38^{-}$ and the $CD34^{+}CD38^{+}$ cells for the 8 normal patients.

For graphical visualisation on the same scale, standardisation of MFI expression for all antigens was performed

20 antigens were assessed for differences in the median fluorescence intensity (MFI). Although 10 antigens were initially noted to be significantly different, following Bonferroni correction to control the familywise error rate, 4 antigens, CD13, CD49d, CD84, and CD133, still showed significantly different expression between groups. A phenotypic signature of strong expression of CD133 and weak CD49d and CD84 on the $CD34^{+}CD38^{-}$ cells was consistent with phenotypic features previously reported on stem cells confirming that the gating strategy identified a $CD34^{+}CD38^{-}$ stem cell enriched population and the $CD34^{+}CD38^{+}$ committed myeloid population (Yin et al., 1997; Zaiss et al., 2003). Surprisingly, the stem cell marker CD90 did not appear to be expressed by the $CD34^{+}CD38^{-}$ population. In this panel, the CD90 antibody was conjugated to FITC, which has weak fluorescent, and CD90 expression may have been weak and masked by background noise.

4.3.2 $CD34^{-}CD117^{+}$ Myeloid progenitors

There have been few reports of the evaluation of this population in MDS, or myeloid malignancy, with two studies by Matarraz *et al.* mentioning the use of HLA-DR and CD45 to identify erythroid, granulocytic and monocytic differentiation pathways (Matarraz et al., 2010; Matarraz et al., 2015). CD71, CD64, and CD24 were used to validate the gating strategy for the proposed erythroid, monocytic, and granulocytic subpopulations as shown in Figure 4.2 and Figure 4.3 .

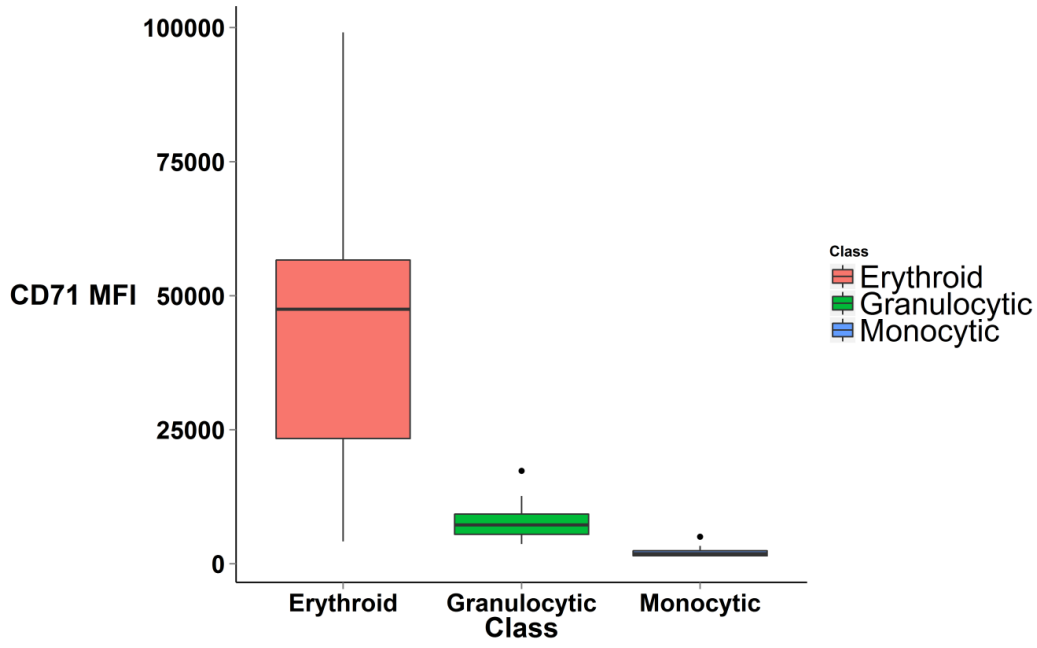


Figure 4.2. Box and whisker plots showing CD71 antigen expression for the $CD34^{-}CD117^{+}$ Erythroid, Granulocytic, and Monocytic populations for the 8 normal control patients.

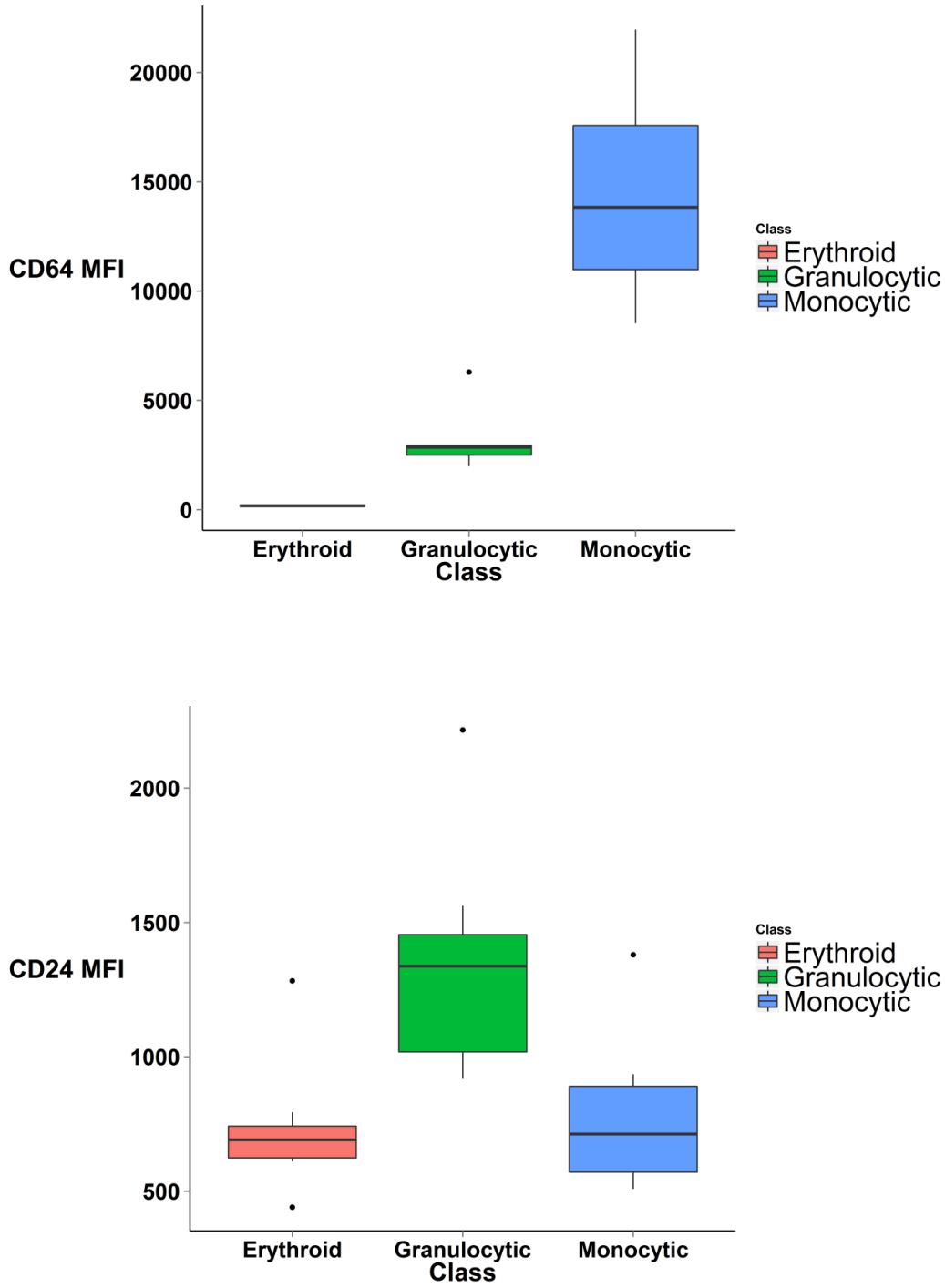


Figure 4.3. Box and whisker plots showing CD64 and CD24 antigen expression for the $CD34^{-}CD117^{+}$ Erythroid, Granulocytic, and Monocytic populations for the 8 normal control patients.

CD71 shows the highest expression in the proposed erythroid differentiating compartment and is reported as showing restricted expression by erythroid precursors (Marsee et al., 2010). CD64 is expressed by both granulocytes and monocytes although monocytic progenitors express CD64 at a higher level (Matarraz et al., 2015). CD24 is reportedly

expressed by granulocytes from the myelocyte stage of differentiation, but not on monocytic cells. The differential expression of these 3 antigens is shown in Figure 4.3 (Elghetany and Patel, 2002; Elghetany, 2002).

4.3.3 Other haematopoietic populations

Although the use of CD64 alone to identify monocytic cells is not the approach outlined for the standardisation of immunophenotyping of peripheral blood monocytes, in bone marrow CD64 is lineage specific for granulo-monocytic cells (Olweus et al., 1995; Maecker et al., 2012). Furthermore, the expression of both HLA-DR and of the monocyte specific antigen CD300e confirmed the monocytic lineage of these cells (Aguilar et al., 2004)

The identification of mast cells by the strong expression of CD117 has previously been reported, and is used as a gating strategy in the immunophenotypic analysis of mast cells in systemic mastocytosis CD117 (Orfao et al., 1996; Escribano et al., 2004). Plasmacytoid dendritic cells (pDCs) were identifying by strong expression of CD123 and HLA-DR as previously reported (McKenna et al., 2005). Basophils were identified by two different strategies: The first strategy was by the use of strong expression of CD203c (Buhring et al., 1999). An alternative strategy for identifying basophils was assessed using strong expression of CD123 and HLA-DR negativity (Han et al., 2008).

4.4 Identification of numerical population differences between the MDS and normal control groups

In total, 18 haematopoietic populations or sub-populations were assessed for percentage differences between the MDS and the normal group (Table 4.3). In the MDS group, a number of cases demonstrated an insufficient number of events (50) for population percentages to be calculated and were reported as below the limit of detection. This included a RAEB patient with $CD34^-$ myeloid progenitors. No control group cases showed this feature.

Although 11 of the 18 populations showed a significant differences between the MDS and normal control group including increased $CD34^+$ (myeloid) progenitors, subpopulations differences within the $CD34^-CD117^+$ population, percentage of plasmacytoid dendritic cells, and monocytic subpopulations, following Bonferroni correction for multiple comparison only 4 populations retained significance. These were the percentage of B-progenitors of $CD34^+$ and of $CD45^+$ cells, the percentage of $CD34^-CD117^+$ cells, and the percentage of monocytes which express CD300e.

Population	MDS			Normal			*P* value	Bonferroni correction
	Min%	Max%	Median%	Min%	Max%	Median%		
$CD34^+$ Progenitors	N/A	31.975	2.600	0.664	3.005	1.39	0.2863	1.0000
$CD34^+$ B-lymphoid	N/A	0.651	0.003	0.018	0.750	0.26	0.0003	0.0054
$CD34^+$ myeloid cells	0.377	31.648	2.275	0.472	1.700	0.92	0.0280	0.5040
B-lymphoid progenitors of $CD34^+$ cells	N/A	27.242	0.769	2.002	40.146	24.04	0.0001	0.0018
$CD34^-CD117^+$	1.321	18.660	7.118	2.089	5.083	2.91	0.0025	0.0450
CD117 Erythroid	1.366	60.131	13.491	21.802	45.589	39.50	0.0250	0.4500
CD117 Myeloid	27.624	98.633	73.169	43.621	71.484	52.32	0.0387	0.6966
CD117 Monocytic	N/A	47.119	5.997	4.874	11.616	9.87	0.1704	1.0000
Granulocytes	16.408	96.452	70.208	49.107	95.676	75.11	0.3064	1.0000
CD123Basophils	0.0002	3.502	0.031	0.028	0.211	0.12	0.2863	1.0000
CD203cBasophils	0.021	3.082	0.180	0.118	0.454	0.29	0.4727	1.0000
pDC's	0.001	4.542	0.058	0.275	0.745	0.33	0.0123	0.2214
$CD117^+$ Mast cells	N/A	0.329	0.017	0.004	0.10	0.016	0.9151	1.0000
$CD64^+$ Monocytes	N/A	75.583	4.836	4.188	9.518	5.01	0.9134	1.0000
$CD14^+$ of $CD64^+$ Monocytes	N/A	91.678	52.399	63.982	78.357	71.56	0.0096	0.1728
$CD300e^+$ of $CD64^+$ Monocytes	N/A	71.955	16.578	29.487	45.827	37.89	0.0016	0.0288
$CD300e^+$ of $CD14^+$ Monocytes	N/A	78.487	40.288	44.702	61.067	51.25	0.0348	0.6264
$CD14^+$ Monocytes	N/A	16.480	2.472	3.000	6.559	3.67	0.1027	1.0000
$CD300e^+$ Monocytes	N/A	10.285	0.639	1.405	2.932	1.95	0.0280	0.5040

Table 4.3. Percentage of haematopoietic populations and sub-populations in the bone marrow of MDS and normal control groups

NA = below the limit of detection. All percentages are reported as a percentage of $CD45^+$ cells unless otherwise stated. Antigens showing significantly different expression between groups by both the Wilcoxon signed rank test and the Bonferroni corrected *p* value are shown in red, whilst those significant by the Wilcoxon signed rank alone are shown in blue.

A numerical statistical comparison between the CD38 compartments of the CD34 expressing cells between the MDS and the normal control group was not performed. Although all patients within the control group had an identifiable $CD34^{+}CD38^{-}$ subpopulation, 10 of the MDS patients were unsuitable for assessment of this population due to either (a) an insufficient number of events in the $CD34^{+}CD38^{-}$ compartment (n=2) or (b) a downregulated, heterogeneous pattern of CD38 expression which rendered identification of an obvious CD38 cut-off impossible (n=8) as shown in Figure 4.4.

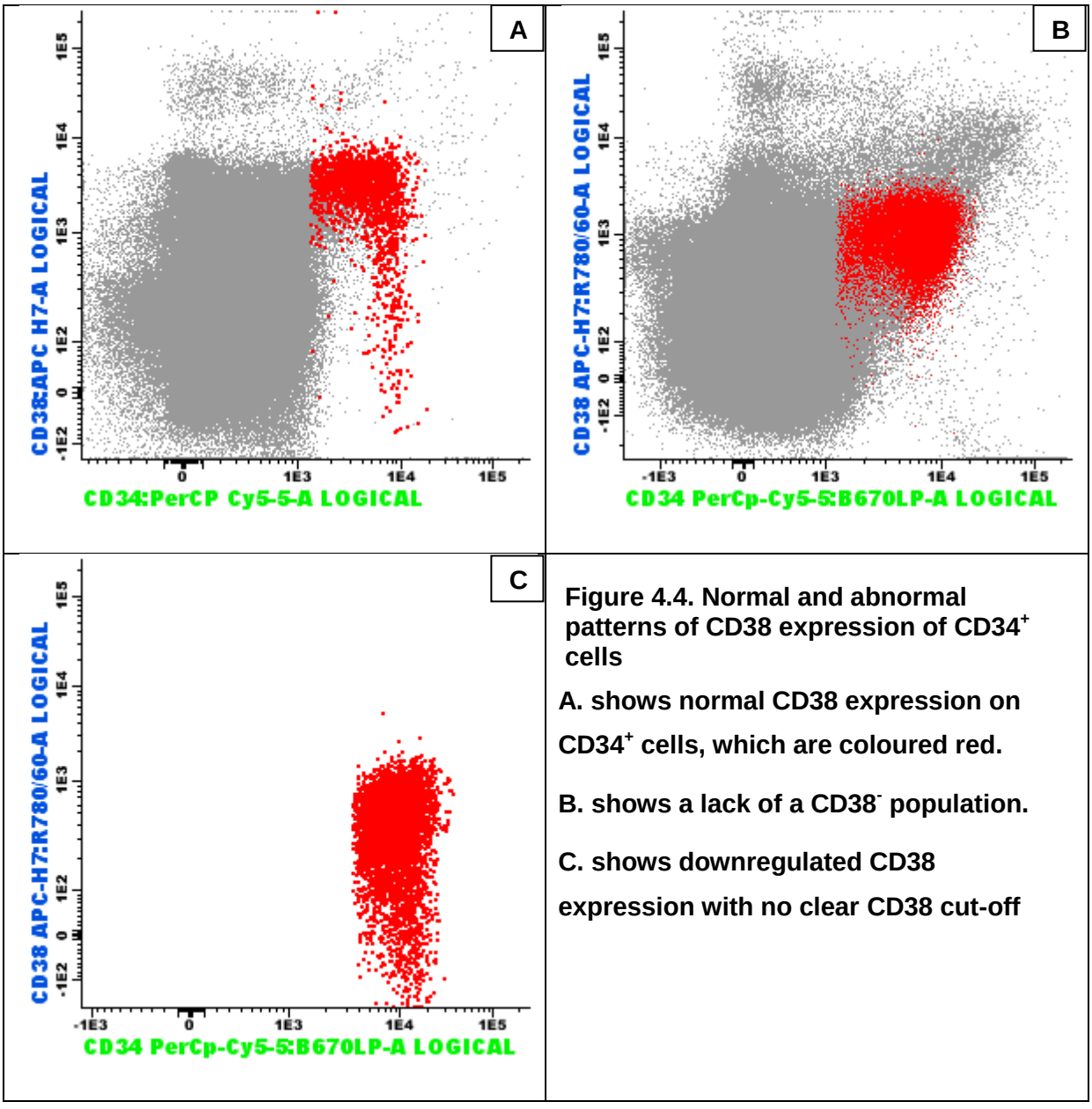


Figure 4.4. Normal and abnormal patterns of CD38 expression of $CD34^{+}$ cells

A. shows normal CD38 expression on $CD34^{+}$ cells, which are coloured red.

B. shows a lack of a $CD38^{-}$ population.

C. shows downregulated CD38 expression with no clear CD38 cut-off

These 10 patients and the RAEB case with no $CD34^+$ myeloid progenitors were removed for statistical analysis of the CD38 populations within the $CD34^+$ cells and for this reduced cohort, there was no significant difference for the percentage of $CD34^+CD38^-$ cells between the MDS group and the normal group (p=0.2381,Wilcoxon signed ranks).

4.5 Do the MDS patients with CD38 abnormalities on the $CD34^+$ cells have a stem cell or myeloid progenitor cell signature?

Although the 10 MDS patients with indiscriminate CD38 expression on their $CD34^+$ cells were removed from a numerical statistical comparison, it was hypothesized that comparing the phenotype of these cells against normal $CD34^+CD38^-$ and $CD34^+CD38^+$ populations would give an insight into whether these cells had a immunophenotypic signature closer to stem cells or to myeloid progenitors.

The earlier confirmation of immunophenotypic MFI differences between the normal $CD34^+CD38^-$ and $CD34^+CD38^+$ populations would allow the use of unsupervised hierarchical clustering to assess immunophenotypic signatures of the 2 normal $CD34^+$ populations and the $CD34^+$ populations from MDS patients in a systematic fashion. This approach would determine whether either individual patients or the group of MDS patients as a whole showed immunophenotypic features more in keeping with stem cells or myeloid progenitors.

The results of this unsupervised clustering approach showed that the $CD34^+$ cells in the majority of MDS patients demonstrated a unique signature which did not correspond to the immunophenotype of either the $CD34^+CD38^-$ and $CD34^+CD38^+$ populations (Figure 4.5). 8 MDS patients formed a discrete cluster and did not cluster with either of the normal the $CD34^+CD38^-$ and $CD34^+CD38^+$ populations. The other 2 MDS patients formed a cluster with each other within 2 $CD34^+CD38^-$ populations and 1 $CD34^+CD38^+$ population.

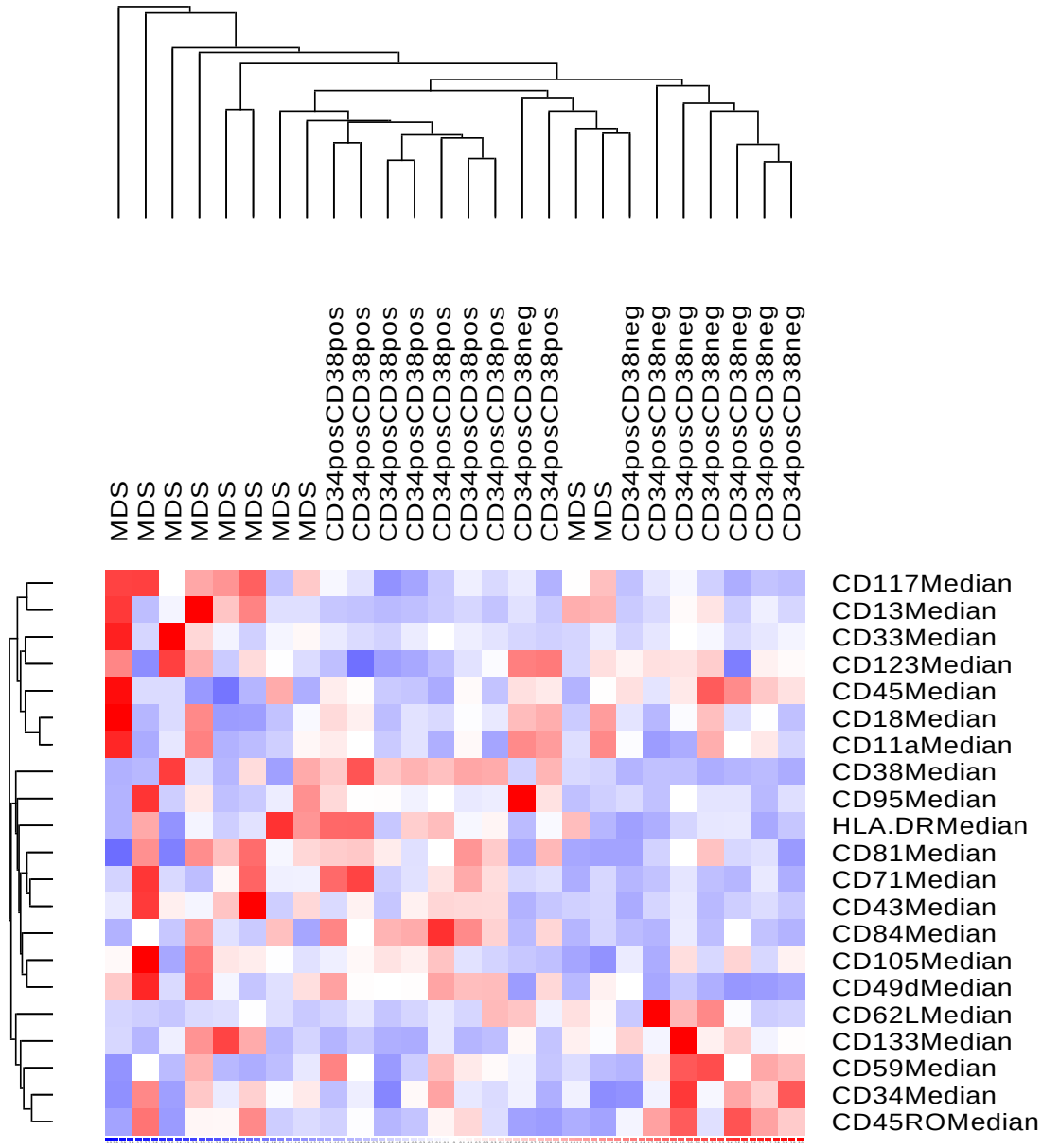


Figure 4.5. Unsupervised hierarchical clustering of MDS cases with indiscriminate CD38 expression and normal $CD34^{+}CD38^{-}$ and $CD34^{+}CD38^{+}$ cells

The rows represent immunophenotypic expression patterns using the MFI whilst each column represents each individual MDS patient or specified control population. Red represents expression greater than the mean whilst blue represents expression lower than the mean.

4.6 Identification of immunophenotypic population differences between the MDS and normal control groups

4.6.1 Identification of the coefficient of variation as a feature of interest

The coefficient of variation (CV) was first noted as a feature of interest in MDS patients when evaluating CD13 expression on $CD34^+$ cells as shown in Figure 4.6.

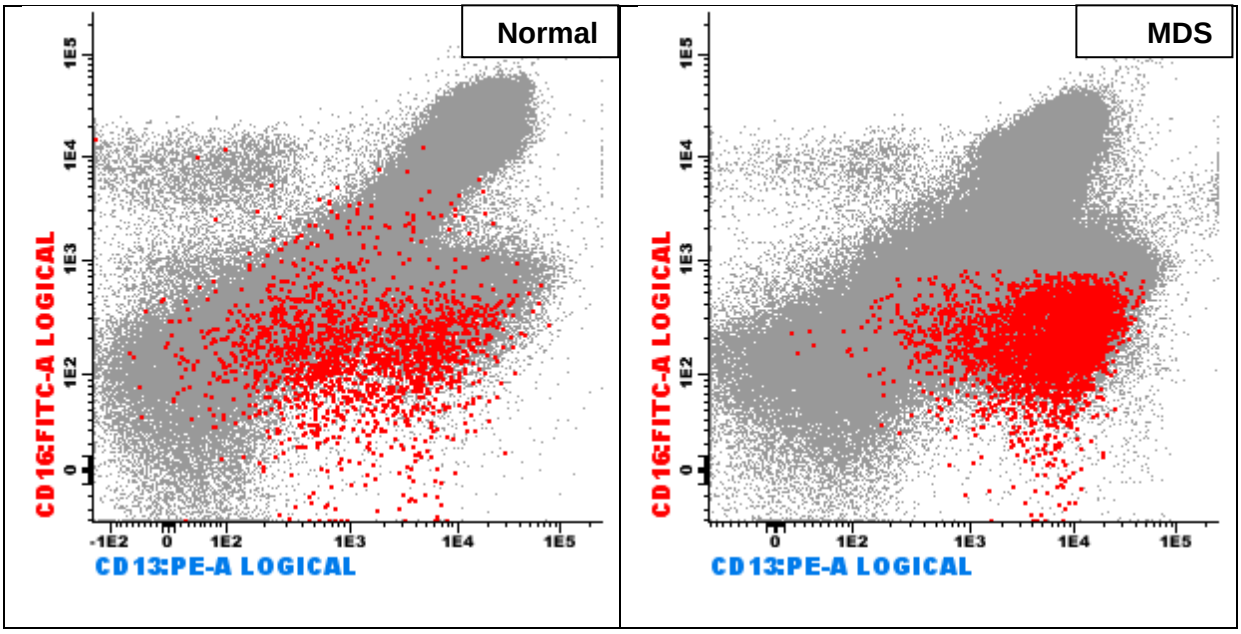


Figure 4.6. An example of a lower CD13 CV on $CD34^+$ cells in an MDS patient in comparison to a normal control patient

The perturbed CV values for CD13 expression on $CD34^+$ cells was a consistent finding across MDS patients and prompted further investigation of other antigens. It was noted that this finding could be extended to other antigens, most notably CD117 and CD123 on the $CD34^+$ cells and CD64 on the $CD34^-CD117^+$ populations (Figure 4.7). Therefore, for populations where there were sufficient events for immunophenotypic studies, both the MFI and the CV were recorded.

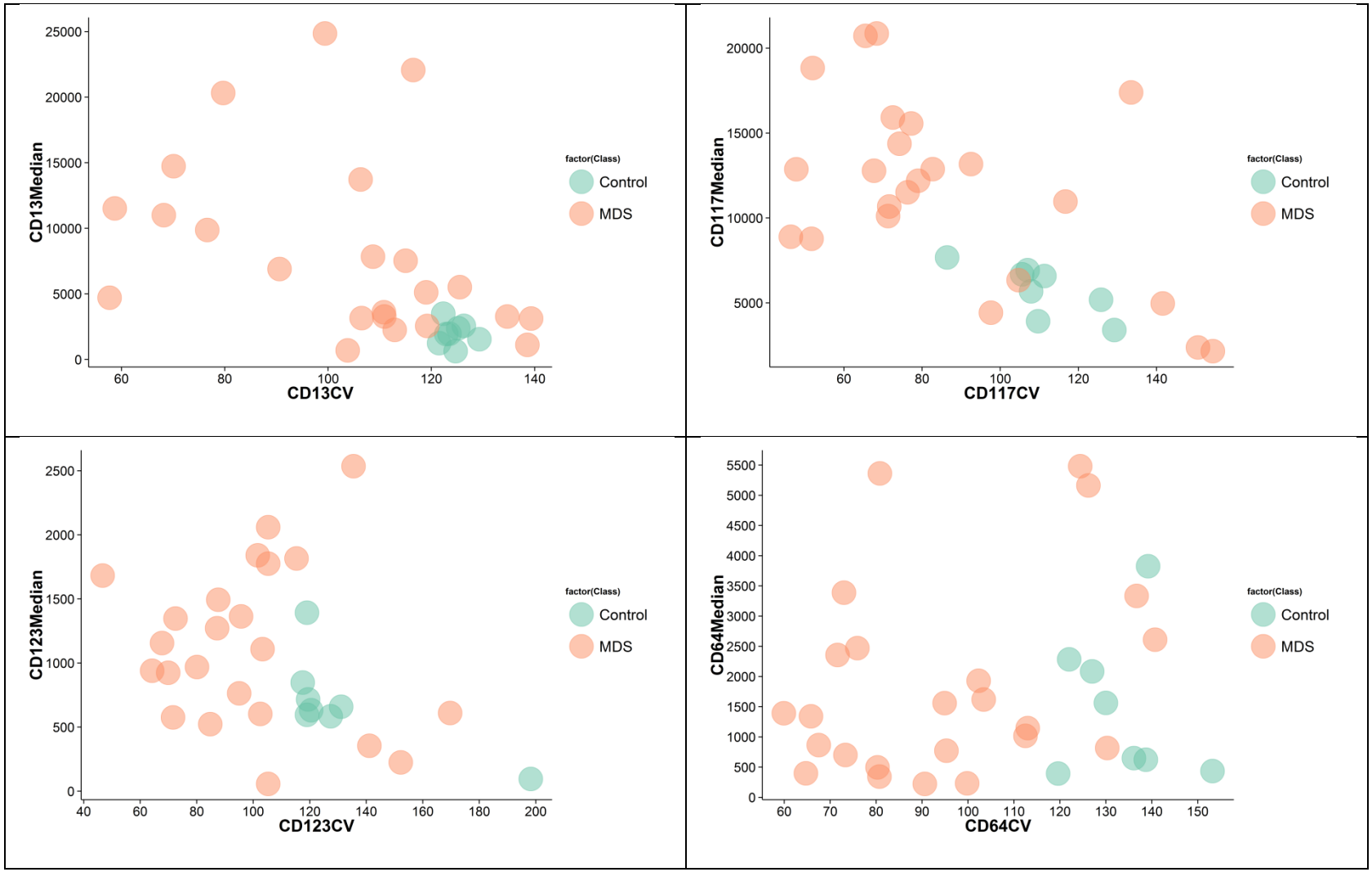


Figure 4.7. CV versus Median MFI for CD13, CD117, and CD123 on $CD34^{+}$ cells and for CD64 on $CD34^{-}CD117^{+}$ cells. For all antigens, the majority of MDS patients have lower CV values than the control group, irrespective of the Median MFI.

4.6.2 Immunophenotypic MFI and CV differences between the MDS and the normal control group

12 main haematopoietic populations were assessed for the presence of immunophenoptypic differences between the MDS group and the normal control group. These were: $CD34^+$ cells, of which $CD34^+CD38^+$ and $CD34^+CD38^-$ cells were also assessed; $CD34^-CD117^+$ cells of which the eythroid, granulocytic, and monocytic differentiating compartments were also assessed; granulocytes; and $CD64^+$ monocytes of which the $CD14^-$ and $CD14^+$, and the $CD300e^+$ populations were also assessed. These latter monocytic populations were assessed for the backbone antigens only. For the other populations (mast cells, basophils, and pDC's), although sufficient cells were present in the majority of cases to quantify the populations, there were usually insufficient events (<300) for immunophenotypic studies and were excluded from further analysis.

In total 388 immunophenotypic attributes (MFI and CV) were recorded and compared for statistical differences between the MDS group and the normal control group. Using the Wilcoxon signed rank test to compare attributes between groups resulted in 74 attributes showing a significantly difference at the $p<0.05$ level. MFI differences accounted for 24 significant differences whilst 50 were CV differences. The statistical analysis for the MFI and CV of expressed antigens for all populations are shown in the appendices as Appendix Tables 4.1 to 4.18.

However, due to the number of attributes assessed and the multiple comparison problem, it was assumed that there would be numerous false positives (type I error) amongst these 74 attributes. The Bonferroni correction reduces the number of type I errors but, in doing so, increases the number of false negatives (type II error). As the primary purpose of this chapter was an exploratory data approach to identify immunophenotypic features which could be applied to a larger cohort, the Bonferroni correction was deemed too conservative and an alternative approach was sought.

Therefore, a false discovery rate (FDR) strategy was implemented using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). This method reports an adjusted FDR *p*-value based on the reported *p* values and the number and rank of comparisons performed. For example, for the data in this chapter, the adjusted FDR *p* values were calculated on the reported Wilcoxon signed ranks *p* values on the basis of 388 comparisons. An FDR is then chosen and this equals the fraction of significant tests which are false positives. For example an FDR of 0.5 would result in 50% of the attributes with a value of <0.5 being false positives. As this was an exploratory approach which was to be combined with pre-existing biological

knowledge, an arbitrary FDR of 0.2 (20%) was chosen, meaning that 20% of attributes with an adjusted FDR *p* value of <0.2 would be false positives.

45 attributes were identified from this dataset as having an FDR adjusted *p* value of <0.2. Differences in the CV between the MDS and normal control group accounted for 30 attributes, and the remaining 15 were MFI differences (Table 4.4.below). Although 45 attributes were identified, due to redundancy and expression by multiple populations, only 22 individual antigens were identified, of which 14 had more than one entry on the list. These were: CD13, CD33, CD36, CD38, CD49d, CD59, CD62L, CD64, CD71, CD81, CD84, CD117, CD123, and CD300e. These 14 antigens represented a restricted set of attractive candidates for further evaluation on a larger cohort of cytopenic cases.

4.6.3 The frequency of lymphoid expression on progenitor cells in MDS

The presence of lineage infidelity expression of the lymphoid antigens CD5, CD7, CD19, and CD56 on myeloid cells is a well-reported phenomenon in MDS. In this cohort, no expression of CD5, CD7, CD19, or CD56 was found on the CD34 expressing cells of any of the normal cases. In the MDS group, 11 cases (46%) demonstrated expression by >20% of $CD34^+$ cells of at least one of CD5, CD7, or CD56. 7 cases showed CD7 expression of which 3 demonstrated concurrent CD5 expression. 2 MDS cases demonstrated sole expression of CD5. The remaining 2 cases showed sole CD56 expression. No case showed CD19 expression.

4.6.4 Visual assessment of the CD11b/CD13 and CD13/CD16 differentiation patterns

Stetler-Stevenson *et al.* reported that abnormalities in the CD13/CD16 and CD11b/CD13 differentiation patterns have been reported as occurring frequently in MDS patients and occur in 70% and 78% of MDS patients, respectively (Stetler-Stevenson et al., 2001). None of the normal group displayed an abnormal pattern, whilst the frequency of these abnormalities in the MDS cohort was 37.5% for the CD11b/CD16 abnormality and 29% for the CD13/CD16 abnormality, which was more in keeping with recently reported figures of 34.5% and 31%, respectively (Chung et al., 2012) Seven MDS cases demonstrated both an abnormal CD11b/CD16 and an abnormal CD13/CD16 pathway and two cases solely demonstrated an abnormal CD11b/CD16 pathway. No case demonstrated a sole CD13/CD16 pathway abnormality which implies that CD16 is the critical abnormality in these pathways. Example images showing normal and abnormal differentiation patterns are shown in Figure 4.8 below.

Population	Attribute	Wilcoxon *p* value	FDR *p* value
CD117Erythroid	CD105CV	0.000195751	0.057
CD117Erythroid	CD59CV	0.000563443	0.057
CD117Erythroid	CD49dCV	0.00049806	0.057
CD117Erythroid	CD81CV	0.000653838	0.057
MatureGrans	CD81CV	0.001021418	0.057
CD117Gran	CD71Median	0.001017236	0.057
CD117Total	CD64CV	0.001017236	0.057
Monocytic	CD14posCD300eCV	0.001244427	0.06
CD117Total	CD13CV	0.001604115	0.069
MatureGrans	CD59CV	0.00203891	0.072
Monocytic	TotalCD64CD300eCV	0.002486335	0.072
CD34Total	CD13Median	0.001985374	0.072
CD34Total	CD123CV	0.00228507	0.072
CD34posCD38pos	CD13Median	0.002651809	0.072
CD34posCD38pos	CD33CV	0.002801666	0.072
CD34posCD38neg	CD49dMedian	0.004334354	0.105
CD117Total	CD62LCV	0.005701074	0.123
CD34Total	CD13CV	0.005496475	0.123
MatureGrans	CD43CV	0.006528915	0.127
CD34Total	CD84CV	0.006813056	0.127
CD34posCD38pos	CD123CV	0.007283437	0.127
CD34posCD38pos	CD18CV	0.008188098	0.127
Monocytic	CD300eposCD38CV	0.008820854	0.127
CD34Total	CD117Median	0.007228943	0.127
CD34posCD38pos	CD33Median	0.008628478	0.127
MatureGrans	CD81Median	0.008144547	0.127
CD117Monocytic	CD95Median	0.007834104	0.127
CD34Total	CD71CV	0.00943655	0.129
CD117Total	CD71Median	0.009614191	0.129
CD117Erythroid	CD36CV	0.010061215	0.13
CD117Total	CD84CV	0.011643996	0.143
CD34posCD38pos	CD123Median	0.011787107	0.143
Monocytic	TotalCD64CD14MFI	0.012226783	0.144
Monocytic	CD14negCD38CV	0.013002184	0.148
CD34posCD38pos	CD45RACV	0.013774032	0.148
MatureGrans	CD33Median	0.013443888	0.148
CD117Total	CD15CV	0.014218423	0.149
CD34Total	CD62LCV	0.015724794	0.153
CD34Total	CD33Median	0.015762456	0.153
Monocytic	CD300eposCD300eMFI	0.015724794	0.153
CD117Total	CD45CV	0.017701283	0.164
CD117Total	CD36Median	0.017794849	0.164
CD117Total	CD33CV	0.020385259	0.18
MatureGrans	CD64CV	0.019896782	0.18

Table 4.4. The immunophenotypic attributes with an FDR adjusted *p* value of <0.2. 45 attributes have a Wilcoxon signed rank *p* value <0.05, denoting a significant difference between the normal control group and the MDS group, and have an FDR *p* value of <0.2. The attribute are ranked from top to bottom on the basis of the FDR *p* value. The haematopoietic population group is also given as it the original Wilcoxon signed ranks *p* value.

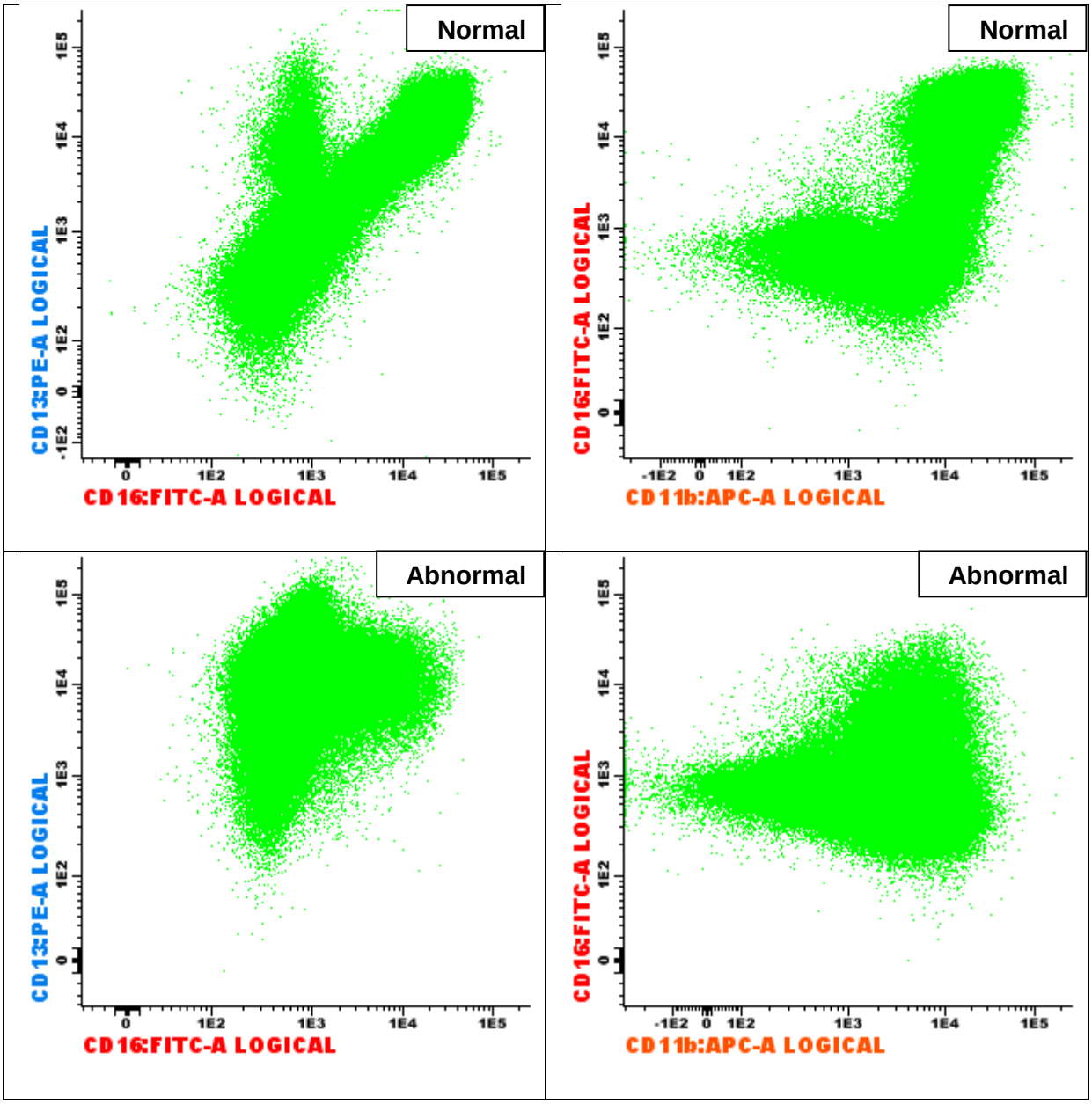


Figure 4.8. Examples of normal and abnormal granulocyte CD13/CD16 and CD11b/CD16 differentiation patterns

The top two plots show normal granulocyte CD13/CD16 and CD11b/CD16 patterns with characteristic visual differentiation patterns. The bottom two plots are from an MDS patient with abnormal patterns.

4.7 Discussion

In this chapter, the numerical and immunophenotypic properties of different haematological populations of a cohort of MDS patients were compared to a normal control group in an attempt to identify a series of attributes which might best discriminate MDS from other, Reactive cytopenic conditions. Two methods for correcting for multiple comparisons (Bonferroni and the Benjamini-Hochberg false discovery rate) were employed to identify those attributes which could be employed in a smaller diagnostic panel.

4.7.1 Numerical differences

Unsurprisingly, given the number of populations evaluated, there were initial significant differences between the MDS and the normal control group, some of which (differences in $CD34^+$ B-lymphoid and myeloid populations, increases in $CD34^-CD117^+$ cells, and a shift towards undifferentiated monocytes) are present in ELN guidelines (Westers et al., 2012). Following the Bonferroni correction, this number understandably decreased due to the conservative nature of this correction method.

The decrease in B-progenitors in MDS was noted again as an important feature and this has been discussed in the previous results chapter. This decrease is well reported and forms a component of guidelines and scoring schemes, with a cut-off of <5% of $CD34^+$ cells used in the FCM scoring scheme (Sternberg et al., 2005; Della Porta et al., 2012; Westers et al., 2012). However, the discovery that B-progenitors were below the limit of detection in 20 out of 24 patients in the MDS group was unexpected and this absence, as opposed to a general decrease, was not a feature of the normal control group. Three out of 4 of the remaining MDS cases had detectable B-progenitors but these were below the 5% cut-off. Therefore a reduction from the oft-employed 5% cut-off may help distinguish additional cases of MDS from cytopenic cases. Whether those MDS cases with detectable B-progenitors, albeit below 5%, represent a different biological or prognostic group than those with B-progenitors below the limit of detection is unknown, and would require clinical monitoring studies or correlation with any cytogenetic abnormalities for further elucidation.

An increase in the $CD34^-CD117^+$ population in MDS patients, as noted in this chapter, has been previously reported by Matarraz *et al.*, but this population does not form part of scoring schemes or guidelines (Matarraz et al., 2010). From a diagnostic perspective, the increase in the $CD34^-CD117^+$ population offers a reproducible MDS abnormality to monitor. Furthermore, subpopulation analysis of this $CD34^-CD117^+$ population hinted at an increased granulocytic and decreased erythroid compartment with this population in MDS patients which should be evaluated on a larger cohort. As this population is not found in peripheral

blood, the different percentage composition within the sub-compartments would be useful as this population would be independent of any blood-dilution of the bone marrow aspirate.

Despite the inclusion of 3 CMML cases within the MDS group, there was no difference between the overall percentage of monocytes between the MDS group and the normal control group. However, the distibution of subsets within this population with respect to the proportions expressing CD300e did differ and indicated a maturation shift towards less differentiated forms in the MDS group. A maturation shift in the monocytes is included as a feature for MDS flow cytometric assessment in the ELN guidelines (Westers et al., 2012). This shift has also been reported as a differentiating feature between CMML and acute monblastic and monocytic leukaemia (Matarraz et al., 2015). Furthermore, as CD300e is expressed by circulating monocytes, the presence of a shift in maturation stages offers a potential target as an aid to MDS diagnosis by the use of peripheral blood monocytes. Indeed, an approach using CD14 and CD16 expression on peripheral blood monocytes has already been adopted for helping to distinguish CMML from a reactive monocytosis (Selimoglu-Buet et al., 2015).

4.7.2 Immunophenotypic differences

Perhaps the most surprising feature of these studies was the more frequent finding that the CV of both common and lineage specific antigens was perturbed across all stages of differentiation. Furthermore, this appeared to be independent of the percentage of cells and of antigenic MFI with CV differences noted for population specific antigens when there was no difference for the MFI e.g. CD105 CV and MFI on $CD117^{+}$ erythroid cells. The use of population percentages and the MFI to report antigenic under- and over-expression are both widely reported in MDS, and differences in these parameters between MDS and normal controls have been demonstrated here. However, the potential benefit of using the CV to aid in the discrimination of MDS from normal was, at this time point, unreported with no studies reported which have evaluated the utility of this approach. A recent publication regarding the use of the CV when assessing flow cytometric erythroid dysplasia may potentially represent a change in practice (Mathis et al., 2013).

The underlying biological basis of this change in CV is unknown. The CV represents the relative variability within a population and any change may represent an increase or decrease in minor subpopulations within the population of interest. For example, this can be seen in the normal control group when the $CD34^{-}CD117^{+}$ compartment is further broken down into the erythroid, granulocytic, and monocytic sub-compartments, each with different levels of expression of antigens. Therefore, a relative increase in one of these subpopulations at the expense of another would result in a change in overall antigen

expression which may cause alterations in the CV. Indeed, this hypothesis may be broadened to other haematopoietic populations as haematopoiesis is a dynamic process with changes in protein (antigen) expression in response to stimuli. A more comprehensive immunophenotypic evaluation of the generic haematopoietic groups may reveal further immunophenotypic subpopulations with distinct expression profiles. This may help to identify in which subpopulations these potential increases or decreases may reside.

An alternatively explanation for the perturbed CV may be that, instead of representing expansion or contraction of subpopulations, a neoplastic cell may express an antigen at an inappropriate level. Should a cell show inappropriate antigen expression which is close to the mean of normal expression, this would also result in a reduced CV in comparison to normal, despite a normal MFI. Evidence for an inappropriate level of antigen expression in MDS was highlighted by those cases of MDS with indiscriminate CD38 expression on the $CD34^+$ cells. 8 out of 10 of these cases showed a distinct immunophenotypic profile by hierarchical clustering which corresponded to neither the $CD38^+$ nor the $CD38^-$ sub-compartment.

4.7.3 Panel design for further testing

A primary aim of this chapter was to identify numerical and phenotypic attributes for inclusion in an antibody panel for further evaluation on cytopenic patients. Therefore, the challenge was to design a small panel which could distil the numerical and phenotypic aberrancies discovered from this MDS cohort into a reduced panel which could be simple enough to be implemented in routine practice without recourse to a lengthy analytical process and further tested on a series of MDS and non-malignant cytopenic cases.

In this study cohort, the comparison of phenotypic features has shown 74 significant differences at the $p<0.05$ level. One reason for this high number is that assessment of the CV and MFI, compared to MFI, alone doubles the available data for each antigen. Indeed, 24 of the 74 differences were MFI differences and 50 were CV differences. This number of differences highlights multiple issues. Firstly, there would be an issue when attempting to incorporate these features in, or building upon, traditional points based MDS diagnostic scoring schemes and classification schemes using more complex data handling features would be required. Secondly, the reality that 74 significant differences were found highlights the challenge of evaluating statistical differences when assessing a large number of attributes. This encapsulates the so called *multiple comparisons problem*.

To overcome the multiple comparison problem and aid in the selection of antigens for a smaller panel, a false discovery rate (FDR) strategy was implemented. As there is an immense number of potential antibody combinations for further testing, an approach

identifying antigens in whom the FDR was <0.2, combined with pre-existing knowledge of MDS biology was adopted.

Due to the number of immunophenotypic attributes tested, even the use of an FDR approach resulted in 45 attributes of potential interest, although this represented only 22 different antibodies. Specific backbone markers were required for population identification (CD45, CD34, CD19, HLA-DR, and CD38), which were included irrespective of any significant difference. CD64, CD117 and CD123 were identified more than once by the FDR approach and could be used for haematopoietic population identification. The myeloid antigen CD13 warranted inclusion due to its perturbed CV properties on the $CD34^{+}$ and $CD34^{-}CD117^{+}$ populations. Finally, although the CD16MFI on granulocytes missed inclusion in FDR top ranked list by virtue of having adjusted FDR *p* value of 0.0206 (the Wilcoxon signed rank *p* value was 0.025), this was included as it could be combined in the panel with CD13 and thereby would allow potential visual assessment of the CD3/CD16 granulocytic pathway.

The selection of these antigens in these combinations allowed the panel to be distilled down to a 2 tube panel (Table 2.6) which would be manageable in a routine laboratory setting, would only require a relatively small number of initial cells (2 million in total) and would allow a relatively large amount of population and phenotypic data to be collected on a large series of MDS and non-malignant cytopenic patients. However, even with a 2 tube panel, the potentially large number of attributes available for assessment would require analytical methods which could process the complex immunophenotypic data which would be generated.

5 Classification studies in the identification of MDS from other non-diagnostic cytopenic cases

5.1 Rationale and introduction

The previous results chapters have shown both numerical population differences between MDS and cytopenic patients, and numerical and phenotypic differences between normal patients and MDS. A flow cytometry panel consisting of two tubes of 8 antibodies per tube was therefore designed to exploit some of these differences and, as the panel contained only two tubes, evaluation of pauci-cellular aspirate cases was also feasible.

Due to the number of lineages and subpopulations affected in MDS, even relatively small flow cytometry panels could yield large amounts of numerical and phenotypic data. Current flow cytometry MDS scoring schemes do not utilise all available information, instead using a smaller number of user-defined parameters. This could lead to the potential omission of diagnostically relevant features which could add to the utility in distinguishing MDS cases from other cytopenic conditions. Furthermore, there is no simple process by which additional parameters can be readily incorporated into these current flow scoring schemes.

In this chapter, the aforementioned two-tube flow cytometry panel was evaluated on a cohort of MDS and cytopenic patients to determine whether a combination of numerical and phenotypic attributes could distinguish MDS cases from non-malignant cytopenic patients. It was further hypothesized that a machine learning approach could be used to (a) overcome the issue of generating diagnostic algorithms for a dataset with a large number of attributes, (b) allow novel attributes and traditional scoring parameters to be used in conjunction, and (c) offer an objective means for maximising the information available to aid in the identification MDS patients.

For the purposes of this chapter, it must be noted that, when referring to immunophenotypic studies, the use of the term CD34 expressing or $CD34^+$ cells refers solely to the myeloid/stem cell CD34 compartment and does not include the $CD34^+$ B-lymphoid progenitors, unless stated otherwise.

5.2 Demographic details, diagnoses, and exclusions

The panel was tested on 183 bone marrow aspirate cases of which 52 patients were diagnosed according to WHO criteria as either MDS or within the MDS/MPN subgroup as shown in Table 5.1.

WHO subgroup (sex)	Number of patients	Median Age (Years)	Age Range (Years)
RARS (Male)	3	81	74-89
RARS-T (Female)	1	70	70-70
RCMD (Female)	5	79	77-87
RCMD (Male)	16	73	58-89
RAEB (Female)	4	74.5	65-84
RAEB (Male)	9	73	63-87
CMML (Female)	3	82	78-86
CMML (Male)	4	77.5	75-80
MDS-U (Female)	1	64	64-64
MDS/MPN-U (Male)	3	76	70-85
5q- (Female)	2	64.5	57-72
Systemic Mastocytosis with associated MDS (Male)	1	77	77-77

Table 5.1. Demographic breakdown of the MDS group by WHO subgroup classification, sex, and age

Of the remaining 131 cases, 52 patients were excluded from final analysis due to the following reasons: 4 patients were reported as having an inadequate sample for morphological evaluation; 1 patient was diagnosed with metastatic carcinoma; 16 patients were diagnosed with a non-MDS haematological malignancy (4xAML, 3xBLPD, 3xPNH, 2xHodgkin, 1xLGL, 1xMPD, 1xCML, and 1xMGUS); 31 patients were excluded, despite having a non-diagnostic bone marrow sample, due to the reported presence of dysplastic features in cells of the myeloid or erythroid lineages, albeit at an insufficient percentage to diagnose as MDS.

The remaining 79 cases consisted of10 cases referred for the staging of lymphoma with no evidence of bone marrow infiltration and 69 patients who were referred for the investigation of cytopenia. These latter patients were further investigated to determine whether subsequent bone marrow samples were referred, with a minimum of 13 months follow-up. For 3 patients, a subsequent bone marrow sample led to a diagnosis of myeloid malignancy (2 patients developed RCMD and 1 patient was diagnosed with atypical CML). The non-diagnostic samples on these cases were, therefore, excluded from the final analysis.

Therefore, in total, 128 patients were used for the final analysis: 76 “Reactive” cases which contained the 10 cases referred for lymphoma staging and the 66 non-diagnostic cytopenic cases with no mention of dysplastic features. The remaining 52 cases were the MDS or overlap, MDS/MPN subgroup cases. The age and sex of the MDS group and the Reactive group is shown in Table 5.2. This shows a typical skew male:female ratio in the MDS group and a younger age for the Reactive group.

Diagnostic Group (sex)	Number of patients	Median Age (years)	Age Range (years)
Reactive (male)	40	73.0	40-90
Reactive (female)	36	66.0	40-91
Total Reactive	*76*	*72.0*	*40-91*
MDS (male)	36	74.5	58-89
MDS (female)	16	78.5	57-87
Total MDS	*52*	*75.5*	*57-89*

Table 5.2. Demographic details for the MDS and the Reactive groups

5.3 Initial assessment of the numerical and phenotypic attributes of the MDS and the Reactive groups

5.3.1 Features of the two tube flow cytometric panel

Although the panel comprised of only two tubes, it was designed to offer numerical percentage assessment of multiple bone marrow haematopoietic populations. It also allowed evaluation of the Median Fluorescent Intensity (MFI) and coefficient of variation (CV) of antigens expressed by those haematopoietic populations with sufficient cells for reliable phenotypic evaluation. The phenotypic method of population identification along with antigen attribute usage is summarised in Table 5.3

Population	Subpopulation	Means of phenotypic Identification	Numerical assessment	Assessment of Phenotypic features
$CD34^+$ Precursor cells		CD34 and CD45	Yes	Yes
	$CD34^+CD19^-$ Progenitors	CD34, CD45, and CD19	Yes	Yes
	$CD34^+CD38^-$	CD34, CD45, CD19, and CD38	Yes	No
	$CD34^+CD38^+$	CD34, CD45, CD19, and CD38	Yes	No
	$CD34^+CD19^+$ B-progenitors	CD34, CD45, and CD19	Yes	No
$CD34^-$ $CD117^+$ Myeloid Precursor cells		CD34, CD117, and CD45	Yes	Yes
	$CD34^-$ $CD117^+$ Erythroid	CD34, CD117, CD38, HLA-DR and CD45	Yes	No
	$CD34^-$ $CD117^+$ Granulocytic	CD34, CD117, CD38, HLA-DR and CD45	Yes	No
	$CD34^-$ $CD117^+$ Monocytic	CD34, CD117, CD38, HLA-DR and CD45	Yes	No
$CD34^-$ $CD117^+$ Granulocytes		CD34, CD117, CD38, HLA-DR and CD45	Yes	Yes
Monocytes		CD64 and CD45	Yes	Yes
Basophils		CD123 and HLA-DR	Yes	No
Plasmacytoid Dendritic Cells		CD123 and HLA-DR	Yes	No
Mast cells		CD117	Yes	No

Table 5.3. Haematopoietic subpopulation phenotypic identification and attribute usage status for MDS and Reactive patients.

5.3.2 Do attributes previously identified as different between MDS and normal also differ between MDS and Reactive patients

Formal statistical comparisons, including correction for multiple comparisons, between the MDS and the Reactive groups for numerical and immunophenotypic attributes are shown in Appendix Table 5.1.

As noted in the previous results chapter, many numerical and immunophenotypic attributes differed statistically between the normal control group and the MDS group. Similarly, the results here demonstrated that a substantial fraction of attributes showed statistically significant differences between the MDS group and Reactive group. Of 65 attributes that were evaluated between the MDS and Reactive groups, a total of 29 differed significantly at the $p<0.05$ level using a standard Wilcoxon signed rank comparison. False discovery rate analysis using the Benjamini Hochberg method showed that, of these 29 attributes, 23 had an adjusted FDR p value of <0.05, 5 attributes had an adjusted FDR p value of <0.1, and only one attribute had an adjusted FDR p value value of >0.1 (CD64 MFI expression on CD34 cells, adjusted FDR p value of 0.108). Use of the conservative adjustment (Bonferroni correction) to account for the family wise error rate/multiple comparison problem yielded 13 significantly different attributes between the MDS and Reactive groups.

The remaining 36 attributes were not found to significantly differ between the MDS and Reactive Groups at the $p<0.05$ level, nor did any achieve an FDR adjusted p value of <0.1.

However, formal statistical analysis of the MDS and Reactive groups only identifies differences *between populations* and cannot be used to determine whether an individual case can be classified as MDS or as Reactive. Therefore, an aim of this results chapter was to develop a classification system which would combine all available attributes and aid in the diagnosis of individual cases of MDS from reactive cytopenic conditions.

5.4 Use of supervised machine learning classifiers to aid in the diagnosis of MDS

The use of machine learning classifiers to aid in the flow cytometric identification of MDS has not been previously reported in studies of MDS. The main MDS flow cytometry scoring systems in use, Ogata and Wells/modified Wells, were formulated using either user defined thresholds from ROC curve analysis (Ogata) or from user defined criteria (Wells/modified Wells) (Wells et al., 2003; van de Loosdrecht et al., 2008; Ogata et al., 2009). With each report of a novel flow cytometric difference between MDS and reactive cases, the number of

potentially evaluable attributes increases. Production of a new, user-defined diagnostic algorithm or incorporation of new features into pre-existing diagnostic scoring schemes is challenging due to the complexity of evaluating multiple features. Additional complexity results from the problems of confounding variables, multicollinearity, and feature redundancy. The use of machine learning algorithms is, thus, an attractive proposition as it offers an objective tool for modelling attributes in classification problems such as the identification of MDS cases from Reactive cases.

5.4.1 Use of this cohort as a training set to evaluate standard classifier performance

Overall, 36 classifiers were evaluated for their performance in separating MDS and Reactive cases in this training set. A brief synopsis of each classifier is shown in Appendix Table 5.2. . All classifiers were initially run with the “Use training set” Test option in WEKA. To evaluate and compare classifiers, 8 performance metrics were assessed and these were: Accuracy, sensitivity, specificity, Kappa statistic, Precision, F-measure, Matthews Correlation Coefficient (MCC), and Area under the ROC curve (AUROC). The sensitivity and specificity measured the proportion of MDS which were correctly identified and the proportion of reactive cases which were correctly identified, respectively. Precision is the positive predictive value, whilst the Kappa statistic measures agreement between predicted and observed classification whilst taking into account the agreement occurring by random chance. The F-measure is the harmonic mean of both precision and sensitivity (see Appendix Table 5.3 for calculation of metrics). The formula for calculating each metric is shown in Appendix Table 5.3. Classifier performance for each evaluable metric is shown in Appendix Table 5.4.

5.4.1.1 Evaluation of the Zero R classifier

The Zero R classifier can be thought of as a baseline classifier as it simply classifiers all cases as the most common class and ignores all attributes. In this cohort there was a class imbalance with the reactive class (n=76) as the majority class and the MDS class as the minority class (n=52). As the Reactive class was the predominant class, all cases were labelled as such. Therefore, all 76 Reactive cases were correctly classified and all 52 MDS cases were incorrectly classified. The Zero R classifier had an accuracy of 0.594 and an area under the ROC curve (AUROC) of 0.500.

The MCC was used to evaluate classifier performance due to the class imbalance, as it takes into account both false positive and false negative classification errors. An MCC of 1 represents perfect classification, whilst a value of 0 indicates average random prediction. For the Zero R classifier, the MCC was 0, indicating the underlying Zero R methodology.

5.4.1.2 Evaluation of the One R classifier

The One R classifier is a simple, decision tree based approach which classifies on the basis of the single attribute with the smallest total error. For this cohort, classification was based on the percentage of CD34 cells and resulted in 110 cases being correctly classified. This gave a classifier accuracy of 0.859, an AUROC of 0.836 and an MCC of 0.712. However, although only 18 cases were incorrectly classified, 15 of these 18 cases were MDS cases, therefore highlighting the requirement for the other evaluable performance metrics (sensitivity, specificity, precision, Kappa statistic and F-measure).

For the One R classifier, the sensitivity was 0.712, the specificity was 0.987, precision was 0.925, and F-measure was 0.804. With the obvious exception of specificity, all performance metrics were higher than those produced by the Zero R classifier. As this was the simplest classifier, in the first instance, all other classifiers were compared to One R for evaluation of performance metrics.

5.4.1.3 Evaluation of the Bayesian classifiers

In comparison to One R, the Bayesian classifiers were more accurate and had a higher sensitivity, Kappa, F-measure, MCC and AUROC. Within this group, the Bayesian Logistic Regression classifier had the fewest misclassified cases (2 MDS and 1 reactive) and, whilst the A2DE classifier was less accurate than the Bayesian Logistic Regression classifier, it did not misclassify any reactive case, therefore had a specificity of 1. The Naive Bayes classifier was the only classifier in this group with a lower specificity and precision than One R.

5.4.1.4 Evaluation of the *Functions* group of classifiers

In comparison to One R, all classifiers within this group had higher accuracy, Kappa statistic, MCC, F-measure, AUROC and, with the exception of the S Pegasos classifier, a higher sensitivity. Although the S Pegasos classifier misclassified more MDS cases than One R (16 cases in comparison to 15) it did not, however, misclassify any Reactive case as MDS.

Specificity and precision varied within this group of classifiers between 0.882 and 1, and between 0.833 and 1, respectively. All classifiers except the RBF Classifier, the Simple Logistic and the Voted Perceptron classifiers achieved higher specificity and precision than the One R classifier.

The MLP Classifier and the Multilayer Perceptron both achieved high accuracy and only misclassified 1 reactive case and 2 MDS cases, respectively. Lastly, both the Kernal Logistic Regression and Logistic (multinomial logistic regression model with a ridge estimator) classifiers appeared to show perfect classification. However, in any such analysis the issue of overfitting to the data is a critical consideration and is further evaluated below.

5.4.1.5 Evaluation of the CHIRP and VFI classifiers

Both classifiers equalled or outperformed One R for every evaluable metric. Each classifier misclassified 6 cases, therefore both had the same overall accuracy. However, whilst the CHIRP classifier misclassified 3 MDS and 3 reactive cases, all 6 cases misclassified by VFI belonged to the MDS class.

5.4.1.6 Evaluation of Rule-based classifiers

Both the Conjunctive Rule and Ridor classifiers had lower accuracy than the One R classifier, with the Ridor classifier misclassifying more MDS (19) cases than Conjunctive Rule (12). Ridor was also outperformed by One R for Kappa statistic, F-measure, and AUROC. Although Conjunctive Rule misclassified fewer MDS cases than One R, it misclassified more Reactive cases therefore had a lower Kappa statistic, specificity, precision, and MCC.

The other two classifiers, FURIA and JRip, either equalled or bettered the One R classifier for all evaluable metrics.

5.4.1.7 Evaluation of the Tree-based classifiers

4 classifiers within this group of classifiers showed perfect classification accuracy and overfitting was suspected for the AD Tree, FT, NB Tree, and Random Forest classifiers.

The Decision Stump, REP Tree and CART classifiers all misclassified 18 MDS cases and 1 Reactive case, therefore scored lower than One R for accuracy, Kappa statistic, sensitivity, F-measure, MCC and AUROC.

The BF Tree, J48, and J48 Graft classifiers all outperformed or, in the case of the specificity of the BF Tree classifier, equalled the One R classifier for all evaluable metrics. The Hoeffding Tree classifier misclassified fewer MDS cases than One R (11 cases compared to 15) but misclassified the same number of reactive cases. The LMT classifier had a higher accuracy, Kappa statistic, sensitivity, F-measure, MCC, and AUROC than One R by virtue of misclassifying fewer overall cases and fewer MDS cases. However, as it misclassified more Reactive cases than One R (5 cases in comparison to 3), specificity and precision were both lower.

5.4.2 Are the same MDS cases repeatedly misclassified?

As different classifiers returned similar sensitivities and specificities, it was unclear whether the same cases were repeatedly misclassified by a variety of classifiers or whether different types of classifiers were misclassifying different cases. Each classifier has a different bias, therefore cases repeatedly misclassified by different classifier may have common biological

features. If these features could be identified then alternative methods could be employed in the future similar when similar cases were encountered.

To evaluate whether repeat misclassification occurred, a clustering heatmap was produced to show how cases were classified by individual classifiers as shown in Figure 5.1. 24 cases of MDS and 55 cases of reactive were correctly classified by every classifier. There was, however, heterogeneity between classifiers for accuracy and the ability to determine the class of specific cases. This is shown in Figure 5.1 whereby MDS and Reactive cases towards the middle of the heatmap are differentially classified depending upon classifier.

To evaluate those MDS patients who were misclassified, a second heatmap was produced to show misclassified MDS cases. These MDS cases were labelled by WHO subgroup and were compared to individual classifiers (Figure 5.2). The results show that there is misclassification across all the WHO subgroups by the majority of classifiers, and that no specific WHO subgroup evaded misclassification.

It was found that the two most frequently misclassified MDS cases were an RARS case (misclassified by 22 different classifiers) and a CMML case (misclassified by 18 different classifiers). Although the root causes of misclassification amongst the various different classifiers could not be determined (due to the different underlying mathematical methods), it was notable that both these cases had a CD34 percentage of less than 1% and there were more than 5% B-progenitors within the $CD34^{+}$ compartment. Both an increased CD34 percentage and a decreased proportion of B-progenitors are features used in flow cytometric scoring schemes to discriminate MDS from reactive conditions (Wells et al., 2003; Ogata et al., 2009; Della Porta et al., 2012).

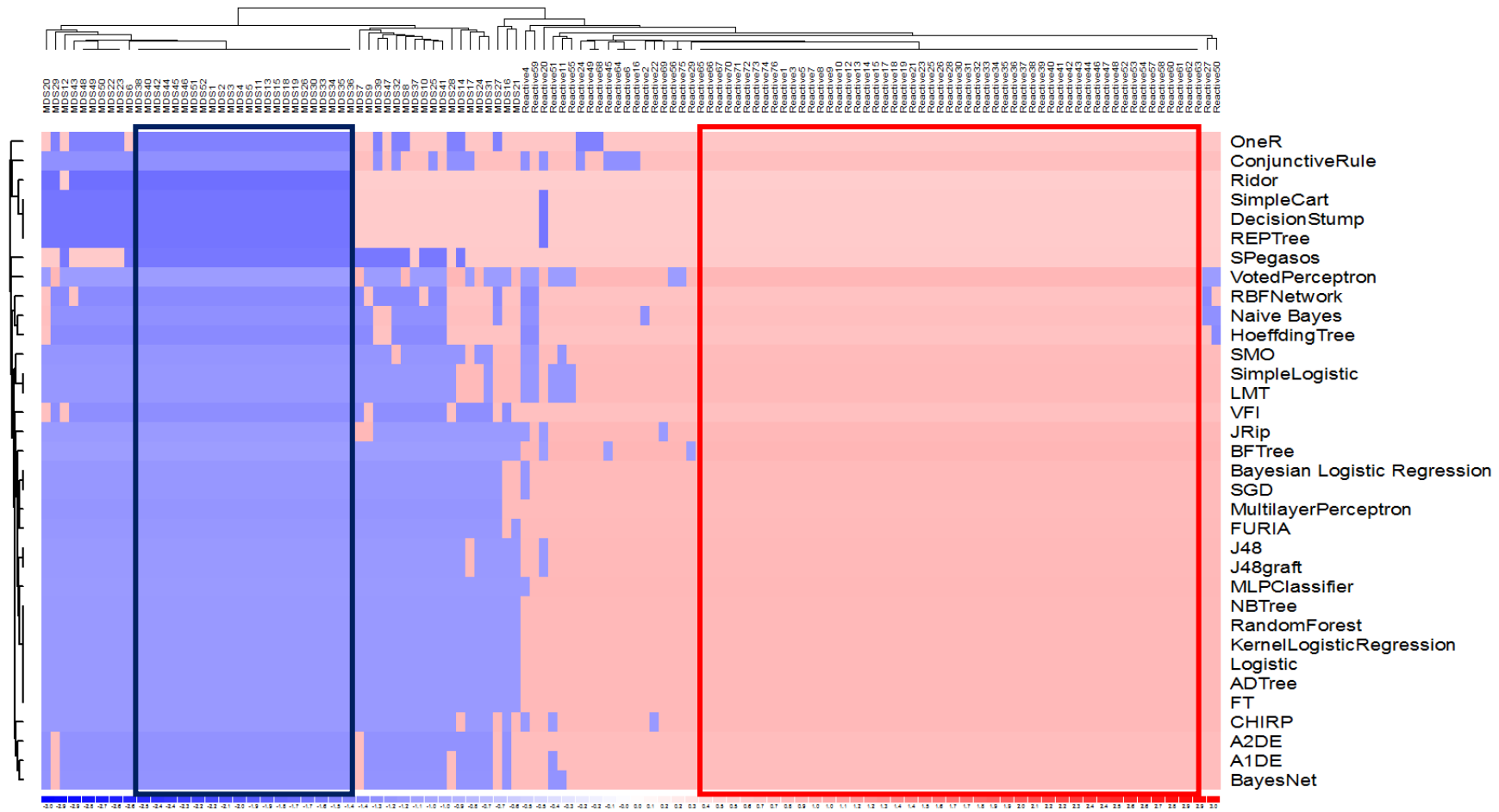


Figure 5.1. Unsupervised hierarchical cluster analysis showing the accuracy of the different classifiers in correctly classifying MDS and Reactive cases. Cases classified as MDS as shown in blue whilst those classified as Reactive are shown in pink. MDS cases correctly classified by all classifiers are enclosed in a blue rectangle. Reactive cases correctly classified by all classifiers are enclosed in a red rectangle.

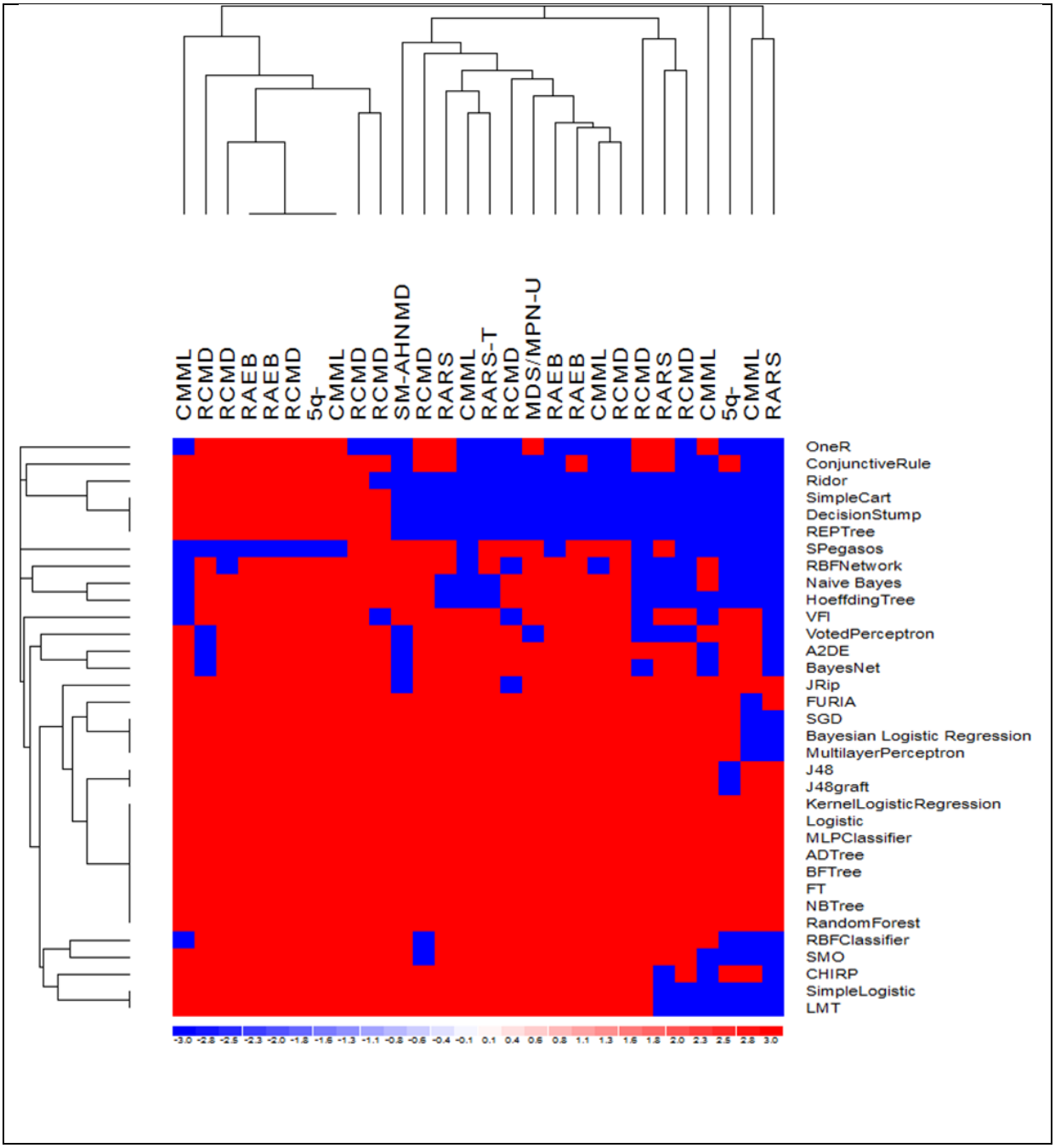


Figure 5.2. Hierarchical cluster analysis of misclassified MDS cases as determined by each classifier.

Squares shown in blue indicates where misclassification has occurred. At least one case from all WHO MDS subgroups was misclassified and misclassification was not restricted to specific MDS subgroups.

5.4.3 Can generalised classifier performance be predicted from the training set?

As 6 classifiers produced no misclassified cases, it was hypothesized that these classifiers were overfitting the data. This phenomenon of overfitting refers to the classifier training on random noise within the training set data as opposed to generalising the data. This results in a classifier which performs accurately on the training set but has poor predictive performance on an unseen, test set. Moreover, this phenomenon may have occurred with the other classifiers.

To overcome the problem of overfitting, 10-fold cross validation was applied to all the classifiers. 10-fold cross validation functions by random partitioning of the dataset into 10 equal sized subsets. The classifier is then trained on 9 subsets and tested on the remaining subset. This is repeated 9 more times and the average accuracy and other metrics are determined. This process is illustrated in figure Figure 5.3 and is used as a technique to assess how well a classifier will generalise on an unseen dataset (Stone, 1974).

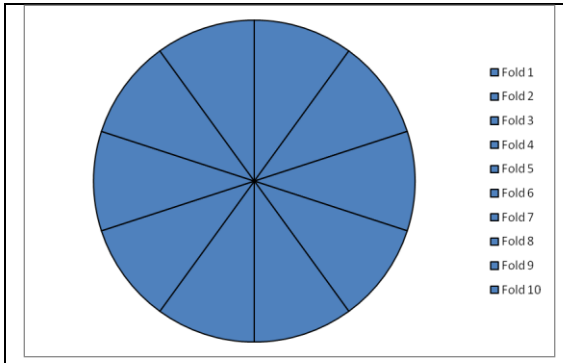
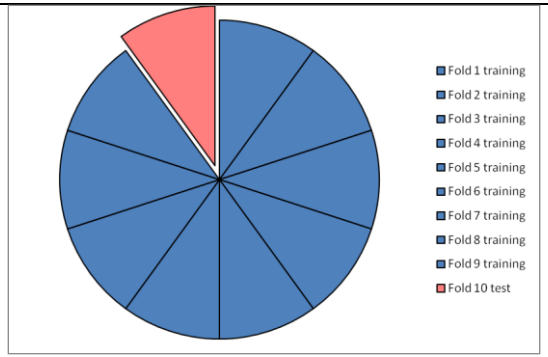
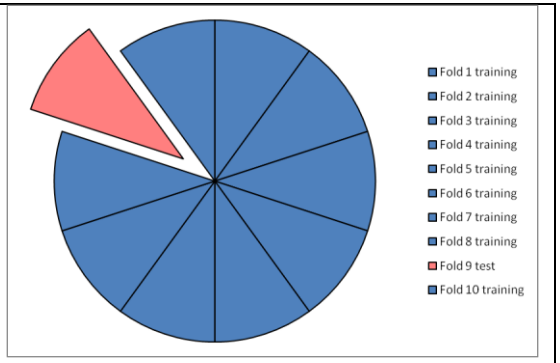
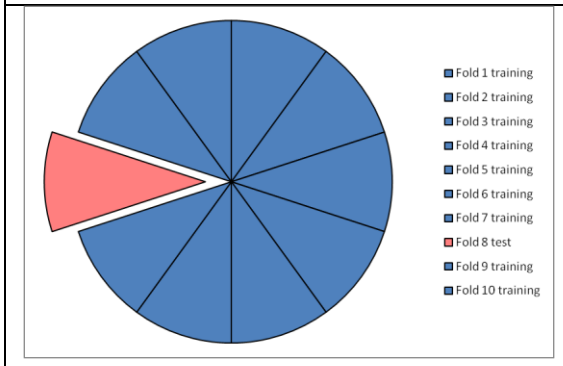
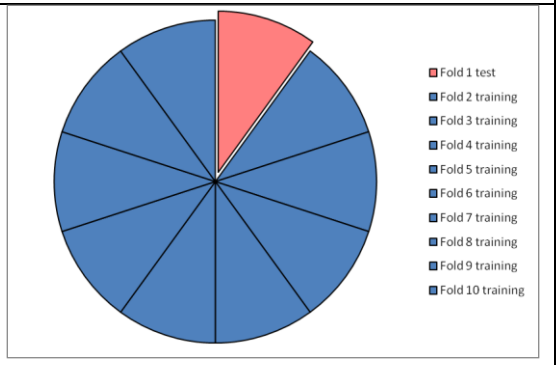
<table>
<tr><td> 
</td><td> 
</td><td> 
</td></tr>
<tr><td>Partition data into 10-folds</td><td>Iteration 1. Train classifier on folds 1-9 and test on fold 10 to obtain accuracy</td><td>Iteration 2. Train classifier on folds 1-8 and 10 and test on fold 9 to obtain accuracy</td></tr>
<tr><td> 
</td><td>  </td><td> 
</td></tr>
<tr><td>Iteration 3. Train classifier on folds 1-7 and 9-10 and test on fold 8 to obtain accuracy</td><td>Repeat iterative process with testing on folds 7, 6, 5, 4, 3, and 2.</td><td>Iteration 10. Train classifier on folds 2-10 and test on fold 1 to obtain accuracy.</td></tr>
<tr><td colspan="3">Final Accuracy = average (iteration 1, iteration 2, iteration 3.....iteration 10)</td></tr>
</table>

Figure 5.3. Schematic of 10-fold cross validation process.

The dataset is randomly partitioned into 10-folds. The classifier is then trained on 9 folds and tested on the remaining 10th fold. This iterative process is repeated 10 times and the classification performance from all 10 iterations is then averaged.

5.4.3.1 Evaluation of classifier performance following 10-fold cross-validation

As expected, the classification performance of all classifiers decreased following 10-fold cross validation (Figure 5.4 and Appendix Table 5.5). This indicates that all classifiers were overfitting the data. No classifier now showed 100% accuracy and none correctly classified either all MDS cases or all Reactive cases.

To evaluate relative performance, classifiers were again compared to One R. In comparison to the One R classifier, 15 classifiers had higher accuracy, although 2 of these (Conjunctive Rule and J48 Graft) misclassified a higher number of MDS cases than One R.

13 classifiers misclassified fewer MDS cases than One R (which misclassified 13 MDS cases) and, with the exception of the VFI classifier, all had higher accuracy than One R. The Bayesian classifiers A1DE, Bayes Net, A2DE, and Naive Bayes, and the Hoeffding Tree classifier were found to have the highest accuracy and misclassified fewer MDS and Reactive cases (or, in the case of A2DE, equal numbers of Reactive cases).

To determine the overall performance of each classifier, a ranking system was used with each classifier ranked for best and worst performance in each of the following 8 categories: accuracy, sensitivity, specificity, kappa statistic, precision, F-measure, MCC, AUROC. This allowed the generation of an average rank for overall performance with the lowest score indicating best overall average classifier performance. The sensitivity and specificity and rank of all the classifiers are shown in figure 5.4.

The Bayesian classifiers performed the best as A1DE, Bayes Net, A2DE, and Naive Bayes were the overall top four classifiers, respectively. The validation of this approach was confirmed by the finding that the top three overall classifiers were also the top 3 ranking classifiers for sensitivity. However, it must be noted that the VFI classifier, which ranked 5th overall for sensitivity performance, was only ranked 20th overall as it had misclassified 16 out of the 76 Reactive cases as MDS, thereby having a lower specificity.

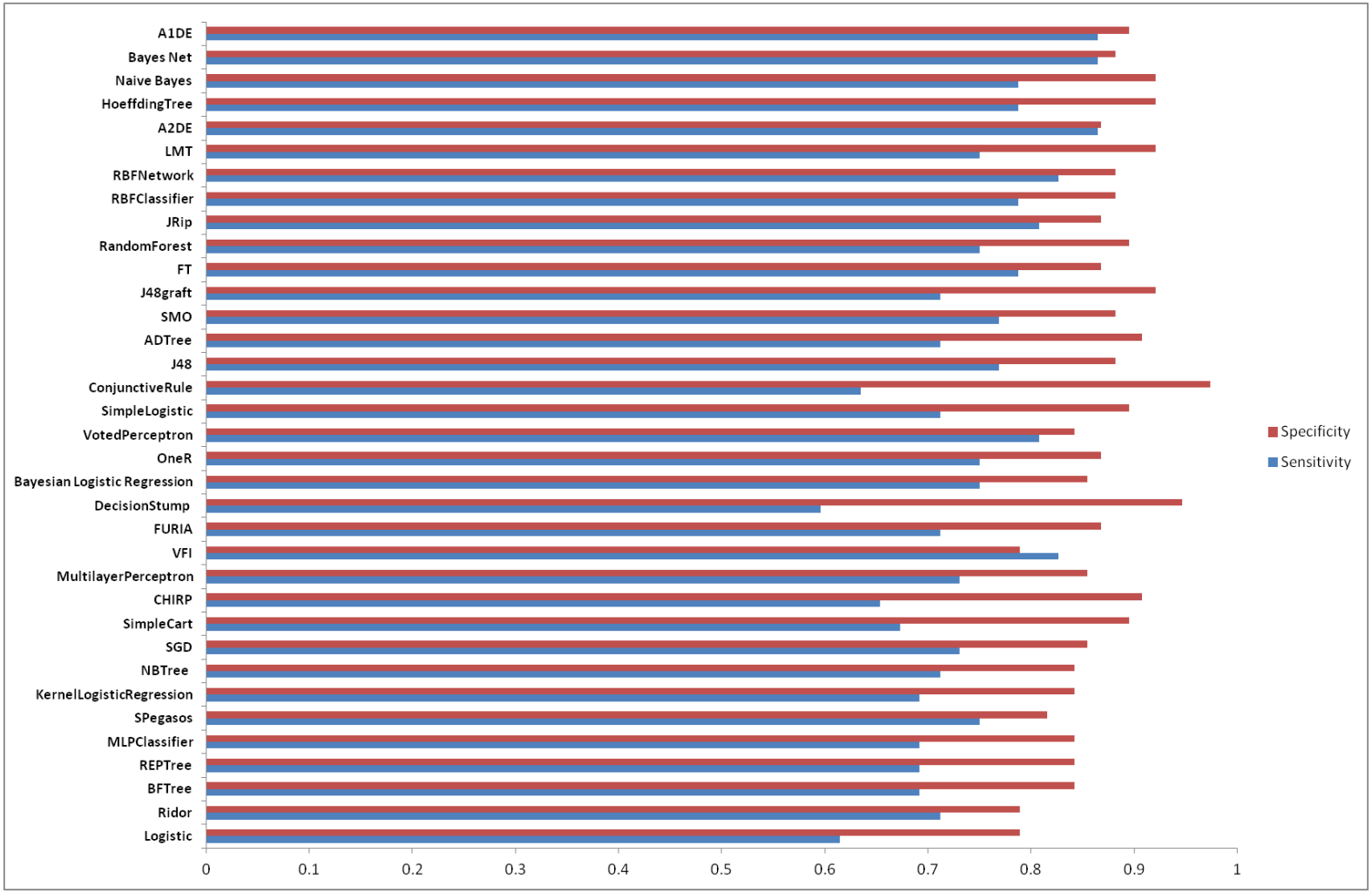


Figure 5.4. Classifier sensitivity and specificity following 10-fold cross validation.

Classifiers are ranked in order from top to bottom with the overall best ranked classifier at the top and the lowest ranked classifier at the bottom. Classifier specificity is shown in red and sensitivity in blue

5.4.4 Can classifier performance be improved through *cost sensitive classification* or *cost sensitive learning*?

For scoring schemes and classifiers, the relative cost of a false positive or a false negative is assumed to be the same. However, in a MDS diagnostic setting it can be argued that these costs are not the same. One might argue that a patient with MDS misclassified as non-MDS (false negative) is denied early clinical intervention and access to medication. This would then outweigh a false positive diagnosis, especially as late diagnosis of cancer has been reported as associated with poorer survival (Richards, 2009). Alternatively, it could be argued that the consequence of falsely classifying a patient with MDS is equally serious. Studies from other fields have shown that there are cost implications associated with false positive screening tests, not to mention to patient anxiety and worry (Lerman et al., 1991; Elmore et al., 1998; Lafata et al., 2004). Unfortunately, a formal analysis of the relative cost of misclassification with respect to financial costs, psychological implications, appropriate treatment and overall survival has not been reported for MDS. For the purposes of evaluation for the cohort of patients in this study, it was assumed that a false negative MDS diagnosis was considered more unfavourable in a diagnostic setting.

A key feature of machine learning classifiers is the ability to modify the relative cost of a false positive or false negative by changing the decision boundary. *Cost sensitive classification* adjusts the output to alter the decision boundary (Witten et al., 2011). For example, to penalise false negative MDS classification, an arbitrary cost of a false negative MDS classification was set at 5 instead of 1. The classifier, therefore, attempts to avoid false negatives as the cost is equivalent to 5 false positives.

Furthermore, an alternative approach known as *cost-sensitive learning* can be adopted. In this procedure, a new classifier could be relearned by duplicating (internally reweighting) instances (Witten et al., 2011). Due to the presence of a class imbalance between the 76 Reactive cases and the 52 MDS cases, an arbitrary classifier cost for the MDS group for this approach was set at 1.46 (ratio of 76 Reactive cases divided by 52 MDS cases), with the cost for Reactive group was retained at 1.0.

For this cohort, the effect of both cost sensitive classification and cost sensitive learning was evaluated for classifier performance with the sensitivity metric considered the most valuable classifier performance indicator for this approach. Furthermore, the cost sensitive approaches were combined with the 10-fold cross validation approach so as to avoid the problem of overfitting.

5.4.4.1 Results of *cost sensitive classification* classifier performance

The same ranking approach as used to evaluate 10-fold cross validation classifier performance was adopted to determine the best overall classifier. Figure 5.5 and Appendix Table 5.6 shows the results of classifier performance in c*ost sensitive classification.*

The *cost sensitive classification* approach improved the accuracy and sensitivity of 5 classifiers over regular 10-fold cross validation and improved the sensitivity alone of another 14 classifiers. 8 classifiers now had a greater sensitivity than the classifiers with the best sensitivity (A1DE and A2DE) by regular 10-fold cross validation. However, this improvement was at the expense of specificity and only 3 of the 8 had a specificity of >0.5 (AD Tree, Simple Logistic, and NB Tree).

The classifier with the highest sensitivity was the RBF Classifier which had a sensitivity of 0.981, but had a specificity of 0.487. This classifier was ranked 26th for overall classifier performance.

The artificial neural network classifier, RBF Network, was the overall best performing classifier. The top ranking classifier which had a sensitivity value higher than the RBF Network classifier was the AD Tree classifier which had a sensitivity of 0.923, and a specificity of 0.711, therefore ranking it 15th in overall classifier performance.

5.4.4.2 Results of *cost sensitive learning* classifier performance

The same ranking approach as used to evaluate regular 10-fold cross validation classifier performance was adopted to determine the best overall classifier. Figure 5.6 and Appendix Table 5.7 shows the results of c*ost sensitive learning* classifier performance.

The cost sensitive learning approach improved the accuracy and sensitivity of 8 classifiers over use of regular 10-fold cross validation. It improved the accuracy alone of 1 classifier and improved the sensitivity alone of another 3 classifiers.

The A1DE classifier had the highest accuracy and sensitivity. However, as the *cost sensitive learning* approach resulted in an extra misclassification of a Reactive case, both the accuracy and specificity of A1DE decreased in comparison to its 10-fold cross validation performance.

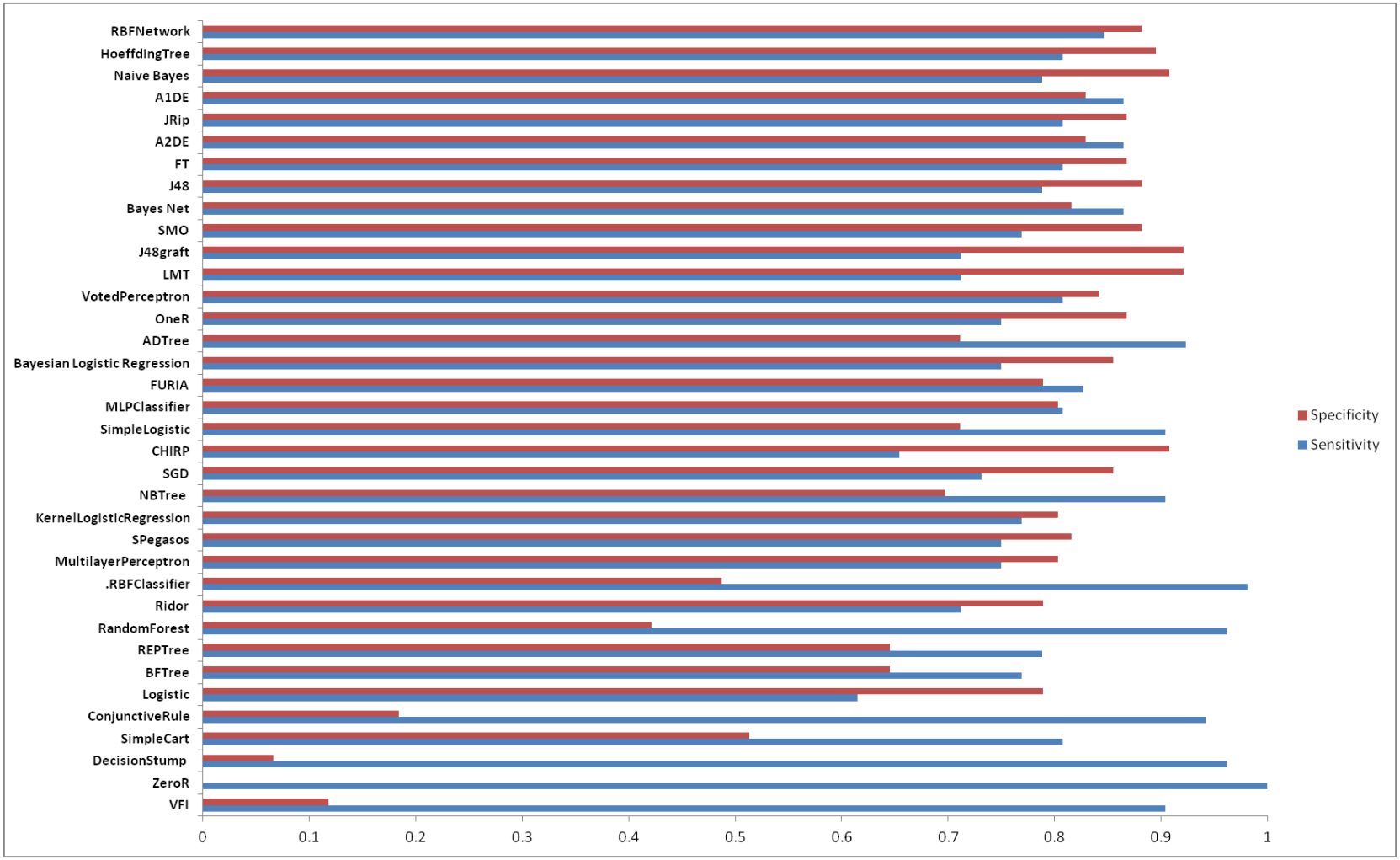


Figure 5.5. Classifier sensitivity and specificity performance following *cost sensitive classification*

Classifiers are ranked in order from top to bottom with the overall best ranked classifier at the top and the lowest ranked classifier at the bottom. Classifier specificity is shown in red and sensitivity in blue

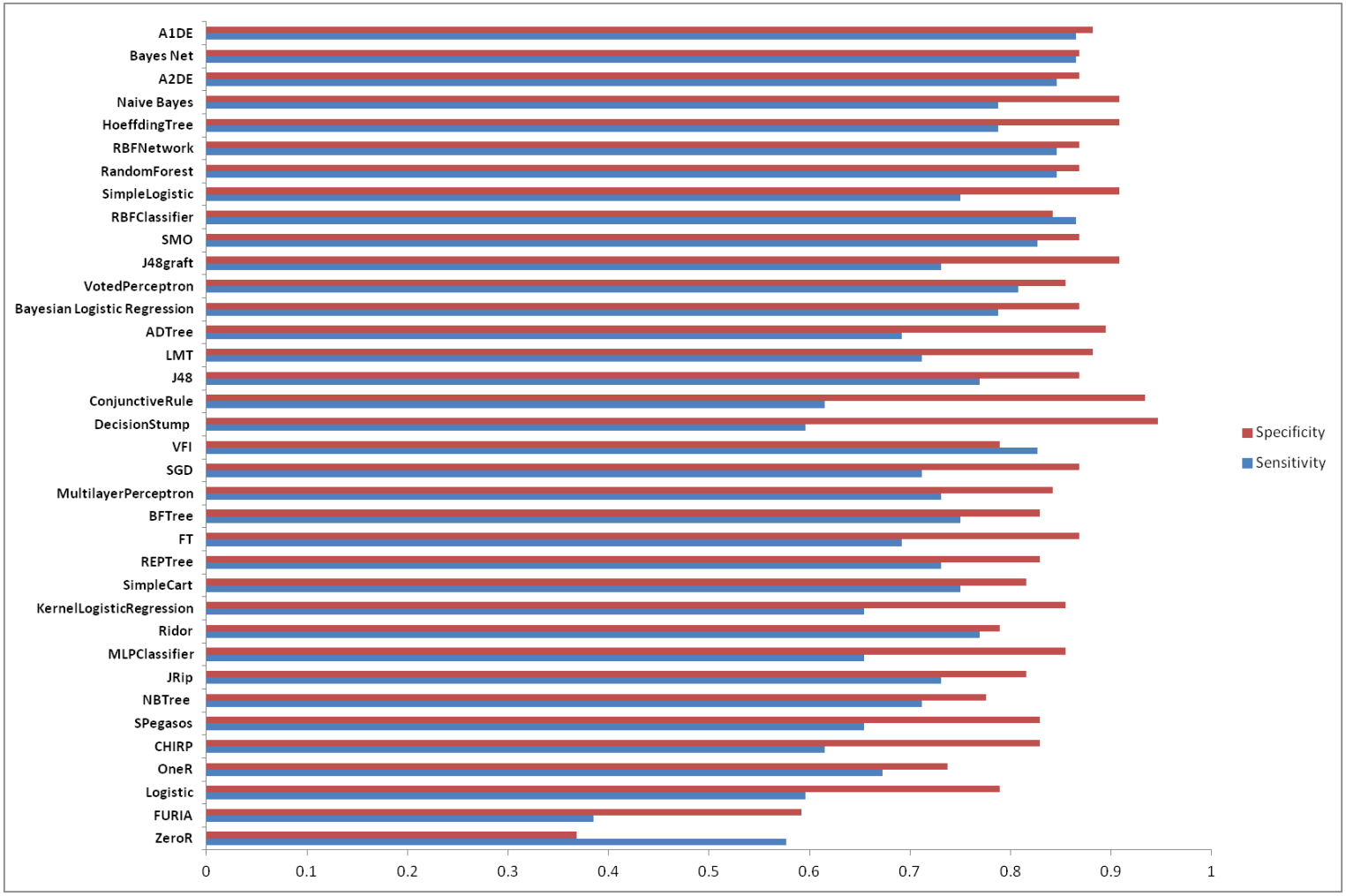


Figure 5.6. Classifier sensitivity and specificity performance following *cost sensitive learning*.

Classifiers are ranked in order from top to bottom with the overall best ranked classifier at the top and the lowest ranked classifier at the bottom. Classifier specificity is shown in red and sensitivity in blue

5.4.5 Does cost sensitive analysis improve classifier performance over regular 10-fold cross validation?

The performance of the best ranking classifier identified from training using either *cost sensitive classification* or *cost sensitive learning* did not improve on the performance of A1DE, which was the best classifier trained with regular 10-fold cross validation (Figure 5.4). The highest ranked classifier using *Cost sensitive classification* was the RBF Network, although none of its metrics outperformed that of A1DE using regular 10-fold cross validation. The highest ranked classifier using *Cost sensitive learning* remained the A1DE classifier, although this showed a decrease in performance in comparison to 10-fold cross validation due to the misclassification of an extra Reactive case.

Approach	Classifier	Accuracy	Kappa Statistic	Sensitivity	Specificity	Precision	F-Measure	MCC
Regular 10-fold cross validation	A1DE	0.882813	0.7578	0.865	0.895	0.849	0.857	0.758
Cost sensitive classification	RBF Network	0.867188	0.7255	0.846	0.882	0.83	0.838	0.726
Cost sensitive learning	A1DE	0.875	0.7425	0.865	0.882	0.833	0.849	0.743

Table 5.4. Performance comparison between the highest ranked classifiers.

Classifiers were trained using 10-fold cross validation, *cost sensitive classification* and *cost sensitive learning* approaches.

Classifier performance was then evaluated by comparison between the classifiers with the highest sensitivity, trained using each approach. The *Cost sensitive classification* approach trained the most sensitive classifier, the RBF Classifier (Table 5.5). This classifier had the highest overall sensitivity (0.981) of any classifiers using any process. However, this sensitivity came at the expense of poor specificity (0.487) resulting in low values for the other performance metrics. The classifier with the highest sensitivity using the *Cost sensitive learning* approach was A1DE and, as stated above, this showed a decrease in performance in comparison its regular 10-fold cross validation performance due to the misclassification of an extra Reactive case.

Approach	Classifier	Accuracy	Kappa Statistic	Sensitivity	Specificity	Precision	F-Measure	MCC
Regular 10-fold cross validation	A1DE	0.882813	0.7578	0.865	0.895	0.849	0.857	0.758
Cost sensitive classification	RBF Network	0.688	0.4192	0.981	0.487	0.567	0.718	0.503
Cost sensitive learning	A1DE	0.875	0.7425	0.865	0.882	0.833	0.849	0.743

Table 5.5. Performance comparison between classifiers with the highest sensitivity.

Classifiers were trained using 10-fold cross validation, *cost sensitive classification* and *cost sensitive learning* approaches.

5.5 Discussion

As previously reported in the first results chapter, a small percentage of non-diagnostic cytopenic cases subsequently develop MDS or other myeloid malignancy. Unsurprisingly, this finding occurred in this cohort with 3 patients subsequently developing a myeloid malignancy. This phenomenon re-iterates both the difficulty in diagnosing MDS and the requirement for follow-up on any case used as a control subject in a training set for MDS diagnosis. Given a longer follow-up period, it is possible that more Reactive cases in the training set will progress to a myeloid malignancy, however, within the scope of this study, these cases remain without evidence of such a transition.

The exclusion of cases which subsequently develop a myeloid malignancy is necessary to produce a dataset which is as untainted as possible. Immunophenotypic changes have been reported in MDS cases in lineages unaffected by morphological dysplasia (van de Loosdrecht et al., 2008; Kern et al., 2010). Therefore, the removal of these cases should improve classifier performance by limiting the inappropriate inclusion of features from cases that subsequently shift between major diagnostic classes into the classifier.

With this in mind, a further 31 cases were excluded from the Reactive control group due to the presence of morphological dysplasia, albeit insufficient for the diagnosis of MDS. There is an understandable ambiguity as to whether these patients can be used as control cases. It can be argued that the morphological dysplasia present is insufficient to diagnose MDS therefore these patients should be included, especially as none of these cases were subsequently found to have been re-referred and diagnosed with a myeloid malignancy with a 13 month minimum timeframe. However, this lack of re-referral does not preclude the presence of a malignancy. Again, the underlying issue here returns to user-to-user variability in diagnosing MDS using a gold standard of subjective morphological assessment. Furthermore, as the aim of any classifier is to be as accurate as possible and is dependent upon the correct labelling of cases in the training set, if a correct label cannot be convincingly assigned then it was reasoned that the case should be excluded.

One approach would be to employ these cases as a test (validation) cohort although, as the clinical follow up and outcome of these cases would be unknown, there would be no means to accurately measure the performance of any classifier on these patients. This diagnostic uncertainty, coupled with the confirmation that a small proportion of cases with a non-diagnostic cytopenia progress to myeloid malignancy, advances the proposal in the first results chapter for the requirement for long-term clinical monitoring of patients referred for the investigation of cytopenia. An alternative approach would be to perform targeted

sequence analysis using a panel of myeloid genes on all cases in a training set and use the presence or absence of mutation(s) as the class-defining feature.

Further regarding the composition of the Reactive group, 4 cases were removed from the initial dataset due to the sample being inadequate for morphological assessment. Due to the limited sample requirement of this two tube panel, these 4 patients generated usable flow cytometric data and were constrained solely by the inadequacy of the sample for a morphological diagnosis. A feature also reported in one of the early flow cytometry studies in MDS (Stetler-Stevenson et al., 2001). It further highlights the potential of flow cytometric evaluation of MDS as, should the flow cytometry results predict a reactive process, the patients may be spared the discomfort of a subsequent bone marrow aspiration.

The use of machine learning classifiers is not widespread in diagnostic flow cytometric studies, although attempts to use machine learning algorithms for the classification of subpopulations using data obtained from the underlying FCS files have been reported (Kalina et al., 2009; Costa et al., 2010). The reason for underuse is unknown and underuse is perhaps surprising given the complexity of multi-colour flow cytometry panels and the number of evaluable attributes. It may be that “black box” approaches such as Artificial Neural Network and Support Vector Machine based classifiers are less appealing than user-defined scoring schemes using transparent biological attributes. However, classifiers can also give an indication of underlying biological features as algorithms such as One R and the J48 tree classifier produce optimal splitting on attributes which differ between two classes. Other factors in classifier underuse may be the issues of classifier choice, the number of parameters which can be manually tweaked within each classifier, and which metric defines the best classifier for a given dataset. Although this latter factor is also true of any user-defined classification scheme.

The advantage of using machine learning algorithms for classification is highlighted in this study. The 2 tube flow cytometry panel generated 11 numerical and 54 phenotypic features for assessment on 76 Reactive and 52 MDS cases. This number of attributes is higher than the number recommended for evaluation by the ELN MDS Flow cytometry guidelines and is despite the fact that many of the ELN recommended attributes were not included in the panel to be assessed (Westers et al., 2012). Incorporation of so many attributes into a user-defined scoring scheme would be problematic. Therefore, alternative methods must be sought and this supports the value of applying machine learning algorithms when assessing so many attributes.

A traditional, standard statistical comparison of the Reactive and MDS groups can still be, and was, undertaken and this showed that 29 attributes significantly differed between these

two groups. Significant differences were also found when both conservative (Bonferroni) and less-conservative (FDR) corrections were applied to take into account multiple comparisons. These differences extend the findings of the previous chapter to show that, as well as differences of haematopoietic cells between MDS and normal cases, differences exist between MDS and non-diagnostic cytopenic cases. These significant differences between the MDS and both the Normal and Reactive control groups indicate that phenotypic and numeric differences in the MDS group did not simply result from a cellular response to a population reduction (cytopenia) or, in the case of MDS/MPN cases, a population increase.

However, a formal statistical comparison approach cannot be used to classify individual cytopenic cases as MDS or Reactive. In this study, individual case classification was proposed by use of a machine learning approach. However, it is a well known adage that no single classifier works best on all datasets, the so-called “No Free Lunch” theory (Wolpert and Macready, 1997). As such, this study was not intended to be a formal comparison of classifier methodologies. The aim was to identify the classifier which best discriminated MDS from Reactive conditions in this training set of case. The chosen classifier could then be tested on a test (validation) cohort to assess generalised performance.

The concept of defining a “best classifier” and measuring best performance for this cohort posed difficulties. For the Ogata FCM scoring scheme, attribute cut-offs were chosen based on ROC curve analysis or mean values of controls +/- 2 standard deviations (Ogata et al., 2009; Della Porta et al., 2012). However, there was no indication as to the method used to chose the ROC curve cut-off (be it Youden’s J statistic, shortest Euclidean distance to (0,1), user-defined, or other (Liu, 2012). Furthermore, no indication was given whether any alterative cut-points could achieve higher sensitivity at the expense of specificity or *vice versa* (Ogata et al., 2009). For the Wells FCSS scoring scheme, with the exception of the percentage of myeloblasts, all abnormalities were weighted as one point each and, accuracy, sensitivity and specificity for MDS and non-MDS cases were all reported for overall scores at single cut-point increments. Cases were then categorised as mild, moderate or severe, depending upon the overall score (Wells et al., 2003).

However, neither of the above studies set out to compare classifiers. For comparison of machine learning classifiers, both speed of computation and accuracy is an important feature. However, as this cohort was a relatively small data set and speed of computational analysis was not an issue, no formal evaluation of classifier processing speed was undertaken. Although it is recognised that this may be more of an issue if a larger cohort with more attributes is to be assessed ultimately the goal would be to develop a classifier implementation that worked on a case-by-case basis, as, for example, previously reported in the context of gene expression data (Care et al., 2013; Sha et al., 2015).

Therefore, to determine the best classifier for this cohort, multiple performance metrics were evaluated. These were accuracy, sensitivity, specificity, Kappa statistics, precision, F-measure, Matthews Correlation Coefficient (MCC) and area under the receiver operating characteristics curve (AUROC). When the accuracy of the standard classifiers was evaluated, 6 classifiers had perfect performance with 100% accuracy. These were the Kernell Logistic Regression, Logistic, AD Tree, FT, NB Tree, and Random Forest classifiers. With the exception of the Zero R classifier, all the remaining classifiers were more accurate than the Zero R classifier, with accuracies ranging from 0.852 to 0.992.

However, as previously noted in this study, the referrals for the investigation of cytopenia result in an imbalanced dataset. This cohort is no different with a slight numerical skew towards Reactive cases. Evaluation of the accuracy metric is based on the assumption that class numbers are balanced. Therefore, in this cohort, accuracy will be skewed by the classification of the Reactive cases. This is illustrated by the performance of the Zero R classifier which has an accuracy of 0.594 and which was obtained simply by classifying all cases in the most frequent class (the Reactive class).

A further issue with accuracy is defining the best classifier when multiple classifiers return the same accuracy. For example, the classifiers A1DE, CHIRP, and VFI all misclassify 6 cases therefore all have an accuracy of 0.953, yet they all misclassify different numbers of MDS cases (5, 3, and 6, respectively). Whilst, between the three classifiers, CHIRP returns the highest Kappa statistic and F-measure, and VFI returns the highest precision, MCC, and AUROC. Therefore, by virtue of scoring highest on the most performance metrics, VFI would be the best classifier of these three. However, this classifier also misclassifies the most MDS case and, therefore, it is important to evaluate sensitivity and specificity.

All classifiers, except the 6 classifiers with perfect accuracy, misclassified at least one MDS case.13 classifiers misclassified 5 or fewer cases, thereby returning a sensitivity of >0.900. The sensitivities of the remaining classifiers ranged from 0.635 to 0.885. 35 classifiers reported a specificity of >0.900, with the Voted Perceptron classifier the sole outlier with a specificity of 0.882. These sensitivities and specificities compare very favourably with those obtained from the Ogata FCM which has reported sensitivities of 0.65/0.89 and specificities of 0.98/0.90 from a two centre study, and a sensitivity of 0.69 and specificity of 0.92 in a multicentre validation study (Ogata et al., 2009; Della Porta et al., 2012).

An interesting result of the approach of comparing multiple classifiers was the separation between MDS cases that could be consistently identified, versus those cases of MDS which were repeatedly misclassified by different types of classifier. Two MDS cases provided extreme examples of this, showing a high degree of misclassification. One case was an

RARS case which was misclassified by 22 classifiers, the other was a CMML case, which was misclassified 18 times. As different classifiers utilise different mathematical modelling, it was unlikely to be an issue with each classifier's methodology. It was hypothesized that both these cases had a high degree of misclassification due to the presence of underlying biological features indistinguishable from those found in cases within the Reactive group. Given the number of attributes evaluated for this cohort, the finding of cases potentially indistinguishable from Reactive is somewhat surprising. Despite it being unfeasible to comparatively evaluate every attribute in these two cases, it was noted that one feature common to both was a relatively low percentage of $CD34^+$ cells, a key feature of all flow cytometry MDS scoring schemes.

It is possible that certain cases with a morphological MDS diagnosis are phenotypically too similar to non-MDS cytopenic cases, irrespective of the number of antigens or numerical attributes assessed. Kern *et al.* reported that 99 out of 804 unselected cytopenic patients had a morphological diagnosis of MDS but with no evidence of dysplastic features by flow cytometry (Kern et al., 2015). These patients had better 2-year and overall survival compared to those patients with both morphological and flow cytometric evidence of dysplasia (Kern et al., 2015).

An alternative explanation is that, in this cohort, there were numerous MDS cases with no, or insufficient, morphological evidence of dysplasia and these were labelled Reactive for classification. If sufficient in number, a classifier may then classify similar cases, albeit with morphological evidence of dysplasia, as Reactive. Kern *et al.* reported that 30 out of 804 unselected cytopenic patients had evidence of dysplastic features by flow cytometry but had insufficient morphological features for a diagnosis of MDS (Kern et al., 2015). The WHO classification provides the MDS-U category for patients with presumptive cytogenetic abnormalities but no morphological evidence of dysplasia (Vardiman et al., 2009). Whilst there is a proposal for patients with genetic mutations but no morphological evidence of dysplasia to be labelled CHIP, there is currently no such diagnostic category for patients with presumptive phenotypic aberrancies (Steensma et al., 2015).

Ultimately, the development of a classifier voting-based scheme which identifies repeatedly misclassified cases may identify MDS patients with potentially better clinical outcomes, or, alternatively, reactive cases which may be at a higher risk of developing MDS. Long-term monitoring of clinical outcome in both types of patients would be necessary.

A central principle of machine learning is that classifiers are trained to obtain the best performance on the training set. However, by maximizing performance this way, it may be the case that any classifier has simply learned the data and is unable to generalise to

unseen data. This concept is known as overfitting. The inclusion of a cross validation step provides a simple process which can be used to limit the effects of overfitting.

In the context of this dataset, the use of 10-fold cross validation to anticipate how a classifier would perform on an unseen dataset was a feature which has not been employed by previously reported user-defined, scoring schemes. As expected, the use of the 10-fold cross validation approach on this cohort resulted in a decrease in performance metrics for all classifiers. Whereas using the standard training set approach, 6 classifiers had an accuracy of 1 and the majority of classifiers had an accuracy >0.9, now the best performing classifier for accuracy following 10-fold cross validation (A1DE classifier) had an accuracy of 0.883.

The decrease in accuracy upon 10-fold cross-validation was not caused by a decrease just in either sensitivity or specificity; rather there was a global decrease in both sensitivity and specificity. The decrease in performance metrics following 10-fold cross validation can be seen as a surrogate for the underlying variability within both the MDS class and the Reactive class. If inter-group attributes were similar across all Reactive or MDS cases, then 10-fold cross validation should not decrease performance metrics. This suggests that the 10-fold cross validation worked effectively to reduce over fitting, and that performance at this level would be transferrable to future data sets. Ultimately, although 10-fold cross validation can give an indication of how a particular classifier may generalise in future studies, it does not replace the requirement for an unseen, validation cohort to test classifier performance.

One feature of machine learning algorithms is their initial training of classifiers on the basis of equal error costs, with false positives and false negatives weighted the same. However, there is the option to amend the relative cost weights. In the context of the diagnosis of MDS, false positive and false negative costs might be considered unequal. For this study, it was assumed that false negative was more costly than false positives. *Cost sensitive classification* was, therefore, combined with 10-fold cross validation and applied to this cohort so that classifiers would penalise a false negative result. The cost of classifying cases as false negative was given a cost of 5, meaning that this cost was 5 times higher than the cost of the misclassification of a case as false positive. Unfortunately, formal studies have not been performed indicating a cost resulting from the non-diagnosis of a genuine MDS, nor the cost of a false positive MDS. Therefore, it is recognised that this value of 5 was arbitrary.

For *cost sensitive classification* analysis, the altered cost matrix changes the threshold of the decision boundary. An alternative approach was to use cost sensitive learning with 10-fold cross validation. For this approach, a value of 1.46 was given for the cost of false negatives. This value was calculated so as to make the class distribution balanced. *Cost sensitive learning* replicates cases via internal weighting and is known as oversampling.

Following *cost sensitive classification*, 18 classifiers increased their sensitivity. The RBF classifier showed the biggest increase with a specificity improving from 0.788 to 0.981. However, this classifier, like all classifiers which increased sensitivity following *cost sensitive classification*, did so at the expense of specificity. Therefore, ultimately, any classifier found to increase sensitivity by the use of *cost sensitive classification* had a decreased overall accuracy in comparison to regular 10-fold cross-validation.

Following *cost sensitive learning*, 11 classifiers increased their sensitivity. However, unlike *cost sensitive classification*, this method of cost sensitive analysis did not decrease classifier specificity to the same extent. Indeed, 9 classifiers improved accuracy, of which 7 showed increased sensitivity. Overall, for *cost sensitive learning*, the classifier with the highest sensitivity was A1DE. However, unlike the RBF classifier learned using the *cost sensitive classification* method, A1DE also had high specificity.

Ultimately, a classifier for further testing had to be chosen. The choice between classifiers is, fundamentally, a trade-off between sensitivity and specificity. For this cohort, correct classification of the MDS class was deemed of greater importance. Therefore, it was preferable to choose a classifier which had high sensitivity, yet retained a reasonable specificity. Standard classification of this training set, without 10-fold cross validation, yielded a number of classifier with seemingly perfect classification accuracy. However, these classifiers were overfitting the data and, as such, would have performed worse on unseen data. The use of 10-fold cross validation decreased performance metrics, but gave a better estimate as to how classifiers would perform on an unseen dataset. Furthermore, although cost sensitive methods improved upon regular 10-fold cross validation sensitivity, this approach either did not improve upon the best classifier identified by regular 10-fold cross validation (A1DE) or, for those classifiers where the sensitivity was higher than for A1DE, the cost sensitive approach vastly reduced the specificity of the classifier.

It is possible that classifier performance could have been further improved as, for all classifiers evaluated, the default WEKA settings were used. Parameter tuning may have improved performance of individual classifiers for this training set. However, the tweaking of multiple parameters per individual classifier would, undoubtedly, have resulted in further overfitting to the data therefore this approach was not pursued.

Given the sensitivity (0.75) and specificity (0.868) obtained from the One R classifier following 10-fold cross validation, it was notable that more complex classifiers only marginally improved over this simple model which classified on a cut off of the percentage of $CD34^+$ cells. Indeed, it has been reported that simpler classifiers perform very well on a variety of datasets (Holte, 1993). It may be the case that the number of attributes used, in

conjunction with a relatively small sample size, led to “noisy” data. The attribute to sample size ratio may diminish classifier performance and an optimal model may use fewer attributes. Due to the mathematical methods used by the different classifiers, it is unknown from this assessment which attributes other than the percentage of CD34+ cells best discriminate MDS from Reactive cases. However, there are algorithmic methods of choosing attributes (Attribute Selection) and attribute evaluation would be possible on this dataset. However, this is beyond the scope of this thesis and would require more samples and independent datasets to prevent overfitting. Any reduction of the number of attributes would, though, have a two-fold benefit. Firstly, it would make classifiers easier to understand and give insights into any underlying biology of MDS. Secondly, it would allow flow cytometry panels to be simpler and could be applicable to smaller laboratories not wanting to perform extensive flow cytometric testing.

Ultimately, the classifier chosen for further testing on a validation cohort was A1DE. It was the highest overall ranked classifier based on 8 performance metrics and had the joint highest sensitivity using the 10-fold cross validation approach. Although, following the *cost sensitive classification* approach the RBF classifier had the highest sensitivity of any classifier, the specificity of this classifier was poor (0.471) and was therefore disregarded. Lastly, although A1DE is a Bayesian classifier, and a problem of Bayesian classifiers is the assumption of independence between attributes. However, A1DE was formulated to have less of an assumption of independence than Naive Bayes (Webb et al., 2005). The choice of this classifier has then followed the standard approach of learning a classifier on a training set and will be used to make predictions on an unseen, validation cohort with its accuracy measured (Langley, 1988).

Finally, the analysis presented here illustrates that the difficulty of evaluating flow cytometric analysis of MDS is shifting from performing extensive flow cytometric tests, to development of algorithms to best use the sizable data that can be generated. Machine learning algorithms can only aid in this respect. Furthermore, the identification of flow cytometric attributes which may be influential in the diagnosis of MDS may be achieved in the future using attribute selection methods, and through the comparison of MDS cases that are consistently classifiable versus those that represent a challenge for separation using metrics derived from current panels. Creation of larger flow cytometric datasets and correlation with molecular studies and clinical outcome should help.

6 Classifier validation for the identification of MDS from non-diagnostic cytopenic cases

6.1 Rationale and introduction

The diagnostic sensitivity using flow cytometric MDS scoring schemes or classifiers remains an area with potential for development. Two flow cytometry MDS scoring schemes, the Ogata FCM and the Wells FCSS, both have demonstrated high specificity on validation sets (Chu et al., 2011; Della Porta et al., 2012). However, the sensitivity for both was lower with values of 0.69 and 0.75, respectively. The previous results chapter saw the mathematical training of alternative classifiers to discriminate between MDS and Reactive cases. The classifier chosen for further testing, A1DE, demonstrated both high sensitivity and high specificity during this training phase. Importantly, it appeared to generalise well when its performance was assessed following 10-fold cross validation.

To further test the performance of the A1DE classifier, a validation cohort was required. As conventional morphological diagnosis of MDS is affected by subjective bias, classification against other methods for assessing the presence of myeloid dysplasia was also desirable. Cross comparison against published flow cytometric MDS scoring schemes would identify non-diagnostic cases with a strong suspicion of MDS. Comparison against the presence of targeted, annotated MDS-related driver mutations could reveal the incidence of mutations in cytopenic patients and determine whether flow cytometric classification correlated with underlying genetic abnormalities. Furthermore, it would address whether a combination of both flow cytometry and targeted sequencing was required to fully identify all cases of MDS.

6.2 Patient selection

A 13 tube, 8 colour flow cytometry panel designed to examine bone marrow haematopoietic populations was acquired on the bone marrow aspirate samples from 50 patients referred to HMDS. The cohort was divided into samples identified as potential control samples and samples from patients referred for the investigation of cytopenia. The samples were selected on the basis of demonstrating good bone marrow aspirate cellularity, combined with the availability of a trephine biopsy. This was a requirement as, unlike the two tube MDS panel, there was a requirement for at least 13 million leucocytes to be present following erythrocyte lysis. A final stipulation was the requirement for samples to be less than 24 hours old. This

was due to an intended comparison with attributes from ELN recommendations for the assessment of dysplasia by flow cytometry (Westers et al., 2012).

6.2.1 Control group patients

For comparison to published flow cytometric MDS scoring schemes, a reference range was required to define normal antigenic expression. Identification of antigen expression in control cases would permit both the visualisation of normal expression and construction of a numerical reference range. This would then allow the identification of antigen under- and over-expression. Numerically this was defined as above or below 2 standard deviations from the mean of the control cases. For defining a reference range, the bone marrow aspirate from 6 patients with normal full blood count parameters who were referred for lymphoma staging was acquired. 5 of the 6 patients were found to have a bone marrow uninvolved by malignancy. One patient showed focal bone marrow involvement by Hodgkin lymphoma, but no morphological features of myeloid dysplasia. This latter patient was excluded from construction of the reference range. The patient was, however, retained for further analysis to determine whether features of flow cytometric dysplasia-related abnormalities were present in other haematological malignancies. The values obtained from the remaining 5 control patients were subsequently used to denote whether antigen expression was within 2 standard deviations of the mean of the reference range.

6.2.2 Patients referred for the investigation of cytopenia

The remaining 44 patients were referred for the evaluation of cytopenia. Two patients presented with a non-MDS haematological malignancy. One patient was diagnosed with AML, whilst the other patient returned a diagnosis of a B-lymphoproliferative disorder. Analogous to the Hodgkin lymphoma patient, these patients were retained for analysis.

A diagnosis of MDS was returned in 13 patients. Nine patients demonstrated dysplastic changes albeit insufficient for a diagnosis of MDS. A further two patients were reported as suspicious of malignancy but not diagnostic. These 11 patients were labelled as equivocal for classification purposes. The remaining 18 patients were considered pathological controls and labelled as Reactive.

All non-diagnostic patients were re-investigated for a subsequent diagnosis of myeloid malignancy, with a minimum follow up of 29 months. Similarly to previous chapters, one patient was subsequently diagnosed with RCMD, and another with CMML. Both patients had previously been labelled as equivocal. The durations between non-diagnostic and diagnostic samples were 7 and 9 months, respectively. One patient labelled as equivocal was subsequently found to have PNH clones present in the peripheral blood. Two further patients

labelled as Reactive were subsequently found to have PNH clones present in the peripheral blood.

6.3 How successfully does each flow cytometry scoring scheme classify cases against conventional diagnostic criteria?

6.3.1 Ogata FCM Scoring scheme

When compared to the initial diagnoses, the FCM had a sensitivity of 0.62. For calculating specificity, the 5 control samples and the 3 haematological malignancy cases (Hodgkin, B-LPD, and AML) were excluded. This resulted in a cohort of 29 cases with an initial non-MDS diagnosis. 21 of these 29 patients had an FCM score of less than 2 (specificity = 0.72). When the two equivocal patients with subsequent MDS diagnoses were included with the MDS group, the sensitivity increased to 0.67 and the specificity increased to 0.78.

All of the normal control samples were correctly classified, although UPN2 had a perturbed granulocyte side scatter ratio and UPN16 had a perturbed $CD34^+$ myeloid precursor CD45 ratio. 8 out of the 13 MDS patients had a score >2 and were classified as suggestive of MDS. 4 of the 18 Reactive cases were classified as suggestive of MDS. 4 equivocal patients were classified as suggestive of MDS. Included in this equivocal group were the two patients who progressed to RCMD and CMML, who both had scores of 2. The AML patient was classified as suggestive of MDS, whilst both the Hodgkin lymphoma patient and the B-LPD patient had a negative FCM score.

When compared to the initial diagnoses, the FCM had a sensitivity of 0.62. For calculating specificity, the 5 control samples and the 3 haematological malignancy cases (Hodgkin, B-LPD, and AML) were excluded. This resulted in a cohort of 29 cases with an initial non-MDS diagnosis. 21 of these 29 patients had an FCM score of less than 2 (specificity = 0.72). When the two equivocal patients with subsequent MDS diagnoses were included with the MDS group, the sensitivity increased to 0.67 and the specificity increased to 0.78.

6.3.2 Wells FCSS Scoring scheme

The Flow Cytometric Scoring System (FCSS) utilises some of the attributes found in the Ogata score and contains numerous additional attributes (Wells et al., 2003). This scoring scheme was formulated in a prognostic setting for MDS patients, but has subsequently been used in a diagnostic setting (Chu et al., 2011). Due to its origins in a prognostic setting, and to the number of attributes assessed, overall scores are partitioned into 3 groups: normal/mild (0 or 1 points), moderate (2 or 3 points), and severe (4 points and above).

All of the 5 normal control patients were classified as normal/mild, with 3 of the 5 having scores of 1 point. All 13 MDS patients were classified as either moderate (3 patients) or severe (10 patients). 10 of the 18 Reactive controls were classified as moderate with the remaining 8 patients classified as normal/mild. Only one of the 11 equivocal patients was classified as normal/mild, the remainder were classified as moderate (5 patients) or severe (5 patients). In this group, the patient who progressed to RCMD was classified as severe, and the patient who progressed to CMML was classified as moderate. The AML patient was classified as severe, the Hodgkin lymphoma patient as normal/mild, and the patient with B-LPD as moderate.

A diagnostic flow score of 2 or above applied to initial diagnoses, excluding the normal control samples and other haematological malignancies, resulted in a sensitivity of 1 and specificity of 0.31. When the two patients with subsequent diagnoses of MDS were included in the MDS group, the sensitivity remained at 1, whilst the specificity increased to 0.33.

6.3.3 A1DE

The A1DE classifier belongs to the class of Aggregating One Dependence Estimators, a naive-Bayes-like classifier which has a reduced dependence upon the assumption of attribute independence (Webb et al., 2005). This classifier was chosen for further testing on a validation cohort due to its overall performance in the previous results chapter.

All of the normal control samples were correctly classified as non-MDS with high predictive probabilities of 0.99 or higher. 8 of the 13 MDS patients were predicted as MDS. 14 of the 18 Reactive controls were classified as non-MDS, with the remaining 4 classified as MDS. The 4 misclassified cases had predictive probabilities of 0.821 and above. 8 out of the 11 patients in the equivocal group were classified as MDS. The two patients who progressed to RCMD and CMML both were classified as MDS. The A1DE classified the AML patient as MDS, whilst both the Hodgkin lymphoma and B-LPD patients were classified as non-MDS.

Classification with the A1DE classifier according to the initial diagnosis, excluding the normal control samples and the other haematological malignancies, resulted in a sensitivity of 0.62 and specificity of 0.59. When the two patients with subsequent diagnoses of MDS were included in the MDS group, the sensitivity increased to 0.67, whilst the specificity increased to 0.63.

6.3.4 Ensemble learning (majority voting) classification scheme

As each classifier predicted class on the basis of both exclusive attributes and by interpreting common attributes differently, an ensemble learning (majority voting) classification scheme was adopted. Class prediction was defined as two or more of the classifiers predicting the class. This ensemble learning approach was then compared to the morphological diagnosis.

Using this ensemble learning approach, all the normal control samples were classified as non-MDS. Sensitivity over both the Ogata and A1DE classifiers were improved upon for the diagnosis of MDS with 9 out of 13 MDS cases now classified as MDS. 14 of the 18 Reactive controls were classified as non-MDS, with the remaining 4 classified as MDS. 8 out of the 11 patients in the equivocal group were classified as MDS. The two patients who progressed to RCMD and CMML were both classified as MDS. The AML patient was classified as MDS, whilst both the Hodgkin lymphoma and B-LPD patients were classified as non-MDS.

Classification using ensemble learning, according to the initial diagnosis, resulted in a sensitivity of 0.69 and specificity of 0.59. When the two patients with subsequent diagnoses of MDS were included in the MDS group, the sensitivity increased to 0.73, whilst the specificity increased to 0.63.

The combined performances of each classifier compared to conventional morphological diagnosis and to class label is shown in Table 6.1.

Overall, the performance of the three classifiers decreased from expected or published sensitivities and specificities, with two of the classifiers (FCM and A1DE) misclassifying over a third of the MDS group, whilst the other (FCSS) misclassified over two-thirds of the Reactive group. As the antibody panel contained the majority of the attributes recommended for flow cytometry dysplasia assessment according to ELN guidelines and contained antibodies used in other scoring schemes, an evaluation of these could be undertaken to determine whether performance could be improved over the A1DE, FCM, and FCSS.

Patient Number	Diagnosis	Class	Progression	Ogata Score	Ogata Score interpretation	Wells Score	Wells Score Interpretation	A1DE prediction	A1DE Prediction probability	Ensemble Learning Meta-Vote
UPN1	AML NOS	Other		3	MDS	5	severe	MDS	1	3
UPN2	Normal Control	Normal		1	negative FCM score	1	normal/mild	Control	1	0
UPN3	RCMD	MDS		2	MDS	7	severe	MDS	1	3
UPN4	No evidence of disease	Reactive		0	negative FCM score	0	normal/mild	Control	0.555	0
UPN5	See comments	Equivocal		1	negative FCM score	3	moderate	MDS	0.994	2
UPN6	See comments	Equivocal	Yes - PNH	3	MDS	4	severe	MDS	0.984	3
UPN7	Normal Control	Normal		0	negative FCM score	0	normal/mild	Control	0.99	0
UPN8	RCMD	MDS		2	MDS	4	severe	Control	0.979	2
UPN9	Reactive changes only	Reactive		1	negative FCM score	1	normal/mild	Control	0.997	0
UPN10	Normal Control	Normal		0	negative FCM score	0	normal/mild	Control	1	0
UPN11	RCMD	MDS		2	MDS	9	severe	MDS	1	3
UPN12	Classical Hodgkin lymphoma	Other		1	negative FCM score	1	normal/mild	Control	0.999	0
UPN13	Suspicious of malignancy but not diagnostic	Equivocal	Yes – RCMD	2	MDS	4	severe	MDS	0.916	3
UPN14	See comments	Equivocal	Yes - CMML	2	MDS	3	moderate	MDS	0.988	3
UPN15	Reactive changes only	Reactive		1	negative FCM score	3	moderate	Control	0.999	1
UPN16	Normal Control	Normal		1	negative FCM score	1	normal/mild	Control	0.999	0
UPN17	Reactive changes only	Reactive	Yes - PNH	1	negative FCM score	3	moderate	Control	0.828	1
UPN18	See comments	Equivocal		0	negative FCM score	3	moderate	MDS	0.561	2

UPN19	RCMD	MDS		1	negative FCM score	2	moderate	MDS	0.684	2
UPN20	Normal Control	Normal		0	negative FCM score	1	normal/mild	Control	0.999	0
UPN21	RCMD	MDS		1	negative FCM score	4	severe	Control	0.792	1
UPN22	RCMD	MDS		1	negative FCM score	3	moderate	Control	0.797	1
UPN23	RCMD	MDS		1	negative FCM score	4	severe	Control	0.981	1
UPN24	Reactive changes only	Reactive		2	MDS	1	normal/mild	Control	1	1
UPN25	See comments	Reactive		2	MDS	3	moderate	MDS	1	3
UPN26	RCMD	MDS		3	MDS	5	severe	MDS	0.923	3
UPN27	CD5-negative B-cell LPD NOS	Other		1	negative FCM score	2	moderate	Control	1	1
UPN28	Reactive changes only	Reactive		1	negative FCM score	2	moderate	MDS	0.98	2
UPN29	Anaemia of Chronic Disease	Reactive		0	negative FCM score	2	moderate	Control	0.993	1
UPN30	RCMD	MDS		3	MDS	2	moderate	MDS	0.577	3
UPN31	Suspicious of malignancy but not diagnostic	Equivocal		1	negative FCM score	2	moderate	Control	0.999	1
UPN32	Reactive changes only	Equivocal		0	negative FCM score	1	normal/mild	MDS	0.693	1
UPN33	See comments	Reactive	Yes - PNH	1	negative FCM score	1	normal/mild	Control	0.992	0
UPN34	Reactive changes only	Reactive		0	negative FCM score	2	moderate	Control	1	1
UPN35	Reactive changes only	Reactive		0	negative FCM score	0	normal/mild	Control	1	0
UPN36	Reactive changes only	Reactive		0	negative FCM score	0	normal/mild	Control	0.931	0
UPN37	See comments	Equivocal		2	MDS	4	severe	Control	1	2

UPN38	See comments	Equivocal		1	negative FCM score	5	severe	MDS	0.998	2
UPN39	Reactive changes only	Reactive		2	MDS	3	moderate	Control	0.999	2
UPN40	Reactive changes only	Reactive		2	MDS	3	moderate	MDS	1	3
UPN41	Reactive changes only	Reactive		0	negative FCM score	1	normal/mild	Control	0.99	0
UPN42	Reactive changes only	Reactive		1	negative FCM score	1	normal/mild	MDS	0.821	0
UPN43	Reactive changes only	Reactive		1	negative FCM score	3	moderate	Control	1	1
UPN44	See comments	Equivocal		1	negative FCM score	5	severe	Control	0.785	1
UPN45	No evidence of disease	Reactive		0	negative FCM score	3	moderate	Control	1	1
UPN46	RAEB	MDS		4	MDS	9	severe	MDS	1	3
UPN47	RAEB	MDS		3	MDS	6	severe	MDS	1	3
UPN48	RARS	MDS		1	negative FCM score	4	severe	Control	0.683	1
UPN49	RCMD	MDS		2	MDS	6	severe	MDS	1	3
UPN50	See comments	Equivocal		1	negative FCM score	2	moderate	MDS	1	2

Table 6.1. Classifier performance versus initial diagnosis.

The 50 patients were labelled as MDS, Reactive, Other, Equivocal, or Normal control and the diagnosis and label was compared against the Ogata FCM, the Wells FCSS, the A1DE classifier, and the ensemble learning scheme. Progression refers to patients who had an initial non-diagnostic bone marrow but who were subsequently diagnosed with a malignancy.

6.3.5 Comparison against other reported flow cytometry abnormalities and evaluation against ELN recommendations

Other flow cytometric abnormalities have been reported and two notable reports show diagnostic utility in distinguishing MDS from other cytopenic conditions. Decreased CD38 expression on CD34 expressing progenitor cells has been shown to have high sensitivity and specificity for MDS (Goardon et al., 2009). Whilst the RED score is a three parameter scoring scheme using CD36 CV, CD71 CV and haemoglobin level to evaluate erythroid dysplasia (Mathis et al., 2013).

6.3.5.1 Use of CD38 expression to distinguish MDS from cytopenic patients

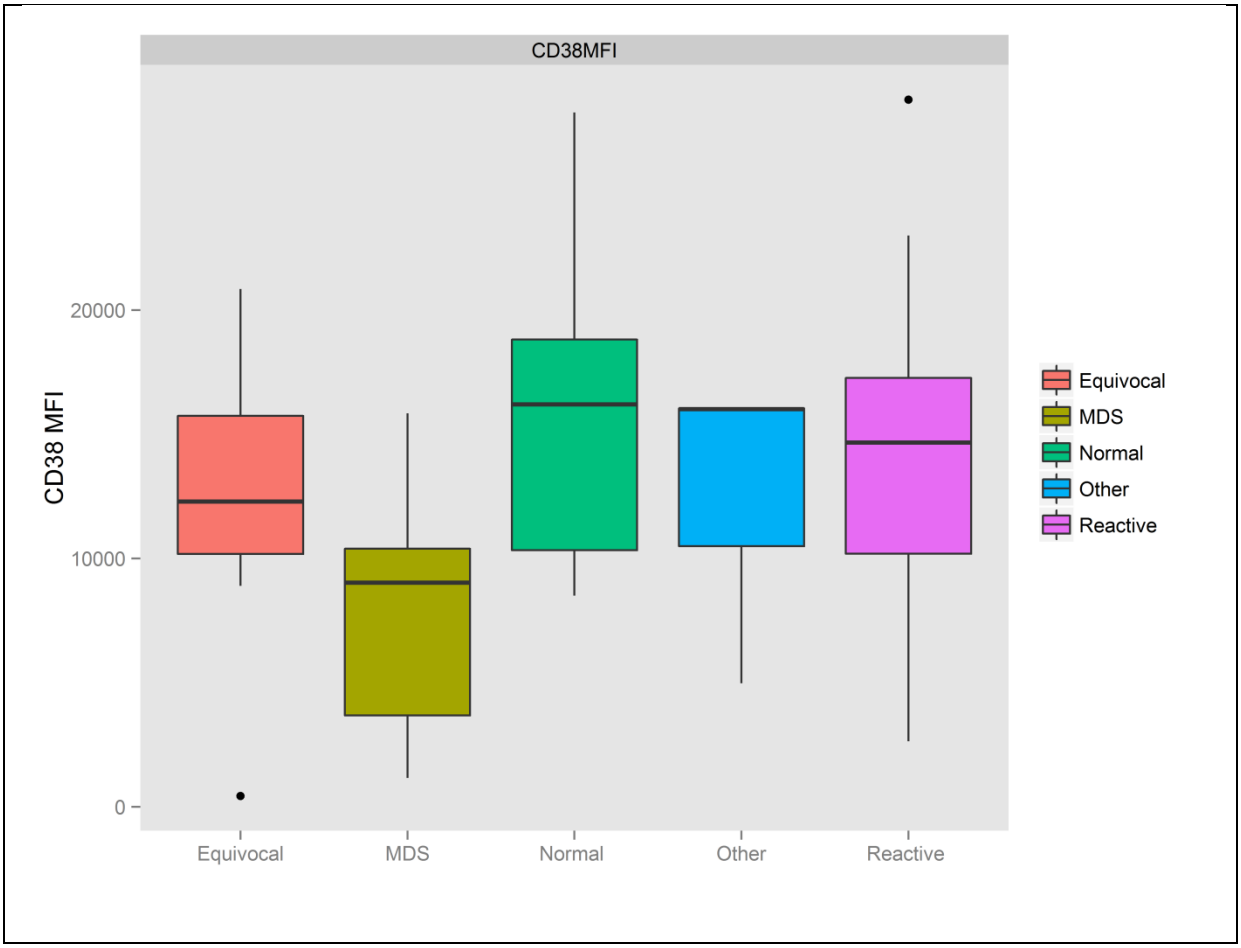


Figure 6.1. Box and whisker plots showing the CD38 Median fluorescent intensity on CD34$^+$ myeloid progenitors.

The groups were labelled as described in Table 6.1.

From the box and whisker plot, there appeared to be good discrimination between the MDS group and the other groups. However, when a reference range was created using the normal controls, no MDS patient had a CD38 MFI below the bottom of the reference range (Mean+/-2SD).

For discriminating between MDS and other cytopenias, Goardon *et al.* used a cut-off value for CD38 MFI (Goardon et al., 2009). However, for that study, CD38 was used as a PE-Cy5 fluorochrome and the MFI was calculated as a ratio to isotype control. As the CD38 fluorochrome used for this results chapter was PerCP-Cy5-5, and no isotype control was used, a new cut-off for CD38 was required. To prevent overfitting, the cut-off was determined using the training set of MDS and Reactive control groups used in Chapter 5. For the ROC curve analysis itself, the shortest Euclidean distance to (0,1) was used to determine the cut-point Figure 6.2. This gave a CD38 MFI cut-off of 8263. When this cut-off value was applied to the training set, it resulted in a sensitivity of 0.71 and a specificity of 0.79. For this validation cohort, when applied to the initial diagnosis, this cut-off resulted in a sensitivity of 0.46 and a specificity of 0.86. When the two patients with a subsequent MDS diagnosis were included in the MDS class the sensitivity decreased to 0.4 and the specificity decreased slightly to 0.85.

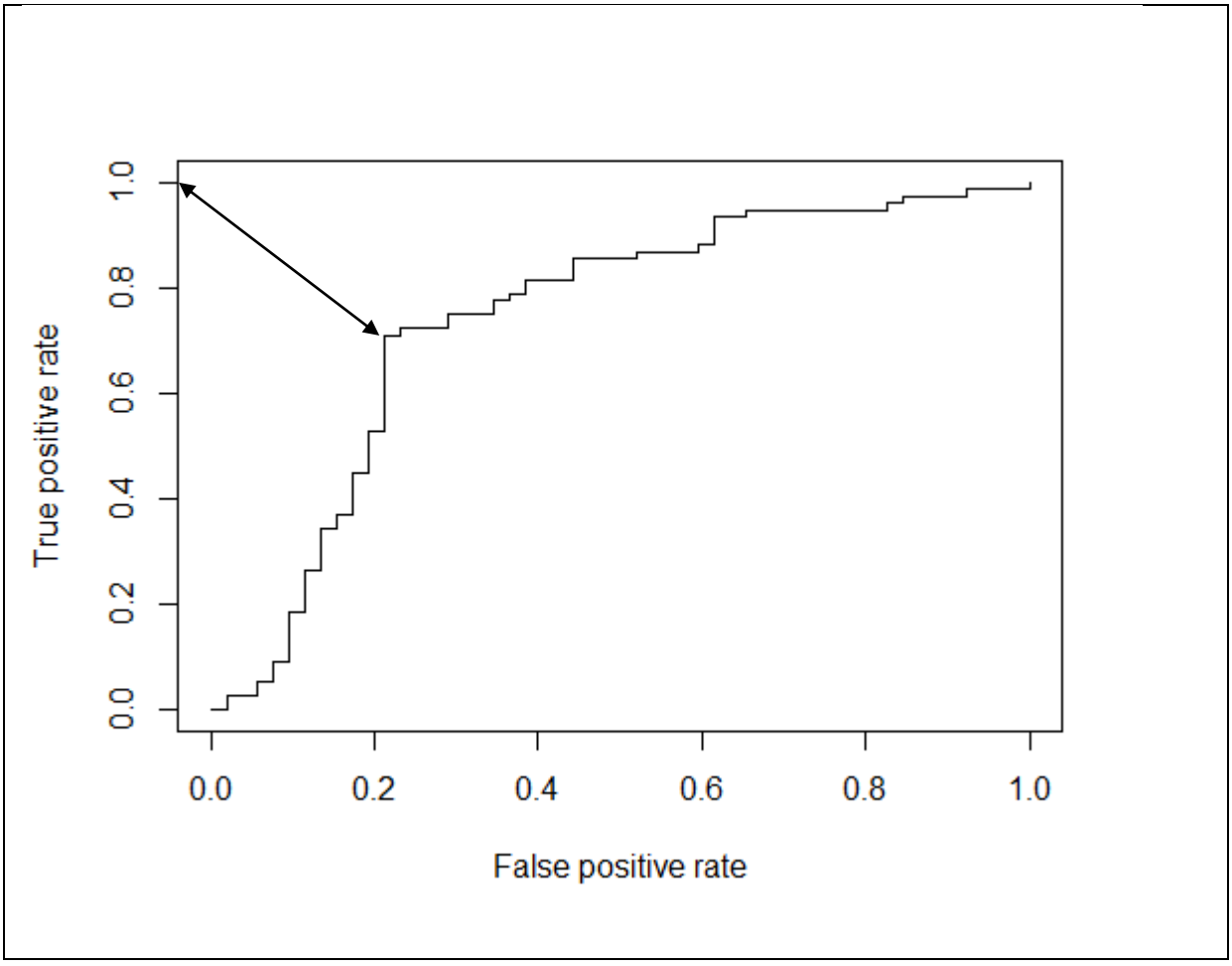


Figure 6.2. ROC curve analysis for CD38 MFI on CD34
The cut-off for CD38 MFI was determined on the training cohort by the shortest Euclidean distance to (0,1), as shown by the double-headed arrow.

6.3.5.2 RED score and evaluation of erythroid dysplasia

Due to an absence of FBC data, a formal comparison to the RED score could not be undertaken. However, a phenotypic comparison using CD36 and CD71 was undertaken to determine the prevalence of erythroid dysplasia in this cohort. Using published CV cut-offs of 65% for CD36 and 80% for CD71, 1 MDS case had an abnormal CD36 and abnormal CD71, no patients had an abnormal CD71 alone, and 8 cases had an abnormal CD36 alone. Of the 8 cases with an abnormal CD36 alone, 6 were MDS cases, 1 case was a patient subsequently demonstrated to have PNH clones and the remaining case was the patient in the *Other* class who was diagnosed with AML.

However, the CD71 antibody for this results chapter was a different fluorochrome (and possibly a different clone; this detail was absent in the publication) to that published (Mathis et al., 2013). It was unclear whether published cut-offs would be valid when using different antibodies and a different flow cytometer. Unlike CD38 expression, CD71 expression could not be assessed in the training cohort and an independent cut-off could not be determined. Therefore, an approach using an upper limit of 2 SD above the mean of the control samples was adopted. This resulted in CV cut-offs for this cohort of 63% and 67%, for CD36 and CD71 respectively. When these new CV cut-offs were applied, 2 patients had abnormal CD36 and CD71 and both patients were diagnosed with RCMD. 5 patients had an abnormal CD71 alone and all belonged to the Reactive class. 10 patients had an abnormal CD36 alone. 6 patients were MDS cases, 1 patient was in the equivocal class and subsequently demonstrated to have PNH clones, 2 patients were in the Reactive class and the remaining case was the patient in the *Other* class who was diagnosed with AML. Table 6.2 shows the results of the assessment of CD38, and of the erythroid antigens CD36 and CD71 per patient.

Patient Number	Class	CD38MFI on CD34	Erythroid CD36CV	Erythroid CD71 CV
UPN1	Other	abnormal	abnormal	normal
UPN2	Normal	normal	normal	normal
UPN3	MDS	abnormal	abnormal	abnormal
UPN4	Reactive	normal	normal	abnormal
UPN5	Equivocal	normal	normal	normal
UPN6	Equivocal	abnormal	abnormal	normal
UPN7	Normal	normal	normal	normal
UPN8	MDS	normal	normal	normal
UPN9	Reactive	normal	normal	normal
UPN10	Normal	normal	normal	normal
UPN11	MDS	abnormal	abnormal	abnormal
UPN12	Other	normal	normal	normal
UPN13	Equivocal	normal	normal	normal
UPN14	Equivocal	normal	normal	normal
UPN15	Reactive	normal	normal	normal
UPN16	Normal	normal	normal	normal
UPN17	Reactive	normal	normal	abnormal
UPN18	Equivocal	normal	normal	normal
UPN19	MDS	abnormal	abnormal	normal
UPN20	Normal	normal	normal	normal
UPN21	MDS	normal	normal	normal
UPN22	MDS	normal	normal	normal
UPN23	MDS	normal	normal	normal
UPN24	Reactive	normal	normal	normal
UPN25	Reactive	abnormal	abnormal	normal
UPN26	MDS	abnormal	abnormal	normal
UPN27	Other	normal	normal	normal
UPN28	Reactive	normal	abnormal	normal
UPN29	Reactive	normal	normal	abnormal
UPN30	MDS	normal	abnormal	normal
UPN31	Equivocal	normal	normal	normal
UPN32	Equivocal	normal	normal	normal
UPN33	Reactive	normal	normal	normal
UPN34	Reactive	normal	normal	abnormal
UPN35	Reactive	abnormal	normal	normal
UPN36	Reactive	normal	normal	normal
UPN37	Equivocal	normal	normal	normal
UPN38	Equivocal	normal	normal	normal

UPN39	Reactive	normal	normal	normal
UPN40	Reactive	abnormal	normal	normal
UPN41	Reactive	normal	normal	abnormal
UPN42	Reactive	normal	normal	normal
UPN43	Reactive	normal	normal	normal
UPN44	Equivocal	normal	normal	normal
UPN45	Reactive	normal	normal	normal
UPN46	MDS	abnormal	abnormal	normal
UPN47	MDS	abnormal	normal	normal
UPN48	MDS	normal	abnormal	normal
UPN49	MDS	normal	abnormal	normal
UPN50	Equivocal	normal	normal	normal

Table 6.2. Assessment of CD38 and erythroid-related flow cytometric aberrant phenotypic features.

A reduction in CD38 expression on $CD34^+$ myeloid cells and decreased CD36 CV and CD71 CV on erythroid cells was defined as abnormal.

6.3.5.3 ELN recommendations

Although in itself not a scoring scheme, the European LeukemiaNet MDS Flow Cytometry Working Group has produced recommended minimal requirements for evaluation of dysplasia in MDS (Westers et al., 2012). These recommendations are a mixture of 31 visual aberrancies and numerical attributes, and include the 4 Ogata FCM attributes and several of the Wells FCSS attributes. The antibody panel used in this results chapter allowed assessment of 28 of the 31 ELN recommended attributes. The 3 non-assessable attributes were: the relationship of CD15 and CD10 on maturing neutrophils; the relationship of CD36 and CD14 on monocytes; and the relationship of CD71 and CD235a in the erythroid compartment.

As the attributes of the ELN recommendations have not been incorporated into a single scoring system, it was hypothesized that summing the total number of aberrancies may be able to distinguish between MDS from other non-MDS cytopenias. Although the normal control samples scored low for the number of phenotypic aberrancies (median = 1), there was overlap between the MDS (median = 9) and equivocal (median = 6) and Reactive (median = 5) groups, as seen in Figure 6.3.

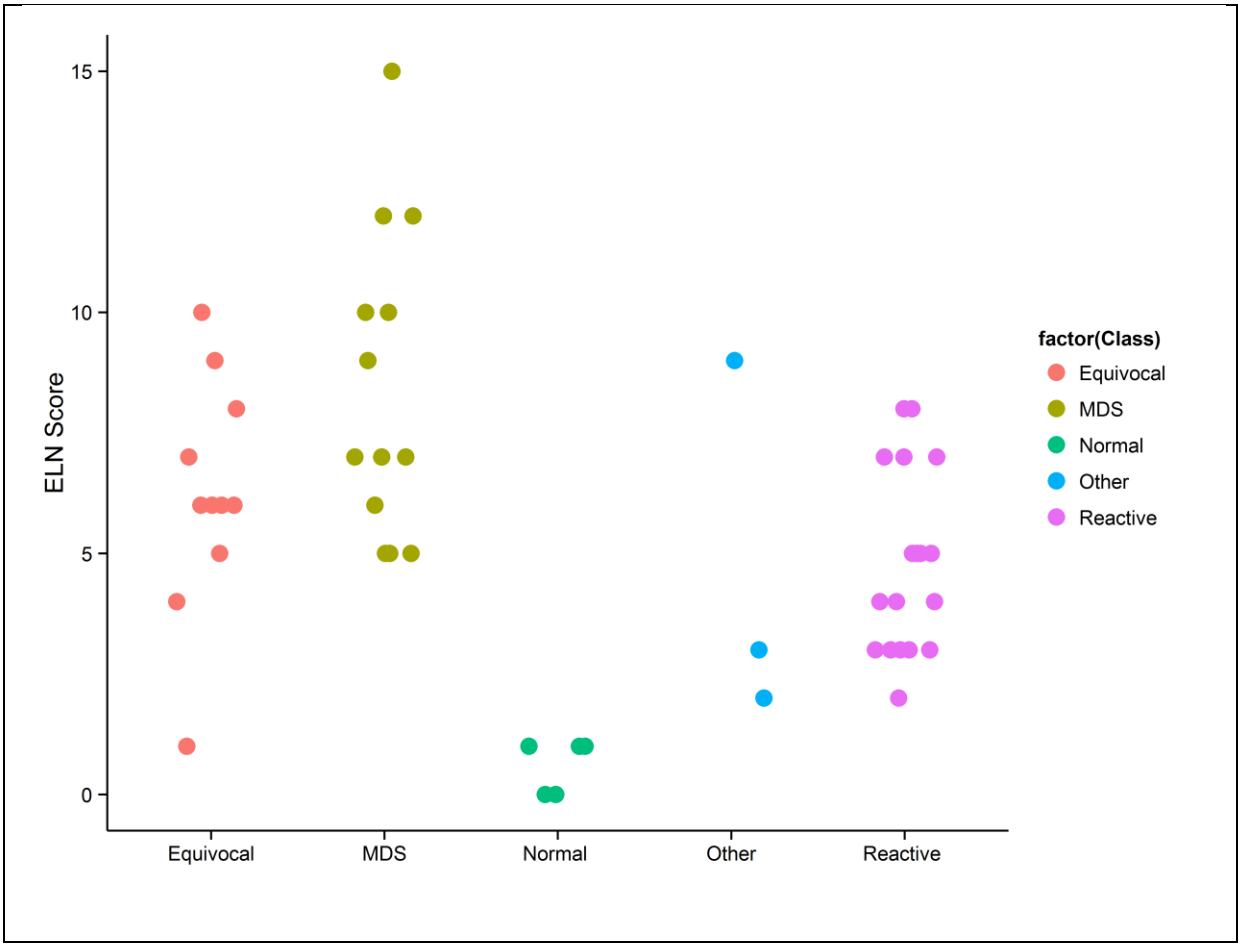


Figure 6.3. The number of ELN phenotypic aberrancies per class.
A total of 28 of the ELN recommended phenotypic aberrancies were assessed to attempt to discriminate between the MDS and the other classes.

Although the number of phenotypic aberrancies did not appear to distinguish MDS from the other classes, it was hypothesized that there may be a phenotypic signature (or signatures) of aberrancies which could identify MDS cases. If such signatures were present, these could, potentially, identify cases with non-dysplastic morphology which were at risk of progression to MDS. To evaluate this further, unsupervised hierarchical clustering was performed and a heatmap generated to determine whether signatures were apparent (Figure 6.4). The clustering showed that MDS cases did not group as a distinct cluster and multiple clusters composed of MDS, reactive, and ambiguous cases existed. The 5 normal control cases were found to cluster together, and these cases were hierarchically distinct from the MDS cases. Therefore, whilst the number of ELN abnormalities was effective at distinguishing Normal from MDS, this approach failed to separate the Reactive or Equivocal groups from the MDS group.

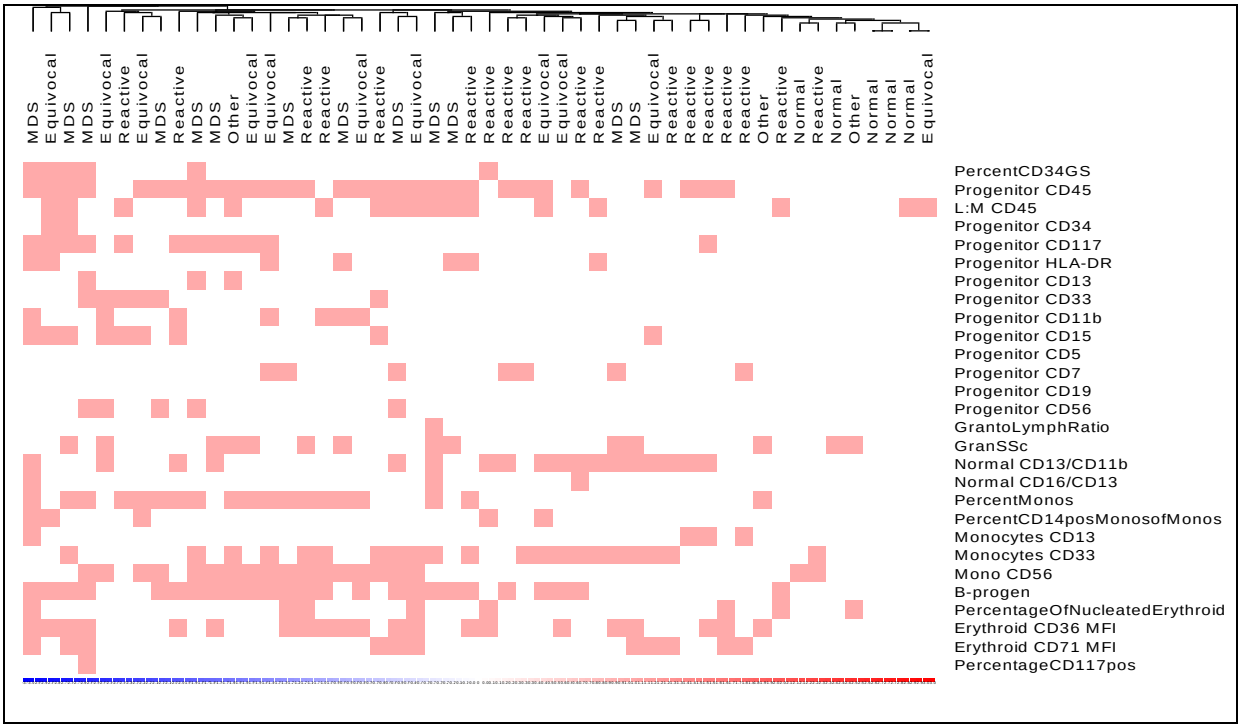


Figure 6.4. Unsupervised hierarchical clustering using the presence of ELN recommended flow cytometric abnormalities.

The normal control samples cluster towards the right of the heatmap with cases in which there is a low number, or absence, of abnormalities. The ELN abnormalities are ordered as encountered in table 1 of the ELN recommendations (Westers et al., 2012). The colour red denotes attribute abnormality, whilst white indicates no abnormality. Average linkage was used as the clustering distance metric and clustering was performed on samples but not flow attributes.

6.4 Comparison of flow classifiers to targeted sequence analysis of MDS-related driver mutations

The presence of a clonal genetic abnormality provides an alternative indicator of abnormality within bone marrow haematopoietic cells. Furthermore, it is less subject to observer bias than conventional morphology. Therefore, to determine if flow cytometry correlated with an underlying MDS-related driver mutation, or if a combination of flow cytometry and presence of mutations better identified MDS cases, targeted sequence analysis of annotated driver mutations was performed on 41 cases: 12 MDS cases, 17 Reactive cases, and 11 equivocal cases. Sequence analysis was not performed on the 5 normal control patients, the Hodgkin lymphoma and B-LPD patients, and UPN8 (MDS class) and UPN25 (Reactive class).

6.4.1 Does initial diagnosis correlate with the presence of a driver mutation?

In the first instance, a comparison of the presence of annotated driver mutation against conventional morphological diagnosis was performed. An annotated mutation was identified in 23/41 cases (56%) with a median of 2 annotated mutations per patient (range 1-7). All 12 MDS patients that were tested showed at least 1 annotated driver mutation with a median of 2 (range 1-7). The AML patient demonstrated 5 annotated mutations.

14 of the 17 Reactive patients had no mutations. Three Reactive patients demonstrated at least one mutation. UPN40 had 3 annotated mutations, whilst UPN42 had 2 annotated mutations. The remaining Reactive case (UPN 43) had an *ASXL1* mutation (4189G>A) which has been reported as both a driver mutation and a SNP. For comparative purposes, this was considered to be a driver mutation.

7 of the 11 equivocal cases demonstrated at least 1 annotated mutation (range 1-5, median of 2). One equivocal patient (UPN32) showed an *ASXL1* variant of undetermined significance. This was not considered to be a driver mutation. The remaining three equivocal patients showed no mutations. The patient who subsequently progressed to RCMD did not demonstrate any mutation, whilst the patient who progressed to CMML showed two mutations in the *TET2* gene and a mutation in *ZRSR2*. All annotated mutations and variants of undetermined significance for each patient in this cohort are shown in Appendix Table 6.1.

6.4.2 Comparison of flow classifiers versus presence of mutation

To evaluate whether abnormalities detected by flow cytometry correlated with the presence of driver mutations, the performance of each of the flow classifiers was determined for the presence (n=23) or absence (n=18) of a driver mutation. For the purposes of performance evaluation, the original class for each case was disregarded. Instead, cases with the presence of a mutation were considered to be the disease (MDS) class, and cases with an absence of mutation to be the control class.

11 patients with annotated mutations had an FCM score of 2 or higher, resulting in a sensitivity of 0.48. The specificity of the FCM was 0.78 with 14 out of 18 patients without an annotated mutation having an FCM score of 0 or 1.

For the FCSS score, 22 patients with annotated mutations were classified as moderate or severe (FCSS score of 2 or above). This resulted in a sensitivity of 0.96. The specificity of the FCSS was 0.44. 8 out of 18 patients without an annotated mutation were classified as normal/mild by the FCSS.

16 out of 23 patients with annotated mutations were classified as MDS by the A1DE classifier (0.70 sensitivity). This classifier had a specificity of 0.78 with 14 out of 18 patients without an annotated mutation predicted to be non-MDS.

Using the ensemble learning approach, 15 out of 23 cases with annotated mutations were classified as MDS, resulting in a sensitivity of 0.65. The specificity was 0.72 with 13 cases without annotated mutations classified as non-MDS. The 8 cases with an annotated mutation who were not classified as MDS demonstrated either 1 (n=4) or 2 (n=4) annotated mutations. Although, these cases did not have a ensemble learning classification of MDS by virtue of scoring less than 2, all 8 cases were classified as MDS by either the FCSS (n=7) or the A1DE classifier (n=1).

Figure 6.5 shows the results of each scoring scheme/classifier versus presence or absence of annotated mutation, and the number of mutations, for each class.

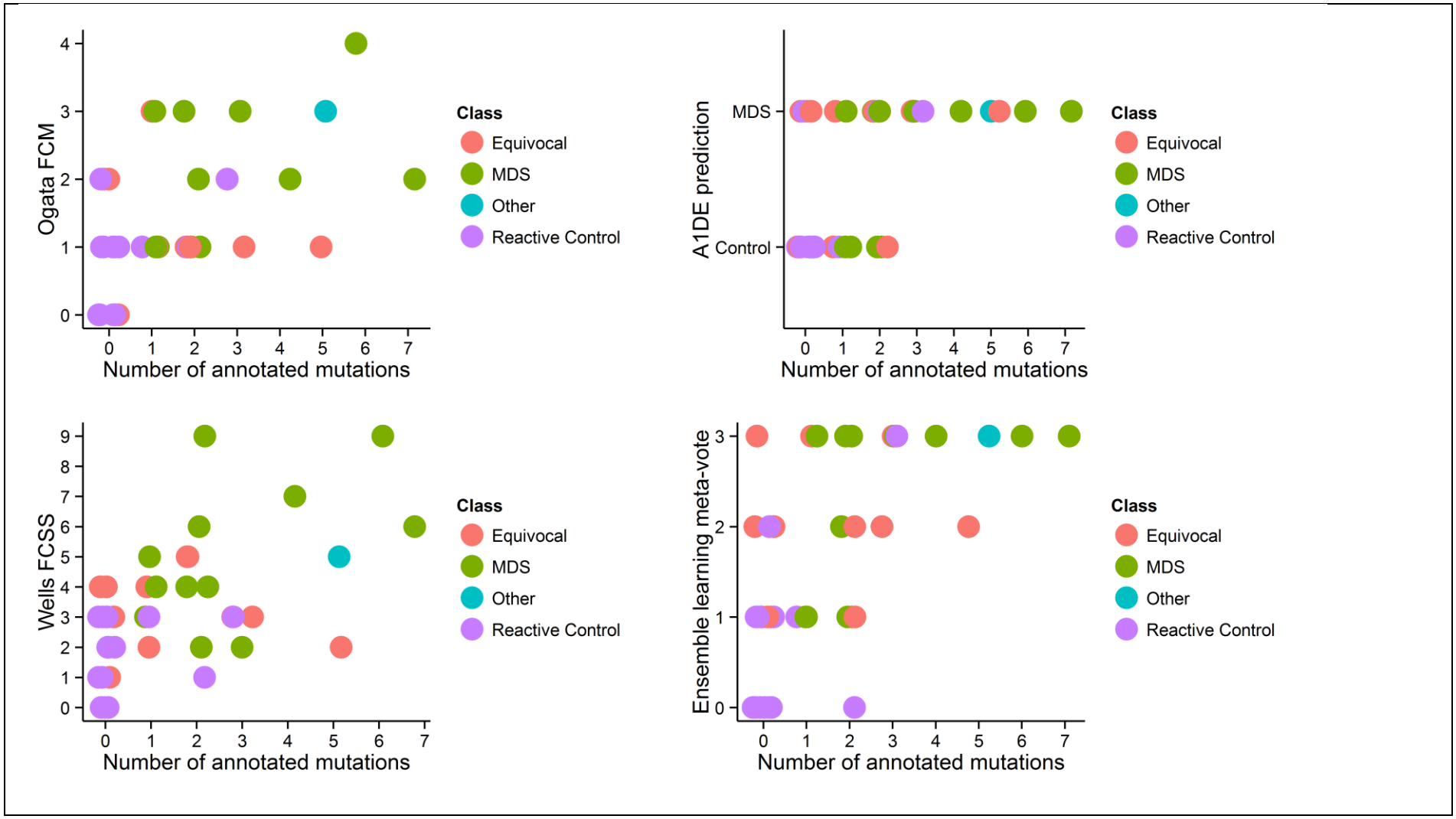


Figure 6.5. Number of annotated mutations versus flow cytometry classifier score/prediction per class label.

Jitter has been added to the x-axis to allow visualisation of all cases.

6.4.3 Does the number of flow abnormalities correlate with the number of annotated mutations

It was noted that 3 of the 5 of MDS cases with the highest Wells FCSS score were also amongst the top 5 cases with the highest number of annotated mutations. It was hypothesized that the number of flow abnormalities might correlate with number of annotated mutations. To assess this, an exploratory analysis comparing the number of ELN recommended flow cytometry abnormalities to the number of annotated mutations was performed as shown in Figure 6.6.

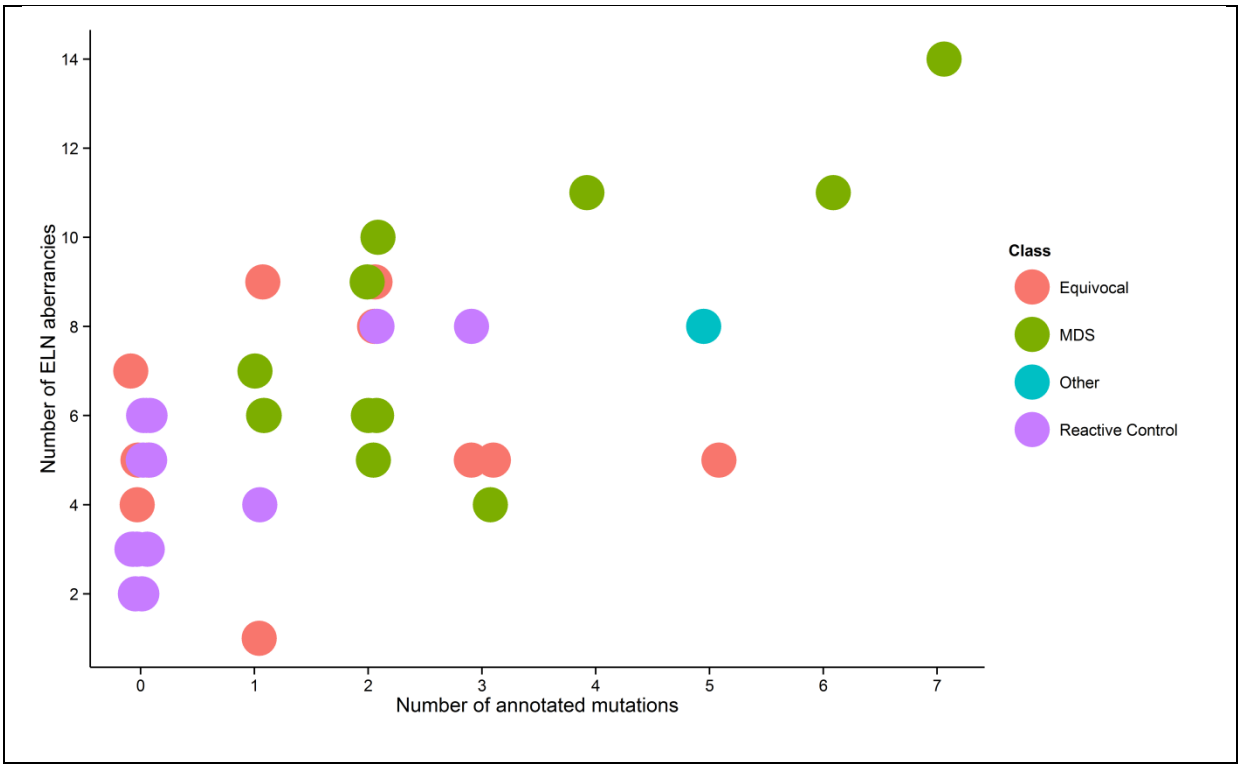


Figure 6.6. The number of annotated mutations versus the number of ELN flow cytometry aberrancies per class.

Jitter has been added to both the x-axis and y-axis to allow visualisation of all cases.

The two cases with the most annotated mutations also had the two highest numbers of flow abnormalities. With the exception of one case (UPN31), all cases with an annotated mutation had 4 or more flow cytometric aberrancies present. The equivocal case (UPN31) with an annotated mutation but only 1 flow cytometric aberrancy demonstrated an SRSF2 mutation (284C>T) with an allelic burden of 9.74%.

6.4.4 Does the presence of specific mutations give rise to phenotypic signatures?

To evaluate whether the presence of mutation in a specific gene correlated with a specific flow abnormality, a visual analysis of these features per patient was performed (Figure 6.7). The plot demonstrated heterogenetity of both flow aberrancies and, to a lesser extent, genetic mutations. No obvious visual pattern was evident. However, it is known that the presence of a mutation in *SF3B1* correlates strongly with the presence of ring sideroblasts (Papaemmanuil et al., 2011). Furthermore, an increase in the CV of CD36 or CD71 on erythroid cells correlates with the presence of morphological dyserythropoiesis (Mathis et al., 2013). Therefore, it was hypothesized that patients with SF3B1 may have a specific pattern of aberrant flow cytometric erythroid attributes and this was examined further.

An identical, annotated *SF3B1* mutation was found in 4 out of the 12 tested MDS cases (UPN5, UPN23, UPN26, and UPN48). One case (UPN5) demonstrated a second annotated *SF3B1* mutation. The erythroid flow cytometric phenotypic features for these cases were compared to determine if there was a phenotypic signature common to cases with an *SF3B1* mutation (Table 6.3). Although there were common features in all cases (the CD71CV, the percentage of erythroid cells, and the percentage of erythroid cells expressing CD117), there were inter-group differences with respect to CD36 MFI and CD71 MFI, and no specific signature was present in these 4 cases.

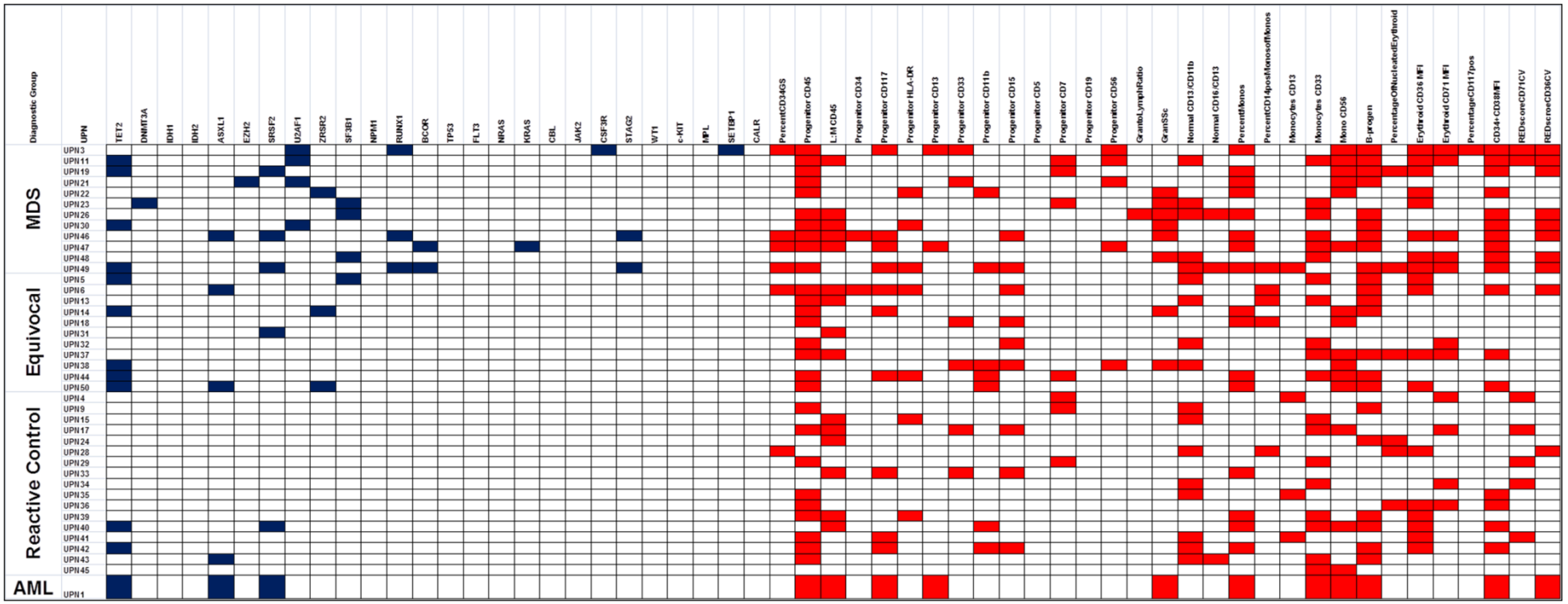


Figure 6.7. Presence of driver mutations and presence of flow cytometry aberrancies per patient.

The patients are grouped by diagnostic category. A blue square denotes the presence of an annotated gene mutation whilst a red square denotes the presence of a flow cytometric abnormality.

UPN	CDS	VAF (%)	Protein	Consequence	CD36 CV	CD71 CV	% nucleated erythroid cells	CD36 MFI	CD71 MFI	%CD117^{+}
UPN5*	2098A>G 1998G>C	40.31 6.74	Lys700Glu Lys666Asn	Missense variant	normal	normal	normal	abnormal	normal	normal
UPN23**	2098A>G	45.09	Lys700Glu	Missense variant	normal	normal	normal	abnormal	normal	normal
UPN26	2098A>G	28.63	Lys700Glu	Missense variant	abnormal	normal	normal	normal	normal	normal
UPN48	2098A>G	48.15	Lys700Glu	Missense variant	abnormal	normal	normal	abnormal	abnormal	normal

Table 6.3. Comparison of the presence of *SF3B1* mutations to the presence of flow cytometric erythroid abnormalities
CDS is coding sequence. VAF is variant allelic fraction. *UPN5 demonstrated a co-existing *TET2* annotated mutation in addition to the two *SF3B1* mutations. **UPN23 demonstrated a co-existing *DNMT3A* mutation.

6.5 Discussion

6.5.1 Defining a typical sample cohort and adequate control samples

The specific sample requirements (cellular material less than 24 hours old) to allow comparison using ELN recommendations placed an artificial restriction on the cases which could be evaluated. The concern was that these restrictions could have resulted in a non-representative cohort of referred samples for the investigation of cytopenia, as many samples in referral practice of the host laboratory will be processed with delay of over 24 hours in routine practice. The proportion of MDS cases within this cohort (13 patients out of 42 cases – 31%) is slightly higher than the proportion in the first results chapter and the figures reported in the literature (Beloosesky et al., 2000; Kwok et al., 2015). This increase may reflect the better certainty when assessing a cellular specimen or is possibly just a chance increase.

One reason for the requirement for testing within 24 hours of aspiration is due to an alteration in both granulocyte scatter characteristics and expression of CD11b with increasing age of sample. Side scatter is a key component of the FCM, whilst examination of CD11b patterns of expression is a key component of the FCSS. However, in the setting of a diagnostic referral centre, a sample may be referred from an external source and be older than 24 hours. This renders the routine use of the FCM or the FCSS to be problematic in the diagnostic setting of HMDS, which is representative of practice across the NHS.

Although all the samples in this cohort were less than 24 hours old, it also appeared that the availability of a cellular bone marrow sample less than 24 hours old does not preclude morphological uncertainty and 11 cases produced an equivocal diagnostic result. Somewhat unsurprisingly, and as discussed in previous results chapters, a proportion of non-diagnostic cases (in this cohort 2 out of the 11 equivocal cases) progressed to MDS.

The lack of recommendations for producing reference ranges for normal bone marrow attributes is a difficulty with respect to the flow cytometric experimental procedure which is not widely discussed. However, the methodology used for the previous results chapters negated the requirement for a pre-determined reference range.

ELN recommendations currently do not specify the number of control patients required to determine a reference range for normal expression of antigens. In published MDS flow cytometry studies, the number (and percentage) of *normal* control cases, independent of pathological controls, varies widely and numerous studies lack inclusion of normal controls (summarised in the ELN recommendations (Westers et al., 2012)). A figure of 120 instances has been cited as the number required for creating a reference interval (Jones and Barker,

2008). This number could theoretically double, if separate reference intervals were required for males and females. This may be unfeasible if normal controls are required, especially when considering the invasive procedure for obtaining a bone marrow aspirate biopsy. This may be further restricted by a potential requirement to obtain age and gender matched controls specific to the MDS.

One difficulty arises from what constitutes a normal control group to determine reference ranges for antigen over- and under-expression. An advantage of using a numerical range lies in the ability to define whether antigen expression is within 2 standard deviations from the mean of the control population. An alternative approach is to use a visual "*deviation from normal pattern"* approach to define antigen over- and under-expression. This approach was adopted by Wells *et al.* for the FCSS (Wells et al., 2003).

6.5.2 Evaluation of flow classifier performance

The cohort in this study differed from those in published flow cytometric studies of MDS by virtue of the initial diagnostic classification. As summarised by Westers *et al*. cases used in published flow cytometry scoring scheme studies are either MDS, so-called pathological controls/Reactive cases, and frequently but not always, control samples (Westers et al., 2012). The number of cases in these studies is predominantly skewed towards MDS relative to pathological controls, a feature inconsistent with findings in a diagnostic setting, where the reverse is generally the case. In previous studies ambiguous or equivocal cases have frequently not been included or addressed. Whilst, understandably, well-defined populations are required when training a classifier, testing of performance on an unseen, real-world, validation cohort including ambiguous cases is desirable. However this comes with the caveat that the ambiguous nature of the cases, precludes a definite conclusion in the absence of prolonged follow up to determine the biological course of disease.

In the cohort in this results chapter, 11 equivocal cases were present. Although this accurately portrayed a real world scenario, it presented a challenge as to how best to compare classifier performances as these cases fall into a "grey zone" between MDS and Reactive cases. These cases were given the label equivocal as they did not have a confirmed diagnosis of MDS. Interestingly, two cases progressed to RCMD and CMML, which underlined the value of follow up and repeat assessments in such instances. Classifier performance as judged by the sensitivity and specificity was calculated twice by including these two cases firstly in the non-MDS class and then in the MDS class.

The use of a single attribute, CD38 MFI expression on $CD34^+$ myeloid cells, to discriminate between MDS and Reactive cases was conceptually similar to the use of the OneR classifier (Holte, 1993). Like most classification schemes, the sensitivity of classification using this

attribute dropped between the training set and the validation cohort, with a resultant sensitivity of under 0.5. Furthermore, use of CD38 did not classify the two equivocal cases which progressed to RCMD and CMML as MDS. However, the specificity of this method was found to be high (>0.85). Overall, the results of this classifier served as a baseline to indicate whether more complex classifiers allowed improved sensitivity through increased complexity.

When compared to the initial morphological diagnosis, the 3 classifiers (FCM, FCSS, and A1DE) all showed better sensitivities than the use of CD38 alone. FCM and A1DE had identical sensitivities of 0.62 whilst the FCSS had a sensitivity of 1. Unlike the use of CD38, all 3 classifiers classified the two equivocal cases, which progressed to RCMD and CMML, as MDS. Again, this highlights the use of flow cytometry in cases with equivocal morphology. However, in contrast to the use of CD38, they all demonstrated lower specificities with the FCM showing the highest specificity. This would indicate that the A1DE was the worst performing classifier with its performance decreasing considerable from the training set.

However, in routine practice, there are drawbacks to the use of the FCM and FCSS, and advantages of using the A1DE classifier. Firstly, both the FCM and the FCSS use attributes which are time sensitive and which should be tested within 24 hours (side scatter and CD11b, respectively). Neither attribute is used in the A1DE classifier, therefore age of sample is not an issue. Secondly, the FCSS requires a visual interpretation of antigen differentiation patterns. This approach requires prior experience of antigenic differentiation patterns and may not be suitable for laboratories with little or no previous experience. Furthermore, unlike the morphological diagnoses of MDS, where the problems of inter-observer variability have been widely reported, there has been no formal evaluation of inter-observer variability in recognising visual aberrancies using flow cytometric antigenic differentiation patterns. Thirdly, unlike the FCM and FCSS, the A1DE classifier outputs a prediction probability, which allows the user to make a judgment upon the confidence of classification. Finally, although the FCSS did not misclassify any MDS case *per se*, its specificity was found to be low (0.31). When assessing the sensitivity and specificity together, it could be seen that the FCSS was over-classifying the majority of cases as MDS.

To dilute this effect of over-classification by FCSS and, as A1DE and FCM differed in the classification of 2 MDS cases (UPN8 and UPN19), an ensemble learning/majority-vote approach was implemented. When this ensemble learning approach was compared to initial diagnosis, the sensitivity and specificity were 0.69 and 0.59, respectively. The sensitivity was improved over both FCM and A1DE and the specificity was improved over FCSS. Following reclassification of the RCMD and CMML cases, the sensitivity and specificity improved further to 0.73 and 0.63, respectively. However, the implementation of this ensemble

learning in a routine setting may not be practical. Moreover, this approach still misclassified over a quarter of MDS cases and over a third of Reactive cases.

6.5.3 ELN recommendations

The ELN recommended minimal requirements to assess flow cytometric dysplasia consist of 31 different attributes generated from a mixture of published studies (Westers et al., 2012). Multiple haematopoietic cells and lineages are evaluated and analyses consist of evaluation of numerical, phenotypic and visual features. Like the FCM and FCSS, assessment of side scatter and CD11b, and evaluation of antigenic differentiation patterns are within these ELN recommendations. Therefore, this leads both to a strict requirement for rapid sample processing and requires a high level of user training, potentially limiting general application.

The rationale that assessing ELN recommended flow cytometric aberrancies could improve on classifier performance was investigated. A “sum of aberrancies” approach was initially adopted. Although there was no overlap between the sum of aberrancies for normal control samples and for MDS cases, there was overlap between the MDS, equivocal, and Reactive cases. A cut-off of 11 aberrancies discriminated MDS from the other classes, but only 3 MDS cases would be classified by this method, rendering it very insensitive. It was further hypothesized that the presence of specific abnormalities giving rise to an MDS phenotypic signature, as opposed to the sum of aberrancies, may better distinguish MDS cases. Unsupervised hierarchical clustering demonstrated phenotypic heterogeneity within this cohort of MDS cases and there was no obvious phenotypic signature, with equivocal and Reactive cases clustered among MDS cases.

Currently, there are no recommendations as to how best to use the ELN attributes. A binary, present or absent approach culminating in a “sum of aberrancies” may not be the optimal technique. The weighting of attributes, as performed by machine learning classification such as logistic regression, may yield better discrimination or identify those cases at risk of progression to MDS. The ELN recommendations were generated from an array of publications and are comprehensive in nature. However, further studies applying attribute selection to large cohorts will be needed to determine which specific attributes better discriminate MDS from non-MDS cases, whilst allowing the removal of attributes which may display multicollinearity.

6.5.4 Driver mutations and classification

At present, there is no literature regarding a comparison, or validation, of flow cytometric scoring schemes against the presence of a genetic mutation. However with the routine application of NGS based sequencing panels for common driver mutations in routine

practice, this is clearly a key area for further development. In this cohort, targeted sequence analysis of 26 commonly mutated genes was available on 41 cases. This included 12 of the 13 MDS cases, 17 Reactive cases, and 11 equivocal cases. This granted the opportunity to begin to study correlation between underlying genetic mutations and phenotypic aberrancies.

Somatic mutations have previously been reported in peripheral blood cells from a large cohort of 17182 persons irrespective of haematological parameters (Jaiswal et al., 2014). The frequency of these mutations increased with age and could be found in patients without cytopenia. To complicate matters, these mutations occurred primarily in *DNMT3A*, *TET2*, and *ASXL1*, all genes which rank in the top 5 genes mutated in MDS (Papaemmanuil et al., 2013). In the cohort in this results chapter, all cases which were initially diagnosed with MDS demonstrated at least one annotated mutation, a finding which supports the morphological diagnosis. Those MDS patients with mutations in *DNMT3A*, *TET2*, or *ASXL1* all had mutations in at least one other gene. In contrast, 2 out of the 3 Reactive patients had solitary mutations in either *TET2* (UPN42) or *ASXL1* (UPN43). The third Reactive patient (UPN40) had a mutation in both *TET2* and in *SRSF2*. These patients would fit the criteria for a diagnosis of *Clonal hematopoiesis of indeterminate potential* (CHIP) (Steensma et al., 2015).

Whether the patient with two mutations is more likely to progress to, or actually be, MDS is difficult to predict. In MDS, poorer outcome correlates with the number of mutations (Papaemmanuil et al., 2013). On the other hand, *SRSF2* was cited as one of the genes found to be mutated in the aforementioned study of 17182 individuals and a small number of individuals in this study (n=49) had 2 mutations (Jaiswal et al., 2014). However, given the finding that the presence of two mutations only occurred in 0.29% of the individuals means that this is a rare occurrence. Therefore, this patient should be a potential candidate for clinical monitoring.

Within the equivocal group, CHIP could also be a straightforward diagnosis for 4 patients who have solitary mutations in either TET2 (UPN38 and UPN44), ASXL1 (UPN6), or SRSF2 (UPN18). However, a further 4 patients within the equivocal group highlight the underlying problematic nature of both CHIP and MDS as diagnoses. Firstly, UPN13 subsequently progressed to RCMD within a relatively short timeframe (7 months). This patient did not have a mutation present, whilst cytogenetic karyotypic analysis demonstrated a non-diagnostic loss of Y, which can be found found in normal, older individuals and is associated with tobacco smoking (Jacobs et al., 1963; Pierre and Hoagland, 1972; Dumanski et al., 2015). It is possible that the patient had a mutation in the “long tail” of mutated genes, or rapidly developed a mutation. However, at initial referral, the patient would not fulfil the criteria for either a diagnosis of CHIP or MDS, therefore may be clinically overlooked. Secondly, UPN 5

had both a *TET2* and an *SF3B1* mutation, and ring sideroblasts were noted as a feature in the bone marrow aspirate. A diagnosis of MDS could not, however, be returned due to the presence of less than 15% ring sideroblasts. Therefore, although this case did not reach the arbitrary cut-off of 15% at diagnostic assessment, the subjective nature of this assessment combined with the presence of any ring sideroblasts and an *SF3B1* mutation suggests that this case should be viewed as satisfying sufficient criteria for a diagnosis of RARS. Thirdly, like UPN13, UPN14 progressed to CMML within a relatively short timeframe (9 months) and was found to have a mutation in both *TET2* and in *ZRSF2,* whilst a fourth patient (UPN50) had a *ZRSF2* mutation coexisting with a *TET2* and an *ASXL1* mutation. ZRSF2 was not reported as mutated in the large cohort of 17182 persons, yet recent studies show this to be the 8th and 12th most mutated gene in MDS (Papaemmanuil et al., 2013; Haferlach et al., 2014). Therefore a mutation in this gene may have clinical relevance and warrant close clinical monitoring.

As an alternative to the number of mutations or specific gene involvement, it may be possible that a combination of flow cytometric analysis and targeted sequence could be used to better identify cytopenic patients with MDS or at higher risk of MDS diagnosis. It appears that presence of flow cytometric aberrancies and presence of mutations may not be absolutely correlated. Cases with up to 7 flow cytometric aberrancies were found to have had no mutations amongst the driver mutations tested. This may therefore reflect the use of a non-exhaustive panel of assessed genes in this cohort and it is possible that whole genome or whole exome sequencing analysis would reveal mutations in other genes. Alternatively, a different genetic abnormality (epigenetic silencing/translocation/deletion/gain/LOH/UPD) may be present to account for the presence of high numbers of flow cytometric aberrancies in such non-diagnostic cases.

Conversely, cases with mutations were found to have low numbers of flow cytometric aberrancies. This discord between the presence of mutation and flow cytometric aberrancies further extends to the flow scoring schemes/classifiers. Both the FCM and the A1DE classifier showed only weak concordance with the presence of mutation. This should come as no surprise considering that both scoring schemes/classifiers were trained on a morphological diagnosis. The FCSS had a higher sensitivity than the FCM and A1DE in predicting presence of mutation, but this was at the expense of specificity. Alternatively, it may be the case that the presence of certain genetic mutations is an age, and not malignancy, related phenomenon as shown by Jaiswal *et al.* (Jaiswal et al., 2014).

It was notable that no common flow cytometric aberrancy signature was evident either overall or, specifically, for the well-defined SF3B1 (genotype) to ring sideroblast (phenotype) relationship. This strengthens the argument that a genetic mutation does not necessarily

give rise to a specific phenotype. Indeed, it is in the ability for flow cytometry and mutation analysis to assess different facets of dysplasia that may be critical in evaluating patients presenting with cytopenia. It has already been demonstrated that flow cytometric aberrancies can be found in lineages unaffected by morphological dysplasia in MDS patients (van de Loosdrecht et al., 2008). Therefore, a combination of mutation(s) plus either flow cytometric aberrancies or a classifier-based MDS diagnosis increases confidence of a diagnosis of MDS or CHIP. The finding that not every case with a mutation has a flow abnormality, and *vice versa,* may reflect the use of a non-exhaustive technique in the results presented in this study. Alternatively, it may reflect normal biological heterogeneity within an elderly population. However, the identification of cases with both a genetic abnormality and a flow cytometric aberrancy would allow future, prospective studies to determine the clinical significance of those cases with either solely genetic mutations or solely flow aberrancies.

In this respect, UPN13 and UPN31 are paradigms for this study. The former was classified as MDS by all three classifiers yet had no mutation and progressed to RCMD. The latter demonstrated an *SRSF2* mutation and evidence of dyserythropoiesis, albeit insufficient for a diagnosis of MDS, but was not classified as MDS by any flow cytometry classifier. Long-term clinical monitoring of patients such as these using a combination of flow cytometry and molecular techniques is essential for two reasons. Firstly, to identify flow cytometric or genetic features genuinely associated with a clinically progressive cytopenia. These features would, hopefully, be distinct from those flow cytometric aberrancies found in Reactive patients who do not progress, or distinct from those mutations found as part of aging process. Secondly, to confirm the finding that accumulation of additional genetic mutations is associated with progression to MDS (Cargo et al., 2015). This could be further extended to discover the relationships between the accumulation of mutations and the development of additional flow cytometric abnormalities.

7 General Discussion

7.1 The accurate diagnosis of MDS and the inherent difficulties therein

Theoretically, a diagnosis of MDS should be straightforward. A textbook example would usually exhibit hallmark features such as the presence of cytopenia, multi-lineage morphological dysplasia in the bone marrow, and a clonal cytogenetic abnormality. However, this scenario belies the complexities involved in diagnosing a heterogeneous disorder which has been divided into arbitrary categories based, in the main, on subjective morphological interpretation. Indeed, even in the presence of classical MDS features, interpretation varies from user-to-user and a consistent classification in accordance with WHO criteria may not be achieved. Furthermore, there is also a corresponding, two-fold challenge when assessing patients referred for the investigation of cytopenia which is to neither over-diagnose MDS, which would abrogate other diagnostic investigations, nor to under-diagnose MDS, thereby restricting access to therapies such as erythropoietin stimulating agents.

Data presented here shows that less than a quarter of patients referred for the investigation of cytopenia are diagnosed with MDS, with the majority having a non-diagnostic bone marrow. This, in itself, may not be a detrimental finding as cases may be referred to exclude a diagnosis of MDS, as opposed to being referred to due to suspicion of MDS. However, a constant theme running through this work has been the finding that patients can be investigated for the presence of cytopenia months and even years before the development of overt morphological signs of dysplasia. Due to the number of cases referred for investigation, it is implausible to attempt to monitor all these patients and, therefore, different methods to diagnosis MDS or to identify patients at risk of MDS are desirable.

7.2 Classification studies based on simple attributes and the identification of immunophenotypic features

Published flow cytometric scoring schemes for the identification of MDS vary from simple 4-parameter schemes to complex scoring schemes involving over 40 different user-defined attributes (Wells et al., 2003; Ogata et al., 2009; Matarraz et al., 2010). Both possess advantages and weaknesses. A simple scoring scheme will be easy to interpret and implement, but may lack sensitivity and, if it includes the evaluation of an attribute which is

time critical, may not be applicable in certain, routine diagnostic setting. It was hypothesized that, due to the finding of significant differences in age and sex ratio between MDS patients and non-diagnostic patients, demographic attributes and the use of a logistic regression model classifier-based approach could improve this sensitivity. However, this approach ultimately suffered the same weakness as other schemes in its lack of sensitivity.

Yet an advantage of the logistic regression model over other schemes was in the calculation of a mathematical probability for class membership. Firstly, this can aid in the confidence of a diagnosis of MDS, or in the exclusion of MDS. The class probability feature also has a further advantage due to its potential application in the triage of cases for a second, more comprehensive method of evaluating MDS. For example, flow cytometric evaluation of dysplasia according to ELN guidelines involves the assessment of 18 antigens on 5 different haematopoietic populations to produce a total of 31 different attributes which can be evaluated. Due to cost and labour required to perform this evaluation, it may be appropriate to exclude those cytopenia patients with a very low probability of MDS from analysis. Therefore, there is the potential for patients who have a very low probability for MDS using a logistic-regression model based classifier to be excluded from a subsequent comprehensive technique, allowing resources to be better focused.

The use of a classifier-based approach also hinted at a solution for some of the issues surrounding the use of multi-attribute, complex scoring schemes, with regards to the number of assessable attributes, redundancy of attributes, the weighting of attributes and incorporation of novel attributes. In some respects, the technique of immunophenoptyping overcomes the inherent subjectivity associated with morphological assessment but, in its own way, it has merely shifted subjectivity to the construction of scoring schemes based on user-defined attributes. The discovery of the coefficient of variation as an important discriminatory feature in the diagnosis of MDS is a case in point. Attempting to incorporate one or two CV attributes into a pre-existing scoring scheme may be achievable, as shown by Mathis *et al.* in attempting to amalgamate the REDDS score and the FCM (Mathis et al., 2013). However, use of the CV potentially doubles the number of antigenic attributes which are evaluable in any flow cytometric panel and, although methods such as Bonferroni and the FDR help to restrict the number of significant attributes, a two-tube, 8-colour flow cytometry panel can still yield a large amount of biological data. In this context, machine learning classifiers can aid in handling the data whilst providing a tool to standardise the approach to classification.

Machine learning classification produces its own series of challenges including classifier evaluation and choice of classifier. For the purposes of this study, a voting system based on numerous evaluable metrics of multiple classifiers tested on the training set was used to

produce a best overall classifier (A1DE) for further performance evaluation. Although defining the best classifier for further evaluation depends upon both the composition of the underlying dataset, and the ultimate requirement of the classifier.

As multiple classifiers were tested on the training set, the feature that each classifier differs in its method of determining class membership could be exploited in a classifier meta-vote scheme to produce an overall confidence in classification. This multi-classifier, meta-vote approach has already been adopted in the machine learning classification of subsets in Diffuse Large B-cell Lymphoma using gene expression array studies (Care et al., 2013). This has subsequently been applied in a routine practice setting in the context of a phase-3 clinical trial, demonstrating the potential for usage of machine learning classifier based tools in clinical practice.

Furthermore, the evaluation of multiple classifiers on the training set also allowed an insight into the underlying cases as there was repeated misclassification by different classifiers of a number of MDS cases. This implies that there was little difference in the population percentages or in immunophenotypic features between the Reactive control group and these cases, at least for the attributes evaluated. The expectation would be that these cases would have a good prognosis due to the presence of features indistinguishable from the control group. This would be analogous to the reported better prognosis of patients with mild flow cytometry abnormalities (Alhan et al., 2014). Furthermore such cases will be interesting to explore in future work aimed at assessing whether there are consistent molecular features that may co-segregate with these “hard to classify” MDS cases.

There are two additional benefits to the use of machine learning classifiers over user-defined scoring schemes. The first is the ability to perform cost sensitive analysis. For this study, it was deemed that a false negative (misclassification of MDS as Reactive) had a higher associated cost. However, it could be argued that, due to the lack of viable treatment options for low-grade MDS that labelling a patient with a false diagnosis of MDS is equally, or more, costly. Secondly, is the use of 10-fold cross validation to try and give an indication of any potential overfitting to the data.

Unfortunately, although a decrease in classifier performance was expected, it appeared that overfitting of the A1DE classifier to the training set did occur. This could be seen when the classifier was tested on the independent test set and the performance metrics decreased more than 10-fold cross validation had suggested. Overfitting is a well described phenomenon when assessing the performance of a classifier which is trained using a large number of attributes relative to the sample size. This problem of overfitting could be improved by the use of more patient samples thereby training a classifier to generalise the

data better. Alternatively, the number of attributes for classifier training could be reduced. This could be performed by manual selection or by the use of statistical attribute selection algorithms (of which numerous are available with the WEKA software). However, another independent dataset would be required to evaluate the effects of attribute selection, otherwise overfitting can again occur. An alternative approach to attribute selection is to choose those attributes which are correlated with overall survival of MDS patients for inclusion in a diagnostic panel. This approach is one recently reported by Alhan *et al.*, albeit for inclusion in a prognostic flow cytometry panel (Alhan et al., 2015).

Two other reasons for the decreased classifier performance on the test set may be related to the control groups. Firstly the panel was design on the basis of attributes identified as significantly different in comparison to a normal control group. It may be the case that performance would have improved for attributes identified by comparison against a Reactive control group, although this is an extension of the aforementioned attribute selection process. Secondly, there was an assumption that all patients in the Reactive group did not have MDS nor did they receive a diagnosis of MDS in the 13 month follow-up timeframe. This may be an erroneous assumption which was dependent upon patients re-presenting to HMDS with a diagnosis MDS. It is entirely feasible that the control group in this study may have contained patients who have already progressed or will progress to MDS, thereby potentially including patients with immunophenotypic abnormalities in the control group and reducing classification accuracy. Therefore, due to the problems of progression to MDS in cytopenic patients, a cohort of age and sex-matched cytopenic patients who underwent flow cytometric testing and on whom there was long-term follow-up data would represent the ideal control group,

7.3 Combining molecular studies with immunophenotyping

The use of contemporary molecular methods to evaluate genetic mutations in patients within an MDS group and within a control group, or a non-diagnostic group, may offer an alternative approach, due to a high proportion of MDS harbouring genetic mutations. Although the use of targeted gene mutation analysis or whole genome or exome sequencing is not without interpretative problems as mutations have been reported in normal individuals, whilst cytopenic patients appear more inclined to demonstrate mutations (Jaiswal et al., 2014; Kwok et al., 2015). Therefore, there is still ambiguity surrounding the significance of gene mutations in the absence of morphological dysplasia.

Although it appears that currently there is much interest in the use of genetic mutation analysis in MDS and that this may well be overtaking the use of flow cytometry in this field, in

a diagnostic setting the two would be complementary, with immunophenotypic studies adding credence to any finding of a genetic mutation. A patient with an absence of morphological dysplasia but the presence of a gene mutation (or mutations) with a flow cytometry classifier indicative of MDS would, at least, suggest close clinical monitoring, if not raise the strong suspicion of MDS. In this respect, case UPN14 in Chapter 6 is the paradigm as this patient demonstrated a mutation in both the *TET2* and the *ZRSR2* genes and was classified as MDS by all the flow scoring schemes. However, this patient did not have morphological features of MDS nor, importantly, a peripheral blood monocytosis and was reported as non-diagnostic. Yet, 9 months later, the patient presented with a confirmed diagnosis of CMML.

Ultimately, this combination of immunophenotyping and NGS could give rise to a new classification scheme which dispenses with morphological based diagnoses and which classify on the basis of the biological features of the disease i.e. cytopenia with flow cytometric aberrancies in the erythroid and progenitors cells with a mutation in ASXL1, or monocytosis with flow cytometric aberrancies in the granulocytic and monocytic lineages and a TET2 mutation. This approach would be less subjective than morphological methods and such a multi-faceted approach to diagnosis would be a fitting tribute to what is a multi-faceted disease.

7.4 Further studies

The issues of classifier overfitting and attribute selection appear to be linked and it is possible that they can both be solved with the acquisition of more cases. DNA would be stored for these cases so that targeted gene mutation sequence analysis using the panel described in Chapter 6 could be performed. Furthermore, as DNA has not been stored on the cases in the training set, it is proposed that DNA is extracted from bone marrow aspirate smears on these patients so that targeted gene mutation sequence can be performed and classifier training can re-occur in the context of the genetic mutation results. There are also currently plans to liaise with HMRN to try to obtain outcome data on those patients within the training and test sets who reside within the HMRN network. This should aid in any future classifier training.

Although the development of a machine learning classifier removes subjectivity regarding flow scoring schemes and can easily incorporate newly discovered attributes, it is unclear whether this approach would work on a multi-centre scale. All analyses were performed on a single cytometer, before attribute values were standardised, therefore it is unknown if different machines, antibody clones, and fluorochrome preferences between laboratories

may restrict this approach. Standardisation, not harmonisation, of flow cytometry protocols across centres could overcome a hurdle in the general applicability of a classifier based approach, as standardisation would for any MDS scoring scheme. However, so far, only the Euroflow panel offers this level of standardisation. Therefore, in the first instance, attempts will be made to evaluated attributes on in-house cytometers to determine whether any cytometer-to-cytometer variability affects classifier performance.

In summary, in this thesis an in-depth exploration of flow cytometric approaches to the diagnosis of MDS has led to the following conclusions:

- Flow cytometry can be used to reproducibly identify cases with a definite abnormal pattern in MDS or with a normal pattern in reactive and normal marrow states.
- A significant “grey zone” exists of cases that cannot be confidently classified by multiple different approaches using flow cytometry.
- Machine learning approaches fail to enhance sensitivity of MDS detection but provide the basis for applying confidence scores which would be of value in sample triage.
- The integration of flow cytometry and targeted gene mutation analysis provides the potential to identify cases which progress to dysplastic states prior to the emergence of confidently identifiable morphological dysplasia.

Aguilar, H., Alvarez-Errico, D., Garcia-Montero, A.C., Orfao, A., Sayos, J. and Lopez-Botet, M. 2004. Molecular characterization of a novel immune receptor restricted to the monocytic lineage. *J Immunol.* **173**(11), pp.6703-6711.

Alhan, C., Westers, T.M., Cremers, E.M., Cali, C., Witte, B.I., Ossenkoppele, G.J. and van de Loosdrecht, A.A. 2014. High flow cytometric scores identify adverse prognostic subgroups within the revised international prognostic scoring system for myelodysplastic syndromes. *Br J Haematol.* **167**(1), pp.100-109.

Alhan, C., Westers, T.M., Cremers, E.M., Cali, C., Witte, B.I., Ossenkoppele, G.J. and van de Loosdrecht, A.A. 2015. The myelodysplastic syndromes flow cytometric score: a three-parameter prognostic flow cytometric scoring system. *Leukemia.*

AML18 Trial UK Clinical Trials Gateway. *A Trial for Older Patients with Acute Myeloid Leukaemia and High Risk Myelodysplastic Syndrome.* [Online]. Available from: https://www.ukctg.nihr.ac.uk/trials/trial-details/trial-details?trialNumber=NCT02272478

Araten, D.J., Swirsky, D., Karadimitris, A., Notaro, R., Nafa, K., Bessler, M., Thaler, H.T., Castro-Malaspina, H., Childs, B.H., Boulad, F., Weiss, M., Anagnostopoulos, N., Kutlar, A., Savage, D.G., Maziarz, R.T., Jhanwar, S. and Luzzatto, L. 2001. Cytogenetic and morphological abnormalities in paroxysmal nocturnal haemoglobinuria. *Br J Haematol.* **115**(2), pp.360-368.

Arnoulet, C., Béné, M.C., Durrieu, F., Feuillard, J., Fossat, C., Husson, B., Jouault, H., Maynadié, M. and Lacombe, F. 2010. Four- and five-color flow cytometry analysis of leukocyte differentiation pathways in normal bone marrow: a reference document based on a systematic approach by the GTLLF and GEIL. *Cytometry. Part B, Clinical Cytometry.* **78**(1), pp.4-10.

Asano, H., Ohashi, H., Ichihara, M., Kinoshita, T., Murate, T., Kobayashi, M., Saito, H. and Hotta, T. 1994. Evidence for nonclonal hematopoietic progenitor cell populations in bone marrow of patients with myelodysplastic syndromes. *Blood.* **84**(2), pp.588-594.

Aul, C., Gattermann, N. and Schneider, W. 1992. Age-related incidence and other epidemiological aspects of myelodysplastic syndromes. *Br J Haematol.* **82**(2), pp.358-367.

Austin, P.C. and Steyerberg, E.W. 2012. Interpreting the concordance statistic of a logistic regression model: relation to the variance and odds ratio of a continuous explanatory variable. *BMC Med Res Methodol.* **12**, p82.

Bach, V., Schruckmayer, G., Sam, I., Kemmler, G. and Stauder, R. 2014. Prevalence and possible causes of anemia in the elderly: a cross-sectional analysis of a large European university hospital cohort. *Clin Interv Aging.* **9**, pp.1187-1196.

Bain, B.J. 1996. The bone marrow aspirate of healthy subjects. *British Journal of Haematology.* **94**(1), pp.206-209.

Bain, B.J. 2005. Bone marrow biopsy morbidity: review of 2003. *Journal of Clinical Pathology.* **58**(4), pp.406-408.

Bakker, A.B., van den Oudenrijn, S., Bakker, A.Q., Feller, N., van Meijer, M., Bia, J.A., Jongeneelen, M.A., Visser, T.J., Bijl, N., Geuijen, C.A., Marissen, W.E., Radosevic, K., Throsby, M., Schuurhuis, G.J., Ossenkoppele, G.J., de Kruif, J., Goudsmit, J. and Kruisbeek, A.M. 2004. C-type lectin-like molecule-1: a novel myeloid cell surface marker associated with acute myeloid leukemia. *Cancer Res.* **64**(22), pp.8443-8450.

Bar-Eli, M., Ahuja, H., Gonzalez-Cadavid, N., Foti, A. and Cline, M.J. 1989. Analysis of N-RAS exon-1 mutations in myelodysplastic syndromes by polymerase chain reaction and direct sequencing. *Blood.* **73**(1), pp.281-283.

Barbui, T., Thiele, J., Vannucchi, A.M. and Tefferi, A. 2015. Rationale for revision and proposed changes of the WHO diagnostic criteria for polycythemia vera, essential thrombocythemia and primary myelofibrosis. *Blood Cancer Journal.* **5**, pe337.

Bardet, V., Wagner-Ballon, O., Guy, J., Morvan, C., Debord, C., Trimoreau, F., Benayoun, E., Chapuis, N., Freynet, N., Rossi, C., Mathis, S., Gourin, M.P., Toma, A., Bene, M.C., Feuillard, J. and Guerin, E. 2015. Multicentric study underlining the interest of adding CD5, CD7 and CD56 expression assessment to the flow cytometric Ogata

score in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms. *Haematologica.* Italy: Ferrata Storti Foundation., pp.472-478.

Barrett, J., Sauntharajah, Y. and Molldrem, J. 2000. Myelodysplastic syndrome and aplastic anemia: distinct entities or diseases linked by a common pathophysiology? *Seminars in Hematology.* **37**(1), pp.15-29.

Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M. and Peault, B. 1992. Isolation of a candidate human hematopoietic stem-cell population. *Proceedings of the National Academy of Sciences of the United States of America.* **89**(7), pp.2804-2808.

Baxter, E.J., Scott, L.M., Campbell, P.J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G.S., Bench, A.J., Boyd, E.M., Curtin, N., Scott, M.A., Erber, W.N. and Green, A.R. 2005. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet.* **365**(9464), pp.1054-1061.

Beer, P.A., Campbell, P.J., Scott, L.M., Bench, A.J., Erber, W.N. and Bareford, D. 2008. MPL mutations in myeloproliferative disorders: analysis of the PT-1 cohort. **112**, pp.141-149.

Bejar, R., Stevenson, K., Abdel-Wahab, O., Galili, N., Nilsson, B., Garcia-Manero, G., Kantarjian, H., Raza, A., Levine, R.L., Neuberg, D. and Ebert, B.L. 2011. Clinical Effect of Point Mutations in Myelodysplastic Syndromes. *New England Journal of Medicine.* **364**(26), pp.2496-2506.

Beloosesky, Y., Cohen, A.M., Grosman, B. and Grinblat, J. 2000. Prevalence and survival of myelodysplastic syndrome of the refractory anemia type in hospitalized cognitively different geriatric patients. *Gerontology.* **46**(6), pp.323-327.

Benjamini, Y. and Hochberg, Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological).* **57**(1), pp.289-300.

Bennett, J.M., Catovsky, D., Daniel, M.-T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. and Sultan, C. 1976. Proposals for the Classification of the Acute Leukaemias French-American-British (FAB) Co-operative Group. *British Journal of Haematology.* **33**(4), pp.451--458.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A.G., Gralnick, H., Sultan, C. and Cox, C. 1994. The chronic myeloid leukaemias: guidelines for distinguishing chronic granulocytic, atypical chronic myeloid, and chronic myelomonocytic leukaemia: Proposals by the French - American - British Cooperative Leukaemia Group. *British Journal of Haematology.* **87**(4), pp.746-754.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. and C., S. 1982. Proposals for the classification of the myelodysplastic syndromes. *British Journal of Haematology.* **51**(2), pp.189--199.

Bennett, J.M. and Orazi, A. 2009. Diagnostic criteria to distinguish hypocellular acute myeloid leukemia from hypocellular myelodysplastic syndromes and aplastic anemia: recommendations for a standardized approach. *Haematologica.* **94**(2), pp.264-268.

Bessman, D. 1977. Erythropoiesis during recovery from macrocytic anemia: macrocytes, normocytes, and microcytes. *Blood.* **50**(6), pp.995-1000.

Bhatia, M., Wang, J.C., Kapp, U., Bonnet, D. and Dick, J.E. 1997. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A.* **94**(10), pp.5320-5325.

Bianco, T., Farmer, B.J., Sage, R.E. and Dobrovic, A. 2001. Loss of red cell A, B, and H antigens is frequent in myeloid malignancies. *Blood.* **97**(11), pp.3633-3639.

Biernaux, C., Loos, M., Sels, A., Huez, G. and Stryckmans, P. 1995. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. *Blood.* **86**(8), pp.3118-3122.

Bjorkman, S.E. 1956. Chronic refractory anemia with sideroblastic bone marrow; a study of four cases. *Blood.* **11**(3), pp.250-259.

Bonnet, D. and Dick, J.E. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* **3**(7), pp.730-737.

Boultwood, J., Lewis, S. and Wainscoat, J.S. 1994. The 5q-syndrome. *Blood.* **84**(10), pp.3253-3260.

Bowen, D.T., Fenaux, P., Hellstrom-Lindberg, E. and de Witte, T. 2008. Time-dependent prognostic scoring system for myelodysplastic syndromes has significant limitations that may influence its reproducibility and practical application. *J Clin Oncol.* United States, pp.1180; author reply 1181-1182.

Buhring, H.J., Simmons, P.J., Pudney, M., Muller, R., Jarrossay, D., van Agthoven, A., Willheim, M., Brugger, W., Valent, P. and Kanz, L. 1999. The monoclonal antibody 97A6 defines a novel surface antigen expressed on human basophils and their multipotent and unipotent progenitors. *Blood.* **94**(7), pp.2343-2356.

Cafolla, A., Dragoni, F., Girelli, G., Tosti, M.E., Monarca, B., Kendall, R. and Scott, C.S. 1998. Red cell folate in elderly patients with myelodysplastic syndrome. *Eur J Haematol.* **61**(3), pp.160-163.

Cairns, R.A., Iqbal, J., Lemonnier, F., Kucuk, C., de Leval, L., Jais, J.P., Parrens, M., Martin, A., Xerri, L., Brousset, P., Chan, L.C., Chan, W.C., Gaulard, P. and Mak, T.W. 2012. IDH2 mutations are frequent in angioimmunoblastic T-cell lymphoma. *Blood.* **119**(8), pp.1901-1903.

Cantù Rajnoldi, A., Fenu, S., Kerndrup, G., Wering, E.R., Niemeyer, C.M., Baumann, I. and for the European Working Group on Myelodysplastic Syndromes in, C. 2005. Evaluation of dysplastic features in myelodysplastic syndromes: experience from the morphology group of the European Working Group of MDS in Childhood (EWOG-MDS). *Annals of Hematology.* **84**(7), pp.429-433.

Care, M.A., Barrans, S., Worrillow, L., Jack, A., Westhead, D.R. and Tooze, R.M. 2013. A Microarray Platform-Independent Classification Tool for Cell of Origin Class Allows Comparative Analysis of Gene Expression in Diffuse Large B-cell Lymphoma. *PLoS ONE.* **8**(2), pe55895.

Cargo, C.A., Rowbotham, N., Evans, P.A., Barrans, S.L., Bowen, D.T., Crouch, S. and Jack, A.S. 2015. Targeted sequencing identifies patients with preclinical MDS at high risk of disease progression. *Blood.* **126**(21), pp.2362-2365.

Cazzola, M., Della Porta, M.G. and Malcovati, L. 2013. The genetic basis of myelodysplasia and its clinical relevance. *Blood.* **122**(25), pp.4021-4034.

Cazzola, M. and Malcovati, L. 2005. Myelodysplastic syndromes--coping with ineffective hematopoiesis. *The New England Journal of Medicine.* **352**(6), pp.536-538.

Chang, C.C. and Cleveland, R.P. 2000. Decreased CD10-positive mature granulocytes in bone marrow from patients with myelodysplastic syndrome. *Archives of Pathology & Laboratory Medicine.* **124**(8), pp.1152-1156.

Chapman, M.A., Lawrence, M.S., Keats, J.J., Cibulskis, K., Sougnez, C., Schinzel, A.C., Harview, C.L., Brunet, J.-P., Ahmann, G.J., Adli, M., Anderson, K.C., Ardlie, K.G., Auclair, D., Baker, A., Bergsagel, P.L., Bernstein, B.E., Drier, Y., Fonseca, R., Gabriel, S.B., Hofmeister, C.C., Jagannath, S., Jakubowiak, A.J., Krishnan, A., Levy, J., Liefeld, T., Lonial, S., Mahan, S., Mfuko, B., Monti, S., Perkins, L.M., Onofrio, R., Pugh, T.J., Vincent Rajkumar, S., Ramos, A.H., Siegel, D.S., Sivachenko, A., Trudel, S., Vij, R., Voet, D., Winckler, W., Zimmerman, T., Carpten, J., Trent, J., Hahn, W.C., Garraway, L.A., Meyerson, M., Lander, E.S., Getz, G. and Golub, T.R. 2011. Initial genome sequencing and analysis of multiple myeloma. *Nature.* **471**(7339), pp.467-472.

Cherian, S., Moore, J., Bantly, A., Vergilio, J.A., Klein, P., Luger, S. and Bagg, A. 2005. Peripheral blood MDS score: a new flow cytometric tool for the diagnosis of myelodysplastic syndromes. *Cytometry B Clin Cytom.* **64**(1), pp.9-17.

Chu, S.C., Wang, T.F., Li, C.C., Kao, R.H., Li, D.K., Su, Y.C., Wells, D.A. and Loken, M.R. 2011. Flow cytometric scoring system as a diagnostic and prognostic tool in myelodysplastic syndromes. *Leuk Res.* England: 2011 Elsevier Ltd, pp.868-873.

Chung, J.W., Park, C.J., Cha, C.H., Cho, Y.U., Jang, S., Chi, H.S., Seo, E.J., Lee, J.H., Lee, K.H., Im, H.J. and Seo, J.J. 2012. A combination of CD15/CD10, CD64/CD33,

CD16/CD13 or CD11b flow cytometric granulocyte panels is sensitive and specific for diagnosis of myelodysplastic syndrome. *Ann Clin Lab Sci.* **42**(3), pp.271-280.

Civin, C.I., Strauss, L.C., Brovall, C., Fackler, M.J., Schwartz, J.F. and Shaper, J.H. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol.* **133**(1), pp.157-165.

Cohen, J. 1960. A Coefficient of Agreement for Nominal Scales. *Educational and Psychological Measurement.* **20**(1), pp.37-46.

Costa, E.S., Pedreira, C.E., Barrena, S., Lecrevisse, Q., Flores, J., Quijano, S., Almeida, J., del Carmen Garcia-Macias, M., Bottcher, S., Van Dongen, J.J. and Orfao, A. 2010. Automated pattern-guided principal component analysis vs expert-based immunophenotypic classification of B-cell chronic lymphoproliferative disorders: a step forward in the standardization of clinical immunophenotyping. *Leukemia.* **24**(11), pp.1927-1933.

Couronné, L., Bastard, C. and Bernard, O.A. 2012. TET2 and DNMT3A Mutations in Human T-Cell Lymphoma. *New England Journal of Medicine.* **366**(1), pp.95-96.

Craig, F.E. and Foon, K.A. 2008. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood.* **111**(8), pp.3941-3967.

Cremers, E.M.P., Westers, T.M., Alhan, C., Cali, C., Wondergem, M.J., Poddighe, P.J., Ossenkoppele, G.J. and van de Loosdrecht, A.A. 2016. Multiparameter flow cytometry is instrumental to distinguish myelodysplastic syndromes from non-neoplastic cytopenias. *European Journal of Cancer.* **54**, pp.49-56.

Cui, Y., Tong, H., Du, X., Li, B., Gale, R.P., Qin, T., Liu, J., Xu, Z., Zhang, Y., Huang, G., Jin, J., Fang, L., Zhang, H., Pan, L., Hu, N., Qu, S. and Xiao, Z. 2015. Impact of TET2, SRSF2, ASXL1 and SETBP1 mutations on survival of patients with chronic myelomonocytic leukemia. *Exp Hematol Oncol.* **4**, p14.

Curran-Everett, D. 2013. Explorations in statistics: the analysis of ratios and normalized data. *Advances in Physiology Education.* **37**(3), pp.213-219.

Cutler, J.A., Wells, D.A., van de Loosdrecht, A.A., de Baca, M.E., Kalnoski, M.H., Zehentner, B.K., Eidenschink, L., Ghirardelli, K.M., Biggerstaff, J.S. and Loken, M.R. 2010. Phenotypic abnormalities strongly reflect genotype in patients with unexplained cytopenias. *Cytometry. Part B, Clinical Cytometry.*

Damm, F., Kosmider, O., Gelsi-Boyer, V., Renneville, A., Carbuccia, N., Hidalgo-Curtis, C., Della Valle, V., Couronné, L., Scourzic, L., Chesnais, V., Guerci-Bresler, A., Slama, B., Beyne-Rauzy, O., Schmidt-Tanguy, A., Stamatoullas-Bastard, A., Dreyfus, F., Prébet, T., de Botton, S., Vey, N., Morgan, M.A., Cross, N.C.P., Preudhomme, C., Birnbaum, D., Bernard, O.A. and Fontenay, M. 2012. Mutations affecting mRNA splicing define distinct clinical phenotypes and correlate with patient outcome in myelodysplastic syndromes. *Blood.* **119**(14), pp.3211-3218.

Davis, B.H., Holden, J.T., Bene, M.C., Borowitz, M.J., Braylan, R.C., Cornfield, D., Gorczyca, W., Lee, R., Maiese, R., Orfao, A., Wells, D., Wood, B.L. and Stetler-Stevenson, M. 2007. 2006 Bethesda International Consensus recommendations on the flow cytometric immunophenotypic analysis of hematolymphoid neoplasia: medical indications. *Cytometry. Part B, Clinical Cytometry.* **72 Suppl 1**, pp.S5-13.

Dedeepiya, V.D., Rao, Y.Y., Jayakrishnan, G.A., Parthiban, J., Baskar, S., Manjunath, S.R., Senthilkumar, R. and Abraham, S.J.K. 2012. Index of CD34+ Cells and Mononuclear Cells in the Bone Marrow of Spinal Cord Injury Patients of Different Age Groups: A Comparative Analysis. *Bone Marrow Res.* **2012**.

Del Cañizo, M.C., Fernández, M.E., López, A., Vidriales, B., Villarón, E., Arroyo, J.L., Ortuño, F., Orfao, A. and San Miguel, J.F. 2003. Immunophenotypic analysis of myelodysplastic syndromes. *Haematologica.* **88**(4), pp.402-407.

Delhommeau, F., Dupont, S., Valle, V.D., James, C., Trannoy, S., Massé, A., Kosmider, O., Le Couedic, J.-P., Robert, F., Alberdi, A., Lécluse, Y., Plo, I., Dreyfus, F.J., Marzac, C., Casadevall, N., Lacombe, C., Romana, S.P., Dessen, P., Soulier, J., Viguié, F.,

Fontenay, M., Vainchenker, W. and Bernard, O.A. 2009. Mutation in TET2 in Myeloid Cancers. *New England Journal of Medicine.* **360**(22), pp.2289-2301.

Della Porta, M.G., Malcovati, L., Invernizzi, R., Travaglino, E., Pascutto, C., Maffioli, M., Galli, A., Boggi, S., Pietra, D., Vanelli, L., Marseglia, C., Levi, S., Arosio, P., Lazzarino, M. and Cazzola, M. 2006. Flow cytometry evaluation of erythroid dysplasia in patients with myelodysplastic syndrome. *Leukemia.* **20**(4), pp.549-555.

Della Porta, M.G., Picone, C., Pascutto, C., Malcovati, L., Tamura, H., Handa, H., Czader, M., Freeman, S., Vyas, P., Porwit, A., Saft, L., Westers, T.M., Alhan, C., Cali, C., van de Loosdrecht, A.A. and Ogata, K. 2012. Multicenter validation of a reproducible flow cytometric score for the diagnosis of low-grade myelodysplastic syndromes: results of a European LeukemiaNET study. *Haematologica.* Italy, pp.1209-1217.

Della Porta, M.G., Picone, C., Tenore, A., Yokose, N., Malcovati, L., Cazzola, M. and Ogata, K. 2014. Prognostic significance of reproducible immunophenotypic markers of marrow dysplasia. *Haematologica.* **99**(1), pp.e8-e10.

Della Porta, M.G., Travaglino, E., Boveri, E., Ponzoni, M., Malcovati, L., Papaemmanuil, E., Rigolin, G.M., Pascutto, C., Croci, G., Gianelli, U., Milani, R., Ambaglio, I., Elena, C., Ubezio, M., Da Via, M.C., Bono, E., Pietra, D., Quaglia, F., Bastia, R., Ferretti, V., Cuneo, A., Morra, E., Campbell, P.J., Orazi, A., Invernizzi, R. and Cazzola, M. 2015a. Minimal morphological criteria for defining bone marrow dysplasia: a basis for clinical implementation of WHO classification of myelodysplastic syndromes. *Leukemia.* **29**(1), pp.66-75.

Della Porta, M.G., Tuechler, H., Malcovati, L., Schanz, J., Sanz, G., Garcia-Manero, G., Sole, F., Bennett, J.M., Bowen, D., Fenaux, P., Dreyfus, F., Kantarjian, H., Kuendgen, A., Levis, A., Cermak, J., Fonatsch, C., Le Beau, M.M., Slovak, M.L., Krieger, O., Luebbert, M., Maciejewski, J., Magalhaes, S.M., Miyazaki, Y., Pfeilstocker, M., Sekeres, M.A., Sperr, W.R., Stauder, R., Tauro, S., Valent, P., Vallespi, T., van de Loosdrecht, A.A., Germing, U., Haase, D., Greenberg, P.L. and Cazzola, M. 2015b. Validation of WHO classification-based Prognostic Scoring System (WPSS) for myelodysplastic syndromes and comparison with the revised International Prognostic Scoring System (IPSS-R). A study of the International Working Group for Prognosis in Myelodysplasia (IWG-PM). *Leukemia.* **29**(7), pp.1502-1513.

Doulatov, S., Notta, F., Eppert, K., Nguyen, L.T., Ohashi, P.S. and Dick, J.E. 2010. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nat Immunol.* **11**(7), pp.585-593.

Drabick, J.J., Davis, B.J. and Byrd, J.C. 2001. Concurrent pernicious anemia and myelodysplastic syndrome. *Annals of Hematology.* **80**(4), pp.243-245.

Dumanski, J.P., Rasi, C., Lönn, M., Davies, H., Ingelsson, M., Giedraitis, V., Lannfelt, L., Magnusson, P.K.E., Lindgren, C.M., Morris, A.P., Cesarini, D., Johannesson, M., Tiensuu Janson, E., Lind, L., Pedersen, N.L., Ingelsson, E. and Forsberg, L.A. 2015. Smoking is associated with mosaic loss of chromosome Y. *Science.* **347**(6217), pp.81-83.

Döhner, H., Weisdorf, D.J. and Bloomfield, C.D. 2015. Acute Myeloid Leukemia. *New England Journal of Medicine.* **373**(12), pp.1136-1152.

Elghetany, M.T. 1998. Surface marker abnormalities in myelodysplastic syndromes. *Haematologica.* **83**(12), pp.1104-1115.

Elghetany, M.T. 2002. Surface antigen changes during normal neutrophilic development: a critical review. *Blood Cells Mol Dis.* **28**(2), pp.260-274.

Elghetany, M.T., Ge, Y., Patel, J., Martinez, J. and Uhrova, H. 2004. Flow cytometric study of neutrophilic granulopoiesis in normal bone marrow using an expanded panel of antibodies: correlation with morphologic assessments. *Journal of Clinical Laboratory Analysis.* **18**(1), pp.36-41.

Elghetany, M.T. and Patel, J. 2002. Assessment of CD24 expression on bone marrow neutrophilic granulocytes: CD24 is a marker for the myelocytic stage of development. *Am J Hematol.* **71**(4), pp.348-349.

Elmore, J.G., Barton, M.B., Moceri, V.M., Polk, S., Arena, P.J. and Fletcher, S.W. 1998. Ten-year risk of false positive screening mammograms and clinical breast examinations. *N Engl J Med.* **338**(16), pp.1089-1096.

Emanuel, P.D. 2008. Juvenile myelomonocytic leukemia and chronic myelomonocytic leukemia. *Leukemia.* England, pp.1335-1342.

Ernst, T., Chase, A.J., Score, J., Hidalgo-Curtis, C.E., Bryant, C., Jones, A.V., Waghorn, K., Zoi, K., Ross, F.M., Reiter, A., Hochhaus, A., Drexler, H.G., Duncombe, A., Cervantes, F., Oscier, D., Boultwood, J., Grand, F.H. and Cross, N.C. 2010. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet.* **42**(8), pp.722-726.

Escribano, L., Diaz-Agustin, B., López, A., Núñez López, R., García-Montero, A., Almeida, J., Prados, A., Angulo, M., Herrero, S. and Orfao, A. 2004. Immunophenotypic analysis of mast cells in mastocytosis: When and how to do it. Proposals of the Spanish Network on Mastocytosis (REMA). *Cytometry Part B: Clinical Cytometry.* **58B**(1), pp.1-8.

Escribano, L., Ocqueteau, M., Almeida, J., Orfao, A. and San Miguel, J.F. 1998. Expression of the c-kit (CD117) molecule in normal and malignant hematopoiesis. *Leuk Lymphoma.* **30**(5-6), pp.459-466.

Estey, E., Thall, P., Beran, M., Kantarjian, H., Pierce, S. and Keating, M. 1997. Effect of Diagnosis (Refractory Anemia With Excess Blasts, Refractory Anemia With Excess Blasts in Transformation, or Acute Myeloid Leukemia [AML]) on Outcome of AML-Type Chemotherapy. *Blood.* **90**(8), pp.2969-2977.

Falini, B., Mecucci, C., Tiacci, E., Alcalay, M., Rosati, R., Pasqualucci, L., La Starza, R., Diverio, D., Colombo, E., Santucci, A., Bigerna, B., Pacini, R., Pucciarini, A., Liso, A., Vignetti, M., Fazi, P., Meani, N., Pettirossi, V., Saglio, G., Mandelli, F., Lo-Coco, F., Pelicci, P.-G. and Martelli, M.F. 2005. Cytoplasmic Nucleophosmin in Acute Myelogenous Leukemia with a Normal Karyotype. *New England Journal of Medicine.* **352**(3), pp.254-266.

Fawcett, T. 2006. An introduction to ROC analysis. *Pattern Recognition Letters.* **27**(8), pp.861-874.

Fenaux, P. and Adès, L. 2013. How we treat lower-risk myelodysplastic syndromes. *Blood.* **121**(21), pp.4280-4286.

Florian, S., Sonneck, K., Hauswirth, A.W., Krauth, M.-T., Schernthaner, G.-H., Sperr, W.R. and Valent, P. 2006. Detection of molecular targets on the surface of CD34+/CD38-- stem cells in various myeloid malignancies. *Leukemia & Lymphoma.* **47**(2), pp.207-222.

Font, P., Loscertales, J., Benavente, C., Bermejo, A., Callejas, M., Garcia-Alonso, L., Garcia-Marcilla, A., Gil, S., Lopez-Rubio, M., Martin, E., Munoz, C., Ricard, P., Soto, C., Balsalobre, P. and Villegas, A. 2013. Inter-observer variance with the diagnosis of myelodysplastic syndromes (MDS) following the 2008 WHO classification. *Ann Hematol.* **92**(1), pp.19-24.

Font, P., Loscertales, J., Soto, C., Ricard, P., Novas, C.M., Martin-Clavero, E., Lopez-Rubio, M., Garcia-Alonso, L., Callejas, M., Bermejo, A., Benavente, C., Ballesteros, M., Cedena, T., Calbacho, M., Urbina, R., Villarrubia, J., Gil, S., Bellon, J.M., Diez-Martin, J.L. and Villegas, A. 2015. Interobserver variance in myelodysplastic syndromes with less than 5 % bone marrow blasts: unilineage vs. multilineage dysplasia and reproducibility of the threshold of 2 % blasts. *Ann Hematol.* **94**(4), pp.565-573.

Font, P., Subirá, D., Mtnez-Chamorro, C., Castañón, S., Arranz, E., Ramiro, S., Gil-Fernández, J.J., López-Pascual, J., Alonso, A., Pérez-Sáenz, M.A., Alaez, C., Renedo, M., Blas, C., Escudero, A. and Fdez-Rañada, J.M. 2006. Evaluation of CD7 and terminal deoxynucleotidyl transferase (TdT) expression in CD34+ myeloblasts from patients with myelodysplastic syndrome. *Leukemia Research.* **30**(8), pp.957-963.

Frederiksen, H. and Schmidt, K. 1999. The Incidence of Idiopathic Thrombocytopenic Purpura in Adults Increases With Age. *Blood.* **94**(3), pp.909-913.

Frisch, B. and Bartl, R. 1986. Bone marrow histology in myelodysplastic syndromes. *Scandinavian Journal of Haematology.* **36**(S45), pp.21-37.

Fureder, W., Schernthaner, G.H., Ghannadan, M., Hauswirth, A., Sperr, W.R., Semper, H., Majlesi, Y., Zwirner, J., Gotze, O., Buhring, H.J., Lechner, K. and Valent, P. 2001. Quantitative, phenotypic, and functional evaluation of basophils in myelodysplastic syndromes. *Eur J Clin Invest.* **31**(10), pp.894-901.

Garcia-Manero, G., Shan, J., Faderl, S., Cortes, J., Ravandi, F., Borthakur, G., Wierda, W.G., Pierce, S., Estey, E., Liu, J., Huang, X. and Kantarjian, H. 2008. A prognostic score for patients with lower risk myelodysplastic syndrome. *Leukemia.* **22**(3), pp.538-543.

Gaskell, H., Derry, S., Andrew Moore, R. and McQuay, H.J. 2008. Prevalence of anaemia in older persons: systematic review. *BMC Geriatr.* **8**, p1.

Geiger, H., de Haan, G. and Florian, M.C. 2013. The ageing haematopoietic stem cell compartment. *Nat Rev Immunol.* **13**(5), pp.376-389.

Gelsi-Boyer, V., Trouplin, V., Adelaide, J., Bonansea, J., Cervera, N., Carbuccia, N., Lagarde, A., Prebet, T., Nezri, M., Sainty, D., Olschwang, S., Xerri, L., Chaffanet, M., Mozziconacci, M.J., Vey, N. and Birnbaum, D. 2009. Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. *Br J Haematol.* **145**(6), pp.788-800.

Gelsi-Boyer, V., Trouplin, V., Roquain, J., Adelaide, J., Carbuccia, N., Esterni, B., Finetti, P., Murati, A., Arnoulet, C., Zerazhi, H., Fezoui, H., Tadrist, Z., Nezri, M., Chaffanet, M., Mozziconacci, M.J., Vey, N. and Birnbaum, D. 2010. ASXL1 mutation is associated with poor prognosis and acute transformation in chronic myelomonocytic leukaemia. *Br J Haematol.* **151**(4), pp.365-375.

Genovese, G., Kähler, A.K., Handsaker, R.E., Lindberg, J., Rose, S.A., Bakhoum, S.F., Chambert, K., Mick, E., Neale, B.M., Fromer, M., Purcell, S.M., Svantesson, O., Landén, M., Höglund, M., Lehmann, S., Gabriel, S.B., Moran, J.L., Lander, E.S., Sullivan, P.F., Sklar, P., Grönberg, H., Hultman, C.M. and McCarroll, S.A. 2014. Clonal Hematopoiesis and Blood-Cancer Risk Inferred from Blood DNA Sequence. *New England Journal of Medicine.* **371**(26), pp.2477-2487.

Germing, U., Gattermann, N., Strupp, C., Aivado, M. and Aul, C. 2000. Validation of the WHO proposals for a new classification of primary myelodysplastic syndromes: a retrospective analysis of 1600 patients. *Leukemia Research.* **24**(12), pp.983-992.

Giagounidis, A.A., Germing, U., Wainscoat, J.S., Boultwood, J. and Aul, C. 2004. The 5q-syndrome. *Hematology.* **9**(4), pp.271-277.

Goardon, N., Nikolousis, E., Sternberg, A., Chu, W.-K., Craddock, C., Richardson, P., Benson, R., Drayson, M., Standen, G., Vyas, P. and Freeman, S. 2009. Reduced CD38 expression on CD34(+) cells as a diagnostic test in myelodysplastic syndromes. *Haematologica.* **94**(8), pp.1160-1163.

Goasguen, J.E., Bennett, J.M., Bain, B.J., Brunning, R., Vallespi, M.T., Tomonaga, M., Zini, G. and Renault, A. 2014. Proposal for refining the definition of dysgranulopoiesis in acute myeloid leukemia and myelodysplastic syndromes. *Leuk Res.* **38**(4), pp.447-453.

Goasguen, J.E., Bennett, J.M., Bain, B.J., Vallespi, T., Brunning, R. and Mufti, G.J. 2009. Morphological evaluation of monocytes and their precursors. *Haematologica.* **94**(7), pp.994-997.

Gondek, L.P., Tiu, R., O'Keefe, C.L., Sekeres, M.A., Theil, K.S. and Maciejewski, J.P. 2008. Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, and MDS-derived AML. *Blood.* **111**(3), pp.1534-1542.

Gonzalez-Medina, I., Bueno, J., Torrequebrada, A., Lopez, A., Vallespi, T. and Massague, I. 2002. Two groups of chronic myelomonocytic leukaemia: myelodysplastic and myeloproliferative. Prognostic implications in a series of a single center. *Leuk Res.* **26**(9), pp.821-824.

Grand, F.H., Hidalgo-Curtis, C.E., Ernst, T., Zoi, K., Zoi, C., McGuire, C., Kreil, S., Jones, A., Score, J., Metzgeroth, G., Oscier, D., Hall, A., Brandts, C., Serve, H., Reiter, A., Chase, A.J. and Cross, N.C. 2009. Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. *Blood.* **113**(24), pp.6182-6192.

Graubert, T.A., Shen, D., Ding, L., Okeyo-Owuor, T., Lunn, C.L., Shao, J., Krysiak, K., Harris, C.C., Koboldt, D.C., Larson, D.E., McLellan, M.D., Dooling, D.J., Abbott, R.M., Fulton, R.S., Schmidt, H., Kalicki-Veizer, J., O'Laughlin, M., Grillot, M., Baty, J., Heath, S., Frater, J.L., Nasim, T., Link, D.C., Tomasson, M.H., Westervelt, P., DiPersio, J.F., Mardis, E.R., Ley, T.J., Wilson, R.K. and Walter, M.J. 2012. Recurrent mutations in the U2AF1 splicing factor in myelodysplastic syndromes. *Nat Genet.* **44**(1), pp.53-57.

Greco, M., Capello, D., Bruscaggin, A., Spina, V., Rasi, S., Monti, S., Ciardullo, C., Cresta, S., Fangazio, M., Gaidano, G., Foà, R. and Rossi, D. 2013. Analysis of SF3B1 mutations in monoclonal B-cell lymphocytosis. *Hematological Oncology.* **31**(1), pp.54-55.

Greenberg, P., Anderson, J., de Witte, T., Estey, E., Fenaux, P., Gupta, P., Hamblin, T., Hellstrom-Lindberg, E., List, A., Mufti, G., Neuwirtova, R., Ohyashiki, K., Oscier, D., Sanz, G., Sanz, M. and Willman, C. 2000. Problematic WHO reclassification of myelodysplastic syndromes. Members of the International MDS Study Group. *J Clin Oncol.* **18**(19), pp.3447-3452.

Greenberg, P., Cox, C., LeBeau, M.M., Fenaux, P., Morel, P., Sanz, G., Sanz, M., Vallespi, T., Hamblin, T., Oscier, D., Ohyashiki, K., Toyama, K., Aul, C., Mufti, G. and Bennett, J. 1997. International Scoring System for Evaluating Prognosis in Myelodysplastic Syndromes. *Blood.* **89**(6), pp.2079-2088.

Greenberg, P.L., Attar, E., Bennett, J.M., Bloomfield, C.D., Borate, U., De Castro, C.M., Deeg, H.J., Frankfurt, O., Gaensler, K., Garcia-Manero, G., Gore, S.D., Head, D., Komrokji, R., Maness, L.J., Millenson, M., O'Donnell, M.R., Shami, P.J., Stein, B.L., Stone, R.M., Thompson, J.E., Westervelt, P., Wheeler, B., Shead, D.A. and Naganuma, M. 2013. Myelodysplastic syndromes: clinical practice guidelines in oncology. *J Natl Compr Canc Netw.* **11**(7), pp.838-874.

Greenberg, P.L., Tuechler, H., Schanz, J., Sanz, G., Garcia-Manero, G., Solé, F., Bennett, J.M., Bowen, D., Fenaux, P., Dreyfus, F., Kantarjian, H., Kuendgen, A., Levis, A., Malcovati, L., Cazzola, M., Cermak, J., Fonatsch, C., Le Beau, M.M., Slovak, M.L., Krieger, O., Luebbert, M., Maciejewski, J., Magalhaes, S.M.M., Miyazaki, Y., Pfeilstöcker, M., Sekeres, M., Sperr, W.R., Stauder, R., Tauro, S., Valent, P., Vallespi, T., van de Loosdrecht, A.A., Germing, U. and Haase, D. 2012. Revised International Prognostic Scoring System for Myelodysplastic Syndromes. *Blood.* **120**(12), pp.2454-2465.

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G.L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Stevens, C., Edkins, S., O/'Meara, S., Vastrik, I., Schmidt, E.E., Avis, T., Barthorpe, S., Bhamra, G., Buck, G., Choudhury, B., Clements, J., Cole, J., Dicks, E., Forbes, S., Gray, K., Halliday, K., Harrison, R., Hills, K., Hinton, J., Jenkinson, A., Jones, D., Menzies, A., Mironenko, T., Perry, J., Raine, K., Richardson, D., Shepherd, R., Small, A., Tofts, C., Varian, J., Webb, T., West, S., Widaa, S., Yates, A., Cahill, D.P., Louis, D.N., Goldstraw, P., Nicholson, A.G., Brasseur, F., Looijenga, L., Weber, B.L., Chiew, Y.-E., deFazio, A., Greaves, M.F., Green, A.R., Campbell, P., Birney, E., Easton, D.F., Chenevix-Trench, G., Tan, M.-H., Khoo, S.K., Teh, B.T., Yuen, S.T., Leung, S.Y., Wooster, R., Futreal, P.A. and Stratton, M.R. 2007. Patterns of somatic mutation in human cancer genomes. *Nature.* **446**(7132), pp.153-158.

Gregg, X.T., Reddy, V. and Prchal, J.T. 2002. Copper deficiency masquerading as myelodysplastic syndrome. *Blood.* **100**(4), pp.1493-1495.

Guerrettaz, L.M., Johnson, S.A. and Cambier, J.C. 2008. Acquired hematopoietic stem cell defects determine B-cell repertoire changes associated with aging. *Proc Natl Acad Sci U S A.* **105**(33), pp.11898-11902.

Gyan, E., Andrieu, V., Sanna, A., Caille, A., Schemenau, J., Sudaka, I., Siguret, V., Malet, M., Park, S., Bordessoule, D., Mairesse, J., Gelsi-Boyer, V., Cheze, S., Beyne-Rauzy, O., Sebert, M., Sapena, R., Zerazhi, H., Legros, L., Guerci-Bresler, A., Ame, S.N., Germing, U., Santini, V., Salvi, F., Gioia, D., Lunghi, M., Dreyfus, F. and Fenaux, P. 2016. Myelodysplastic syndromes with single neutropenia or thrombocytopenia are rarely refractory cytopenias with unilineage dysplasia by World Health Organization 2008 criteria and have favourable prognosis. *Br J Haematol.*

Haase, D., Germing, U., Schanz, J., Pfeilstocker, M., Nosslinger, T., Hildebrandt, B., Kundgen, A., Lubbert, M., Kunzmann, R., Giagounidis, A.A.N., Aul, C., Trumper, L., Krieger, O., Stauder, R., Muller, T.H., Wimazal, F., Valent, P., Fonatsch, C. and Steidl, C. 2007. New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. *Blood.* **110**(13), pp.4385-4395.

Haferlach, T., Nagata, Y., Grossmann, V., Okuno, Y., Bacher, U., Nagae, G., Schnittger, S., Sanada, M., Kon, A., Alpermann, T., Yoshida, K., Roller, A., Nadarajah, N., Shiraishi, Y., Shiozawa, Y., Chiba, K., Tanaka, H., Koeffler, H.P., Klein, H.U., Dugas, M., Aburatani, H., Kohlmann, A., Miyano, S., Haferlach, C., Kern, W. and Ogawa, S. 2014. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia.* **28**(2), pp.241-247.

Hall, M., Frank, E., Holmes, G., Pfahringer, B., Reutemann, P. and Witten, I.H. 2009. *The WEKA Data Mining Software: An Update*. SIGKDD Explorations. 11.

Hamilton-Paterson, J.L. 1949. Pre Leukaemic Anaemia. *Acta Haematologica.* **2**(5), pp.309-316.

Han, X., Jorgensen, J.L., Brahmandam, A., Schlette, E., Huh, Y.O., Shi, Y., Awagu, S. and Chen, W. 2008. Immunophenotypic study of basophils by multiparameter flow cytometry. *Arch Pathol Lab Med.* **132**(5), pp.813-819.

Hanson, C., Hoyer, J., Zakko, L., Hodnefield, J. and Steensma, D. 2009. P089 Is idiopathic cytopenia of undetermined significance (ICUS) a valid clinical concept? A longitudinal clinicopathological study. *Leukemia Research.* **33**, pp.S109-S111.

Harrison, P., Mackie, I., Mumford, A., Briggs, C., Liesner, R., Winter, M. and Machin, S. 2011. Guidelines for the laboratory investigation of heritable disorders of platelet function. *Br J Haematol.* **155**(1), pp.30-44.

Heuser, M., Schlarmann, C., Dobbernack, V., Panagiota, V., Wiehlmann, L., Walter, C., Beier, F., Ziegler, P., Yun, H., Kade, S., Kirchner, A., Huang, L., Koenecke, C., Eder, M., Brummendorf, T.H., Dugas, M., Ganser, A. and Thol, F. 2014. Genetic characterization of acquired aplastic anemia by targeted sequencing. *Haematologica.* Italy, pp.e165-167.

Hirai, H., Kobayashi, Y., Mano, H., Hagiwara, K., Maru, Y., Omine, M., Mizoguchi, H., Nishida, J. and Takaku, F. 1987. A point mutation at codon 13 of the N-ras oncogene in myelodysplastic syndrome. *Nature.* **327**(6121), pp.430-432.

HMRN. 2016. *HMRN region: participating hospitals.* [Online]. [Accessed 20th February]. Available from: https://www.hmrn.org/about/info

HMRN. 2016. *HMRN - Incidence.* [Online]. [Accessed 20th February

]. Available from: https://www.hmrn.org/statistics/incidence

Holte, R. 1993. Very Simple Classification Rules Perform Well on Most Commonly Used Datasets. *Machine Learning.* **11**(1), pp.63-90.

Howe, R.B., Porwit-MacDonald, A., Wanat, R., Tehranchi, R. and Hellström-Lindberg, E. 2003. The WHO classification of MDS does make a difference. *Blood.* **103**(9), pp.3265-3270.

Imai, Y., Kurokawa, M., Izutsu, K., Hangaishi, A., Takeuchi, K., Maki, K., Ogawa, S., Chiba, S., Mitani, K. and Hirai, H. 2000. Mutations of the AML1 gene in myelodysplastic

syndrome and their functional implications in leukemogenesis. *Blood.* **96**(9), pp.3154-3160.

Itzykson, R., Kosmider, O., Renneville, A., Gelsi-Boyer, V., Meggendorfer, M., Morabito, M., Berthon, C., Ades, L., Fenaux, P., Beyne-Rauzy, O., Vey, N., Braun, T., Haferlach, T., Dreyfus, F., Cross, N.C., Preudhomme, C., Bernard, O.A., Fontenay, M., Vainchenker, W., Schnittger, S., Birnbaum, D., Droin, N. and Solary, E. 2013. Prognostic score including gene mutations in chronic myelomonocytic leukemia. *J Clin Oncol.* **31**(19), pp.2428-2436.

Izumi, M., Takeshita, A., Shinjo, K., Naito, K., Matsui, H., Shibata, K., Ohnishi, K., Kanno, T. and Ohno, R. 2001. Decreased amount of mpl and reduced expression of glycoprotein IIb/IIIa and glycoprotein Ib on platelets from patients with refractory anemia: analysis by a non-isotopic quantitative ligand binding assay and immunofluorescence. *European Journal of Haematology.* **66**(4), pp.245-252.

Jabbar, K.J., Medeiros, L.J., Wang, S.A., Miranda, R.N., Johnson, M.R., Verstovsek, S. and Jorgensen, J.L. 2014. Flow cytometric immunophenotypic analysis of systemic mastocytosis involving bone marrow. *Arch Pathol Lab Med.* **138**(9), pp.1210-1214.

Jacobs, P.A., Brunton, M., Court Brown, W.M., Doll, R. and Goldstein, H. 1963. Change of human chromosome count distribution with age: evidence for a sex differences. *Nature.* **197**, pp.1080-1081.

Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P.V., Mar, B.G., Lindsley, R.C., Mermel, C.H., Burtt, N., Chavez, A., Higgins, J.M., Moltchanov, V., Kuo, F.C., Kluk, M.J., Henderson, B., Kinnunen, L., Koistinen, H.A., Ladenvall, C., Getz, G., Correa, A., Banahan, B.F., Gabriel, S., Kathiresan, S., Stringham, H.M., McCarthy, M.I., Boehnke, M., Tuomilehto, J., Haiman, C., Groop, L., Atzmon, G., Wilson, J.G., Neuberg, D., Altshuler, D. and Ebert, B.L. 2014. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med.* **371**(26), pp.2488-2498.

James, C., Ugo, V., Le Couedic, J.P., Staerk, J., Delhommeau, F. and Lacout, C. 2005. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature.* **434**, pp.1144-1148.

Jones, G. and Barker, A. 2008. Reference intervals. *Clin Biochem Rev.* **29 Suppl 1**, pp.S93-97.

Jordan, C.T., Upchurch, D., Szilvassy, S.J., Guzman, M.L., Howard, D.S., Pettigrew, A.L., Meyerrose, T., Rossi, R., Grimes, B., Rizzieri, D.A., Luger, S.M. and Phillips, G.L. 2000. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia: Official Journal of the Leukemia Society of America, Leukemia Research Fund, U.K.* **14**(10), pp.1777-1784.

Kahn, J.D., Chamuleau, M.E.D., Westers, T.M., Van de Ven, P.M., van Dreunen, L., van Spronsen, M., Ossenkoppele, G.J. and van de Loosdrecht, A.A. 2015. Regulatory T cells and progenitor B cells are independent prognostic predictors in lower risk myelodysplastic syndromes. *Haematologica.* **100**(6), pp.e220-e222.

Kalina, T., Stuchly, J., Janda, A., Hrusak, O., Ruzickova, S., Sediva, A., Litzman, J. and Vlkova, M. 2009. Profiling of polychromatic flow cytometry data on B-cells reveals patients' clusters in common variable immunodeficiency. *Cytometry A.* **75**(11), pp.902-909.

Kantarjian, H., O'Brien, S., Ravandi, F., Cortes, J., Shan, J., Bennett, J.M., List, A., Fenaux, P., Sanz, G., Issa, J.P., Freireich, E.J. and Garcia-Manero, G. 2008. Proposal for a new risk model in myelodysplastic syndrome that accounts for events not considered in the original International Prognostic Scoring System. *Cancer.* **113**(6), pp.1351-1361.

Kao, J.M., McMillan, A. and Greenberg, P.L. 2008. International MDS risk analysis workshop (IMRAW)/IPSS reanalyzed: impact of cytopenias on clinical outcomes in myelodysplastic syndromes. *American Journal of Hematology.* **83**(10), pp.765-770.

Kaplan, E., Zuelzer, W.W. and Mouriquand, C. 1954. Sideroblasts: a study of stainable nonhemoglobin iron in marrow normoblasts. *Blood.* **9**(3), pp.203-213.

Karcher, D.S. and Frost, A.R. 1991. The bone marrow in human immunodeficiency virus (HIV)-related disease. Morphology and clinical correlation. *American Journal of Clinical Pathology.* **95**(1), pp.63-71.

Kawakami, Z., Ninomiya, H., Tomiyama, J. and Abe, T. 1990. Deficiency of glycosyl-phosphatidylinositol anchored proteins on paroxysmal nocturnal haemoglobinuria (PNH) neutrophils and monocytes: heterogeneous deficiency of decay-accelerating factor (DAF) and CD16 on PNH neutrophils. *Br J Haematol.* **74**(4), pp.508-513.

Kerbauy, D.B. and Deeg, H.J. 2007. Apoptosis and anti-apoptotic mechanisms in the progression of MDS. *Experimental hematology.* **35**(11), pp.1739-1746.

Kern, W., Bacher, U., Haferlach, C., Alpermann, T., Schnittger, S. and Haferlach, T. 2015. Multiparameter flow cytometry provides independent prognostic information in patients with suspected myelodysplastic syndromes: A study on 804 patients. *Cytometry B Clin Cytom.* **88**(3), pp.154-164.

Kern, W., Haferlach, C., Schnittger, S., Alpermann, T. and Haferlach, T. 2013. Serial assessment of suspected myelodysplastic syndromes: significance of flow cytometric findings validated by cytomorphology, cytogenetics, and molecular genetics. *Haematologica.* **98**(2), pp.201-207.

Kern, W., Haferlach, C., Schnittger, S. and Haferlach, T. 2010. Clinical utility of multiparameter flow cytometry in the diagnosis of 1013 patients with suspected myelodysplastic syndrome: correlation to cytomorphology, cytogenetics, and clinical data. *Cancer.* **116**(19), pp.4549-4563.

Killick, S.B., Carter, C., Culligan, D., Dalley, C., Das-Gupta, E., Drummond, M., Enright, H., Jones, G.L., Kell, J., Mills, J., Mufti, G., Parker, J., Raj, K., Sternberg, A., Vyas, P., Bowen, D. and Haematology, B.C.f.S.i. 2014. Guidelines for the diagnosis and management of adult myelodysplastic syndromes. *British Journal of Haematology.* **164**(4), pp.503--525.

Klampfl, T., Gisslinger, H., Harutyunyan, A.S., Nivarthi, H., Rumi, E., Milosevic, J.D., Them, N.C., Berg, T., Gisslinger, B., Pietra, D., Chen, D., Vladimer, G.I., Bagienski, K., Milanesi, C., Casetti, I.C., Sant'Antonio, E., Ferretti, V., Elena, C., Schischlik, F., Cleary, C., Six, M., Schalling, M., Schonegger, A., Bock, C., Malcovati, L., Pascutto, C., Superti-Furga, G., Cazzola, M. and Kralovics, R. 2013. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med.* **369**(25), pp.2379-2390.

Knudson, A.G. 1971. Mutation and Cancer: Statistical Study of Retinoblastoma. *Proceedings of the National Academy of Sciences of the United States of America.* **68**(4), pp.820-823.

Kordasti, S.Y., Ingram, W., Hayden, J., Darling, D., Barber, L., Afzali, B., Lombardi, G., Wlodarski, M.W., Maciejewski, J.P., Farzaneh, F. and Mufti, G.J. 2007. CD4+CD25high Foxp3+ regulatory T cells in myelodysplastic syndrome (MDS). *Blood.* **110**(3), pp.847-850.

Kosmider, O., Gelsi-Boyer, V., Cheok, M., Grabar, S., Della-Valle, V., Picard, F., Viguie, F., Quesnel, B., Beyne-Rauzy, O., Solary, E., Vey, N., Hunault-Berger, M., Fenaux, P., Mansat-De Mas, V., Delabesse, E., Guardiola, P., Lacombe, C., Vainchenker, W., Preudhomme, C., Dreyfus, F., Bernard, O.A., Birnbaum, D. and Fontenay, M. 2009. TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDSs). *Blood.* **114**(15), pp.3285-3291.

Kralovics, R., Passamonti, F., Buser, A.S., Teo, S.S., Tiedt, R., Passweg, J.R., Tichelli, A., Cazzola, M. and Skoda, R.C. 2005. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med.* **352**(17), pp.1779-1790.

Kronmal, R.A. 1993. Spurious Correlation and the Fallacy of the Ratio Standard Revisited. *Journal of the Royal Statistical Society. Series A (Statistics in Society).* **156**(3), pp.379-392.

Kuiper-Kramer, P.A., Huisman, C.M., Van der Molen-Sinke, J., Abbes, A. and Van Eijk, H.G. 1997. The expression of transferrin receptors on erythroblasts in anaemia of chronic disease, myelodysplastic syndromes and iron deficiency. *Acta Haematologica.* **97**(3), pp.127-131.

Kulasekararaj, A.G., Jiang, J., Smith, A.E., Mohamedali, A.M., Mian, S., Gandhi, S., Gaken, J., Czepulkowski, B., Marsh, J.C. and Mufti, G.J. 2014. Somatic mutations identify a subgroup of aplastic anemia patients who progress to myelodysplastic syndrome. *Blood.* **124**(17), pp.2698-2704.

Kuranda, K., Vargaftig, J., de la Rochere, P., Dosquet, C., Charron, D., Bardin, F., Tonnelle, C., Bonnet, D. and Goodhardt, M. 2011. Age-related changes in human hematopoietic stem/progenitor cells. *Aging Cell.* **10**(3), pp.542-546.

Kussick, S.J., Fromm, J.R., Rossini, A., Li, Y., Chang, A., Norwood, T.H. and Wood, B.L. 2005. Four-color flow cytometry shows strong concordance with bone marrow morphology and cytogenetics in the evaluation for myelodysplasia. *American Journal of Clinical Pathology.* **124**(2), pp.170-181.

Kussick, S.J. and Wood, B.L. 2003. Using 4-color flow cytometry to identify abnormal myeloid populations. *Archives of Pathology & Laboratory Medicine.* **127**(9), pp.1140-1147.

Kwok, B., Hall, J.M., Witte, J.S., Xu, Y., Reddy, P., Lin, K., Flamholz, R., Dabbas, B., Yung, A., Al-Hafidh, J., Balmert, E., Vaupel, C., El Hader, C., McGinniss, M.J., Nahas, S.A., Kines, J. and Bejar, R. 2015. MDS-associated somatic mutations and clonal hematopoiesis are common in idiopathic cytopenias of undetermined significance. *Blood.* **126**(21), pp.2355-2361.

Lacronique-Gazaille, C., Chaury, M.-P., Le Guyader, A., Faucher, J.-L., Bordessoule, D. and Feuillard, J. 2007. A simple method for detection of major phenotypic abnormalities in myelodysplastic syndromes: expression of CD56 in CMML. *Haematologica.* **92**(6), pp.859-860.

Lafata, J.E., Simpkins, J., Lamerato, L., Poisson, L., Divine, G. and Johnson, C.C. 2004. The economic impact of false-positive cancer screens. *Cancer Epidemiol Biomarkers Prev.* **13**(12), pp.2126-2132.

Lamarque, M., Raynaud, S., Itzykson, R., Thepot, S., Quesnel, B., Dreyfus, F., Rauzy, O.B., Turlure, P., Vey, N., Recher, C., Dartigeas, C., Legros, L., Delaunay, J., Visanica, S., Stamatoullas, A., Fenaux, P. and Adès, L. 2012. The revised IPSS is a powerful tool to evaluate the outcome of MDS patients treated with azacitidine: the GFM experience. *Blood.* **120**(25), pp.5084-5085.

Lane, A.A., Odejide, O., Kopp, N., Kim, S., Yoda, A., Erlich, R., Wagle, N., Abel, G.A., Rodig, S.J., Antin, J.H. and Weinstock, D.M. 2013. Low frequency clonal mutations recoverable by deep sequencing in patients with aplastic anemia. *Leukemia.* England, pp.968-971.

Langemeijer, S.M.C., Kuiper, R.P., Berends, M., Knops, R., Aslanyan, M.G., Massop, M., Stevens-Linders, E., van Hoogen, P., van Kessel, A.G., Raymakers, R.A.P., Kamping, E.J., Verhoef, G.E., Verburgh, E., Hagemeijer, A., Vandenberghe, P., de Witte, T., van der Reijden, B.A. and Jansen, J.H. 2009. Acquired mutations in TET2 are common in myelodysplastic syndromes. *Nat Genet.* **41**(7), pp.838-842.

Langley, P. 1988. Machine Learning as an Experimental Science. *Machine Learning.* **3**(1), pp.5-8.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A. and Dick, J.E. 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature.* **367**(6464), pp.645-648.

Lazarevic, V., Horstedt, A.S., Johansson, B., Antunovic, P., Billstrom, R., Derolf, A., Lehmann, S., Mollgard, L., Peterson, S., Stockelberg, D., Uggla, B., Vennstrom, L., Wahlin, A., Hoglund, M. and Juliusson, G. 2015. Failure matters: unsuccessful cytogenetics and unperformed cytogenetics are associated with a poor prognosis in a population-based series of acute myeloid leukaemia. *Eur J Haematol.* **94**(5), pp.419-423.

Lerman, C., Trock, B., Rimer, B.K., Boyce, A., Jepson, C. and Engstrom, P.F. 1991. Psychological and behavioral implications of abnormal mammograms. *Ann Intern Med.* **114**(8), pp.657-661.

Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., Harris, C.C., Lichti, C.F., Townsend, R.R., Fulton, R.S., Dooling, D.J., Koboldt, D.C., Schmidt, H., Zhang, Q., Osborne, J.R., Lin, L., O'Laughlin, M., McMichael, J.F., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Magrini, V.J., Vickery, T.L., Hundal, J., Cook, L.L., Conyers, J.J., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T., Walker, J., Kalicki, J., Watson, M.A., Heath, S., Shannon, W.D., Varghese, N., Nagarajan, R., Westervelt, P., Tomasson, M.H., Link, D.C., Graubert, T.A., DiPersio, J.F., Mardis, E.R. and Wilson, R.K. 2010. DNMT3A Mutations in Acute Myeloid Leukemia. *The New England journal of medicine.* **363**(25), pp.2424-2433.

Ley, T.J., Mardis, E.R., Ding, L., Fulton, B., McLellan, M.D., Chen, K., Dooling, D., Dunford-Shore, B.H., McGrath, S., Hickenbotham, M., Cook, L., Abbott, R., Larson, D.E., Koboldt, D.C., Pohl, C., Smith, S., Hawkins, A., Abbott, S., Locke, D., Hillier, L.W., Miner, T., Fulton, L., Magrini, V., Wylie, T., Glasscock, J., Conyers, J., Sander, N., Shi, X., Osborne, J.R., Minx, P., Gordon, D., Chinwalla, A., Zhao, Y., Ries, R.E., Payton, J.E., Westervelt, P., Tomasson, M.H., Watson, M., Baty, J., Ivanovich, J., Heath, S., Shannon, W.D., Nagarajan, R., Walter, M.J., Link, D.C., Graubert, T.A., DiPersio, J.F. and Wilson, R.K. 2008. DNA sequencing of a cytogenetically normal acute myeloid leukemia genome. *Nature.* **456**(7218), pp.66-72.

Li, C. and Wong, W.H. 2001. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A.* **98**(1), pp.31-36.

Lichtman, M.A. 2013. Does a diagnosis of myelogenous leukemia require 20% marrow myeloblasts, and does <5% marrow myeloblasts represent a remission? The history and ambiguity of arbitrary diagnostic boundaries in the understanding of myelodysplasia. *Oncologist.* **18**(9), pp.973-980.

Limpens, J., Stad, R., Vos, C., de Vlaam, C., de Jong, D., van Ommen, G.J., Schuuring, E. and Kluin, P.M. 1995. Lymphoma-associated translocation t(14;18) in blood B cells of normal individuals. *Blood.* **85**(9), pp.2528-2536.

Linda M. Scott, P.D., Wei Tong, Ph.D., Ross L. Levine, M.D., Mike A. Scott, Ph.D., Philip A. Beer, M.R.C.P., M.R.C.Path., Michael R. Stratton, M.D., Ph.D., P. Andrew Futreal, Ph.D., Wendy N. Erber, M.D., Mary Frances McMullin, F.R.C.P., F.R.C.Path., Claire N. Harrison, M.R.C.P., M.R.C.Path., Alan J. Warren, F.R.C.Path., F.Med.Sci., D. Gary Gilliland, M.D., Ph.D., Harvey F. Lodish, Ph.D., and Anthony R. Green. 2014. JAK2 Exon 12 Mutations in Polycythemia Vera and Idiopathic Erythrocytosis — NEJM.

List, A., Dewald, G., Bennett, J., Giagounidis, A., Raza, A., Feldman, E., Powell, B., Greenberg, P., Thomas, D., Stone, R., Reeder, C., Wride, K., Patin, J., Schmidt, M., Zeldis, J. and Knight, R. 2006. Lenalidomide in the Myelodysplastic Syndrome with Chromosome 5q Deletion. *New England Journal of Medicine.* **355**(14), pp.1456-1465.

Liu, X. 2012. Classification accuracy and cut point selection. *Statistics in Medicine.* **31**(23), pp.2676--2686.

Loken, M.R., Shah, V.O., Dattilio, K.L. and Civin, C.I. 1987. Flow cytometric analysis of human bone marrow: I. Normal erythroid development. *Blood.* **69**(1), pp.255-263.

Longo, L., Bessler, M., Beris, P., Swirsky, D. and Luzzatto, L. 1994. Myelodysplasia in a patient with pre-existing paroxysmal nocturnal haemoglobinuria: a clonal disease originating from within a clonal disease. *Br J Haematol.* **87**(2), pp.401-403.

Lorand-Metze, I., Ribeiro, E., Lima, C.S., Batista, L.S. and Metze, K. 2007. Detection of hematopoietic maturation abnormalities by flow cytometry in myelodysplastic syndromes and its utility for the differential diagnosis with non-clonal disorders. *Leuk Res.* **31**(2), pp.147-155.

Lorenzo, F., Nishii, K., Monma, F., Kuwagata, S., Usui, E. and Shiku, H. 2006. Mutational analysis of the KIT gene in myelodysplastic syndrome (MDS) and MDS-derived leukemia. *Leukemia Research.* **30**(10), pp.1235-1239.

Maciejewski, J.P. and Mufti, G.J. 2008. Whole genome scanning as a cytogenetic tool in hematologic malignancies. *Blood.* **112**(4), pp.965-974.

Maciejewski, J.P., Risitano, A., Sloand, E.M., Nunez, O. and Young, N.S. 2002. Distinct clinical outcomes for cytogenetic abnormalities evolving from aplastic anemia. *Blood.* **99**(9), pp.3129-3135.

Maecker, H.T., McCoy, J.P. and Nussenblatt, R. 2012. Standardizing immunophenotyping for the Human Immunology Project. *Nat Rev Immunol.* **12**(3), pp.191-200.

Malcovati, L., Della Porta, M.G., Lunghi, M., Pascutto, C., Vanelli, L., Travaglino, E., Maffioli, M., Bernasconi, P., Lazzarino, M., Invernizzi, R. and Cazzola, M. 2005. Flow cytometry evaluation of erythroid and myeloid dysplasia in patients with myelodysplastic syndrome. *Leukemia: Official Journal of the Leukemia Society of America, Leukemia Research Fund, U.K.* **19**(5), pp.776-783.

Malcovati, L., Della Porta, M.G., Strupp, C., Ambaglio, I., Kuendgen, A., Nachtkamp, K., Travaglino, E., Invernizzi, R., Pascutto, C., Lazzarino, M., Germing, U. and Cazzola, M. 2011. Impact of the degree of anemia on the outcome of patients with myelodysplastic syndrome and its integration into the WHO classification-based Prognostic Scoring System (WPSS). *Haematologica.* **96**(10), pp.1433-1440.

Malcovati, L., Germing, U., Kuendgen, A., Della Porta, M.G., Pascutto, C., Invernizzi, R., Giagounidis, A., Hildebrandt, B., Bernasconi, P., Knipp, S., Strupp, C., Lazzarino, M., Aul, C. and Cazzola, M. 2007. Time-Dependent Prognostic Scoring System for Predicting Survival and Leukemic Evolution in Myelodysplastic Syndromes. *Journal of Clinical Oncology.* **25**(23), pp.3503-3510.

Malcovati, L., Hellstrom-Lindberg, E., Bowen, D., Ades, L., Cermak, J., Del Canizo, C., Della Porta, M.G., Fenaux, P., Gattermann, N., Germing, U., Jansen, J.H., Mittelman, M., Mufti, G., Platzbecker, U., Sanz, G.F., Selleslag, D., Skov-Holm, M., Stauder, R., Symeonidis, A., van de Loosdrecht, A.A., de Witte, T. and Cazzola, M. 2013. Diagnosis and treatment of primary myelodysplastic syndromes in adults: recommendations from the European LeukemiaNet. *Blood.* **122**(17), pp.2943-2964.

Malcovati, L., Papaemmanuil, E., Ambaglio, I., Elena, C., Galli, A., Della Porta, M.G., Travaglino, E., Pietra, D., Pascutto, C., Ubezio, M., Bono, E., Da Via, M.C., Brisci, A., Bruno, F., Cremonesi, L., Ferrari, M., Boveri, E., Invernizzi, R., Campbell, P.J. and Cazzola, M. 2014. Driver somatic mutations identify distinct disease entities within myeloid neoplasms with myelodysplasia. *Blood.* **124**(9), pp.1513-1521.

Mangi, M.H. and Mufti, G.J. 1992. Primary myelodysplastic syndromes: diagnostic and prognostic significance of immunohistochemical assessment of bone marrow biopsies. *Blood.* **79**(1), pp.198-205.

Mansfield, E.R. and Helms, B.P. 1982. Detecting Multicollinearity. *The American Statistician.* **36**(3a), pp.158-160.

Manz, M.G., Miyamoto, T., Akashi, K. and Weissman, I.L. 2002. Prospective isolation of human clonogenic common myeloid progenitors. *Proceedings of the National Academy of Sciences.* **99**(18), pp.11872-11877.

Mardis, E.R., Ding, L., Dooling, D.J., Larson, D.E., McLellan, M.D., Chen, K., Koboldt, D.C., Fulton, R.S., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Locke, D.P., Magrini, V.J., Abbott, R.M., Vickery, T.L., Reed, J.S., Robinson, J.S., Wylie, T., Smith, S.M., Carmichael, L., Eldred, J.M., Harris, C.C., Walker, J., Peck, J.B., Du, F., Dukes, A.F., Sanderson, G.E., Brummett, A.M., Clark, E., McMichael, J.F., Meyer, R.J., Schindler, J.K., Pohl, C.S., Wallis, J.W., Shi, X., Lin, L., Schmidt, H., Tang, Y., Haipek, C., Wiechert, M.E., Ivy, J.V., Kalicki, J., Elliott, G., Ries, R.E., Payton, J.E., Westervelt, P., Tomasson, M.H., Watson, M.A., Baty, J., Heath, S., Shannon, W.D., Nagarajan, R., Link, D.C., Walter, M.J., Graubert, T.A., DiPersio, J.F., Wilson, R.K. and Ley, T.J. 2009. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med.* **361**(11), pp.1058-1066.

Marsee, D.K., Pinkus, G.S. and Yu, H. 2010. CD71 (transferrin receptor): an effective marker for erythroid precursors in bone marrow biopsy specimens. *Am J Clin Pathol.* **134**(3), pp.429-435.

Matarraz, S., Almeida, J., Flores-Montero, J., Lecrevisse, Q., Guerri, V., Lopez, A., Barrena, S., Van Der Velden, V.H., Te Marvelde, J.G., Van Dongen, J.J. and Orfao, A. 2015. Introduction to the diagnosis and classification of monocytic-lineage leukemias by flow cytometry. *Cytometry B Clin Cytom.*

Matarraz, S., Lopez, A., Barrena, S., Fernandez, C., Jensen, E., Flores, J., Barcena, P., Rasillo, A., Sayagues, J.M., Sanchez, M.L., Hernandez-Campo, P., Hernandez Rivas, J.M., Salvador, C., Fernandez-Mosteirin, N., Giralt, M., Perdiguer, L. and Orfao, A. 2008. The immunophenotype of different immature, myeloid and B-cell lineage-committed CD34+ hematopoietic cells allows discrimination between normal/reactive and myelodysplastic syndrome precursors. *Leukemia.* **22**(6), pp.1175-1183.

Matarraz, S., Lopez, A., Barrena, S., Fernandez, C., Jensen, E., Flores-Montero, J., Rasillo, A., Sayagues, J.M., Sanchez, M.L., Barcena, P., Hernandez-Rivas, J.M., Salvador, C., Fernandez-Mosteirin, N., Giralt, M., Perdiguer, L., Laranjeira, P., Paiva, A. and Orfao, A. 2010. Bone marrow cells from myelodysplastic syndromes show altered immunophenotypic profiles that may contribute to the diagnosis and prognostic stratification of the disease: a pilot study on a series of 56 patients. *Cytometry B Clin Cytom.* **78**(3), pp.154-168.

Mathis, S., Chapuis, N., Debord, C., Rouquette, A., Radford-Weiss, I., Park, S., Dreyfus, F., Lacombe, C., Bene, M.C., Kosmider, O., Fontenay, M. and Bardet, V. 2013. Flow cytometric detection of dyserythropoiesis: a sensitive and powerful diagnostic tool for myelodysplastic syndromes. *Leukemia.* **27**(10), pp.1981-1987.

Maynadie, M., Verret, C., Moskovtchenko, P., Mugneret, F., Petrella, T., Caillot, D. and Carli, P.M. 1996. Epidemiological characteristics of myelodysplastic syndrome in a well-defined French population. *Br J Cancer.* **74**(2), pp.288-290.

McKenna, K., Beignon, A.S. and Bhardwaj, N. 2005. Plasmacytoid dendritic cells: linking innate and adaptive immunity. *J Virol.* **79**(1), pp.17-27.

McKenna, R.W., Washington, L.T., Aquino, D.B., Picker, L.J. and Kroft, S.H. 2001. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry. *Blood.* **98**(8), pp.2498-2507.

Medeiros, B.C., Othus, M., Estey, E.H., Fang, M. and Appelbaum, F.R. 2014. Unsuccessful Diagnostic Cytogenetic Analysis Is a Poor Prognostic Feature in Acute Myeloid Leukaemia. *British journal of haematology.* **164**(2), pp.245-250.

Meggendorfer, M., Roller, A., Haferlach, T., Eder, C., Dicker, F., Grossmann, V., Kohlmann, A., Alpermann, T., Yoshida, K., Ogawa, S., Koeffler, H.P., Kern, W., Haferlach, C. and Schnittger, S. 2012. SRSF2 mutations in 275 cases with chronic myelomonocytic leukemia (CMML). *Blood.* **120**(15), pp.3080-3088.

Mian, S.A., Rouault-Pierre, K., Smith, A.E., Seidl, T., Pizzitola, I., Kizilors, A., Kulasekararaj, A.G., Bonnet, D. and Mufti, G.J. 2015. SF3B1 mutant MDS-initiating cells may arise from the haematopoietic stem cell compartment. *Nat Commun.* **6**, p10004.

Miller, K.B., Kim, K., Morrison, F.S., Winter, J.N., Bennett, J.M., Neiman, R.S., Head, D.R., Cassileth, P.A., O'Connell, M.J. and Kyungmann, K. 1992. The evaluation of low-dose cytarabine in the treatment of myelodysplastic syndromes: a phase-III intergroup study. *Annals of Hematology.* **65**(4), pp.162-168.

Min, H., Montecino-Rodriguez, E. and Dorshkind, K. 2006. Effects of aging on the common lymphoid progenitor to pro-B cell transition. *J Immunol.* **176**(2), pp.1007-1012.

Mishra, A., Corrales-Yepez, M., Ali, N.A., Kharfan-Dabaja, M., Padron, E., Zhang, L., Epling-Burnette, P.K., Pinilla-Ibarz, J., Lancet, J.E., List, A.F. and Komrokji, R.S. 2013. Validation of the revised International Prognostic Scoring System in treated patients with myelodysplastic syndromes. *Am J Hematol.* **88**(7), pp.566-570.

Mohamedali, A., Gaken, J., Twine, N.A., Ingram, W., Westwood, N., Lea, N.C., Hayden, J., Donaldson, N., Aul, C., Gattermann, N., Giagounidis, A., Germing, U., List, A.F. and Mufti, G.J. 2007. Prevalence and prognostic significance of allelic imbalance by

single-nucleotide polymorphism analysis in low-risk myelodysplastic syndromes. *Blood.* **110**(9), pp.3365-3373.

Mohamedali, A.M., Alkhatabi, H., Kulasekararaj, A., Shinde, S., Mian, S., Malik, F., Smith, A.E., Gaken, J. and Mufti, G.J. 2013. Utility of peripheral blood for cytogenetic and mutation analysis in myelodysplastic syndrome. *Blood.* **122**(4), pp.567-570.

Mohamedali, A.M., Gaken, J., Ahmed, M., Malik, F., Smith, A.E., Best, S., Mian, S., Gaymes, T., Ireland, R., Kulasekararaj, A.G. and Mufti, G.J. 2015. High concordance of genomic and cytogenetic aberrations between peripheral blood and bone marrow in myelodysplastic syndrome (MDS). *Leukemia.* **29**(9), pp.1928-1938.

Monreal, M.B., Pardo, M.L., Pavlovsky, M.A., Fernandez, I., Corrado, C.S., Giere, I., Sapia, S. and Pavlovsky, S. 2006. Increased immature hematopoietic progenitor cells CD34+/CD38dim in myelodysplasia. *Cytometry. Part B, Clinical Cytometry.* **70**(2), pp.63-70.

Morin, R.D., Johnson, N.A., Severson, T.M., Mungall, A.J., An, J., Goya, R., Paul, J.E., Boyle, M., Woolcock, B.W., Kuchenbauer, F., Yap, D., Humphries, R.K., Griffith, O.L., Shah, S., Zhu, H., Kimbara, M., Shashkin, P., Charlot, J.F., Tcherpakov, M., Corbett, R., Tam, A., Varhol, R., Smailus, D., Moksa, M., Zhao, Y., Delaney, A., Qian, H., Birol, I., Schein, J., Moore, R., Holt, R., Horsman, D.E., Connors, J.M., Jones, S., Aparicio, S., Hirst, M., Gascoyne, R.D. and Marra, M.A. 2010. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. *Nat Genet.* **42**(2), pp.181-185.

Mufti, G.J., Bennett, J.M., Goasguen, J., Bain, B.J., Baumann, I., Brunning, R., Cazzola, M., Fenaux, P., Germing, U., Hellstrom-Lindberg, E., Jinnai, I., Manabe, A., Matsuda, A., Niemeyer, C.M., Sanz, G., Tomonaga, M., Vallespi, T. and Yoshimi, A. 2008. Diagnosis and classification of myelodysplastic syndrome: International Working Group on Morphology of myelodysplastic syndrome (IWGM-MDS) consensus proposals for the definition and enumeration of myeloblasts and ring sideroblasts. *Haematologica.* **93**(11), pp.1712-1717.

Nagata, H., Worobec, A.S., Oh, C.K., Chowdhury, B.A., Tannenbaum, S., Suzuki, Y. and Metcalfe, D.D. 1995. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci U S A.* **92**(23), pp.10560-10564.

Nakao, M., Yokota, S., Iwai, T., Kaneko, H., Horiike, S., Kashima, K., Sonoda, Y., Fujimoto, T. and Misawa, S. 1996. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia.* **10**(12), pp.1911-1918.

Nangalia, J., Massie, C.E., Baxter, E.J., Nice, F.L., Gundem, G. and Wedge, D.C. 2013. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *New Engl J Med.* **369**, pp.2391-2405.

National Cancer Institute. SEER Cancer Statistics Review 1975-2012. *National Cancer Institute. SEER Cancer Statistics Review 1975-2012. Myelodysplastic Syndromes (MDS), Chronic Myeloproliferative Disorders (CMD), andChronic Myelomonocytic Leukemia (CMML). Available at http://seer.cancer.gov/csr/1975_2012/results_merged/sect_30_mds.pdf.*

NHS, D.o.H. 2007. *Cancer Reform Strategy.* [Online]. [Accessed 23rd February]. Available from: http://www.nhs.uk/NHSEngland/NSF/Documents/Cancer%20Reform%20Strategy.pdf Available from: http://webarchive.nationalarchives.gov.uk/+/www.dh.gov.uk/en/Healthcare/Cancer/DH_091120

NICE. 2003. *Improving outcomes in haemato-oncology cancer.* [Online]. [Accessed February 14]. Available from: https://web.archive.org/web/20111015033031/http://guidance.nice.org.uk/CSGHO/Guidance/pdf/English

Niemeyer, C.M., Aricó, M., Basso, G., Biondi, A., Rajnoldi, A.C., Creutzig, U., Haas, O., Harbott, J., Hasle, H., Kerndrup, G., Locatelli, F., Mann, G., Stollmann-Gibbels, B., Veer-Korthof, E.T.v.t., Wering, E.v., Zimmermann, M. and (EWOG-MDS), M.o.t.E.W.G.o.M.S.i.C. 1997. Chronic Myelomonocytic Leukemia in Childhood: A Retrospective Analysis of 110 Cases.

Nilsson, L., Astrand-Grundström, I., Arvidsson, I., Jacobsson, B., Hellström-Lindberg, E., Hast, R. and Jacobsen, S.E. 2000. Isolation and characterization of hematopoietic progenitor/stem cells in 5q-deleted myelodysplastic syndromes: evidence for involvement at the hematopoietic stem cell level. *Blood.* **96**(6), pp.2012-2021.

Nilsson, L., Edén, P., Olsson, E., Månsson, R., Astrand-Grundström, I., Strömbeck, B., Theilgaard-Mönch, K., Anderson, K., Hast, R., Hellström-Lindberg, E., Samuelsson, J., Bergh, G., Nerlov, C., Johansson, B., Sigvardsson, M., Borg, A. and Jacobsen, S.E.W. 2007. The molecular signature of MDS stem cells supports a stem-cell origin of 5q myelodysplastic syndromes. *Blood.* **110**(8), pp.3005-3014.

Nimer, S.D. and Golde, D.W. 1987. The 5q- abnormality. *Blood.* **70**(6), pp.1705-1712.

Nosslinger, T., Tuchler, H., Germing, U., Sperr, W.R., Krieger, O., Haase, D., Lubbert, M., Stauder, R., Giagounidis, A., Valent, P. and Pfeilstocker, M. 2010. Prognostic impact of age and gender in 897 untreated patients with primary myelodysplastic syndromes. *Ann Oncol.* **21**(1), pp.120-125.

O'Keefe, C., McDevitt, M.A. and Maciejewski, J.P. 2010. Copy neutral loss of heterozygosity: a novel chromosomal lesion in myeloid malignancies. *Blood.* **115**(14), pp.2731-2739.

Ogata, K., Della Porta, M.G., Malcovati, L., Picone, C., Yokose, N., Matsuda, A., Yamashita, T., Tamura, H., Tsukada, J. and Dan, K. 2009. Diagnostic utility of flow cytometry in low-grade myelodysplastic syndromes: a prospective validation study. *Haematologica.* Italy, pp.1066-1074.

Ogata, K., Kishikawa, Y., Satoh, C., Tamura, H., Dan, K. and Hayashi, A. 2006. Diagnostic application of flow cytometric characteristics of CD34+ cells in low-grade myelodysplastic syndromes. *Blood.* United States, pp.1037-1044.

Ogata, K., Nakamura, K., Yokose, N., Tamura, H., Tachibana, M., Taniguchi, O., Iwakiri, R., Hayashi, T., Sakamaki, H., Murai, Y., Tohyama, K., Tomoyasu, S., Nonaka, Y., Mori, M., Dan, K. and Yoshida, Y. 2002. Clinical significance of phenotypic features of blasts in patients with myelodysplastic syndrome. *Blood.* **100**(12), pp.3887-3896.

Ojha, J., Secreto, C., Rabe, K., Ayres-Silva, J., Tschumper, R., Dyke, D.V., Slager, S., Fonseca, R., Shanafelt, T., Kay, N. and Braggio, E. 2014. Monoclonal B-cell lymphocytosis is characterized by mutations in CLL putative driver genes and clonal heterogeneity many years before disease progression. *Leukemia.* England, pp.2395-2398.

Olweus, J., Lund-Johansen, F. and Terstappen, L.W. 1995. CD64/Fc gamma RI is a granulo-monocytic lineage marker on CD34+ hematopoietic progenitor cells. *Blood.* **85**(9), pp.2402-2413.

Onida, F., Kantarjian, H.M., Smith, T.L., Ball, G., Keating, M.J., Estey, E.H., Glassman, A.B., Albitar, M., Kwari, M.I. and Beran, M. 2002. Prognostic factors and scoring systems in chronic myelomonocytic leukemia: a retrospective analysis of 213 patients. *Blood.* **99**(3), pp.840-849.

Orfao, A., Escribano, L., Villarrubia, J., Velasco, J.L., Cervero, C., Ciudad, J., Navarro, J.L. and San Miguel, J.F. 1996. Flow cytometric analysis of mast cells from normal and pathological human bone marrow samples: identification and enumeration. *Am J Pathol.* **149**(5), pp.1493-1499.

Osato, M., Asou, N., Abdalla, E., Hoshino, K., Yamasaki, H., Okubo, T., Suzushima, H., Takatsuki, K., Kanno, T., Shigesada, K. and Ito, Y. 1999. Biallelic and Heterozygous Point Mutations in the Runt Domain of theAML1/PEBP2・B Gene Associated With Myeloblastic Leukemias. *Blood.* **93**(6), pp.1817-1824.

Padron, E., Garcia-Manero, G., Patnaik, M.M., Itzykson, R., Lasho, T., Nazha, A., Rampal, R.K., Sanchez, M.E., Jabbour, E., Al Ali, N.H., Thompson, Z., Colla, S., Fenaux, P., Kantarjian, H.M., Killick, S., Sekeres, M.A., List, A.F., Onida, F., Komrokji, R.S.,

Tefferi, A. and Solary, E. 2015. An international data set for CMML validates prognostic scoring systems and demonstrates a need for novel prognostication strategies. *Blood Cancer J.* **5**, pe333.

Pang, W.W., Pluvinage, J.V., Price, E.A., Sridhar, K., Arber, D.A., Greenberg, P.L., Schrier, S.L., Park, C.Y. and Weissman, I.L. 2013. Hematopoietic stem cell and progenitor cell mechanisms in myelodysplastic syndromes. *Proceedings of the National Academy of Sciences.* **110**(8), pp.3011-3016.

Papaemmanuil, E., Cazzola, M., Boultwood, J., Malcovati, L., Vyas, P., Bowen, D., Pellagatti, A., Wainscoat, J.S., Hellstrom-Lindberg, E., Gambacorti-Passerini, C., Godfrey, A.L., Rapado, I., Cvejic, A., Rance, R., McGee, C., Ellis, P., Mudie, L.J., Stephens, P.J., McLaren, S., Massie, C.E., Tarpey, P.S., Varela, I., Nik-Zainal, S., Davies, H.R., Shlien, A., Jones, D., Raine, K., Hinton, J., Butler, A.P., Teague, J.W., Baxter, E.J., Score, J., Galli, A., Della Porta, M.G., Travaglino, E., Groves, M., Tauro, S., Munshi, N.C., Anderson, K.C., El-Naggar, A., Fischer, A., Mustonen, V., Warren, A.J., Cross, N.C.P., Green, A.R., Futreal, P.A., Stratton, M.R. and Campbell, P.J. 2011. Somatic SF3B1 Mutation in Myelodysplasia with Ring Sideroblasts. *New England Journal of Medicine.* **365**(15), pp.1384-1395.

Papaemmanuil, E., Gerstung, M., Malcovati, L., Tauro, S., Gundem, G., Van Loo, P., Yoon, C.J., Ellis, P., Wedge, D.C., Pellagatti, A., Shlien, A., Groves, M.J., Forbes, S.A., Raine, K., Hinton, J., Mudie, L.J., McLaren, S., Hardy, C., Latimer, C., Della Porta, M.G., O'Meara, S., Ambaglio, I., Galli, A., Butler, A.P., Walldin, G., Teague, J.W., Quek, L., Sternberg, A., Gambacorti-Passerini, C., Cross, N.C.P., Green, A.R., Boultwood, J., Vyas, P., Hellstrom-Lindberg, E., Bowen, D., Cazzola, M., Stratton, M.R. and Campbell, P.J. 2013. Clinical and biological implications of driver mutations in myelodysplastic syndromes. *Blood.* **122**(22), pp.3616-3627.

Parker, J.E., Mufti, G.J., Rasool, F., Mijovic, A., Devereux, S. and Pagliuca, A. 2000. The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. *Blood.* **96**(12), pp.3932-3938.

Parmentier, S., Schetelig, J., Lorenz, K., Kramer, M., Ireland, R., Schuler, U., Ordemann, R., Rall, G., Schaich, M., Bornhauser, M., Ehninger, G. and Kroschinsky, F. 2012. Assessment of dysplastic hematopoiesis: lessons from healthy bone marrow donors. *Haematologica.* Italy, pp.723-730.

Passmore, S.J., Chessells, J.M., Kempski, H., Hann, I.M., Brownbill, P.A. and Stiller, C.A. 2003. Paediatric myelodysplastic syndromes and juvenile myelomonocytic leukaemia in the UK: a population-based study of incidence and survival. *Br J Haematol.* England, pp.758-767.

Patnaik, M.M., Itzykson, R., Lasho, T.L., Kosmider, O., Finke, C.M., Hanson, C.A., Knudson, R.A., Ketterling, R.P., Tefferi, A. and Solary, E. 2014. ASXL1 and SETBP1 mutations and their prognostic contribution in chronic myelomonocytic leukemia: a two-center study of 466 patients. *Leukemia.* **28**(11), pp.2206-2212.

Patnaik, M.M., Lasho, T.L., Vijayvargiya, P., Finke, C.M., Hanson, C.A., Ketterling, R.P., Gangat, N. and Tefferi, A. 2016. Prognostic interaction between ASXL1 and TET2 mutations in chronic myelomonocytic leukemia. *Blood Cancer Journal.* **6**, pe385.

Patnaik, M.M., Padron, E., LaBorde, R.R., Lasho, T.L., Finke, C.M., Hanson, C.A., Hodnefield, J.M., Knudson, R.A., Ketterling, R.P., Al-kali, A., Pardanani, A., Ali, N.A., Komroji, R.S. and Tefferi, A. 2013. Mayo prognostic model for WHO-defined chronic myelomonocytic leukemia: ASXL1 and spliceosome component mutations and outcomes. *Leukemia.* **27**(7), pp.1504-1510.

Pellagatti, A., Roy, S., Di Genua, C., Burns, A., McGraw, K., Valletta, S., Larrayoz, M.J., Fernandez-Mercado, M., Mason, J., Killick, S., Mecucci, C., Calasanz, M.J., List, A., Schuh, A. and Boultwood, J. 2016. Targeted resequencing analysis of 31 genes commonly mutated in myeloid disorders in serial samples from myelodysplastic syndrome patients showing disease progression. *Leukemia.* **30**(1), pp.248-250.

Pierre, R.V. and Hoagland, H.C. 1972. Age-associated aneuploidy: loss of Y chromosome from human bone marrow cells with aging. *Cancer.* **30**(4), pp.889-894.

Platzbecker, U., Santini, V., Mufti, G.J., Haferlach, C., Maciejewski, J.P., Park, S., Sole, F., van de Loosdrecht, A.A. and Haase, D. 2012. Update on developments in the diagnosis and prognostic evaluation of patients with myelodysplastic syndromes (MDS): consensus statements and report from an expert workshop. *Leuk Res.* **36**(3), pp.264-270.

Pomeroy, C., Oken, M.M., Rydell, R.E. and Filice, G.A. 1991. Infection in the myelodysplastic syndromes. *The American Journal of Medicine.* **90**(3), pp.338-344.

Porwit, A., van de Loosdrecht, A.A., Bettelheim, P., Brodersen, L.E., Burbury, K., Cremers, E., Della Porta, M.G., Ireland, R., Johansson, U., Matarraz, S., Ogata, K., Orfao, A., Preijers, F., Psarra, K., Subira, D., Valent, P., van der Velden, V.H., Wells, D., Westers, T.M., Kern, W. and Bene, M.C. 2014. Revisiting guidelines for integration of flow cytometry results in the WHO classification of myelodysplastic syndromes-proposal from the International/European LeukemiaNet Working Group for Flow Cytometry in MDS. *Leukemia.* **28**(9), pp.1793-1798.

Pozdnyakova, O., Miron, P.M., Tang, G., Walter, O., Raza, A., Woda, B. and Wang, S.A. 2008. Cytogenetic abnormalities in a series of 1,029 patients with primary myelodysplastic syndromes: a report from the US with a focus on some undefined single chromosomal abnormalities. *Cancer.* **113**(12), pp.3331-3340.

Quesada, V., Conde, L., Villamor, N., Ordonez, G.R., Jares, P., Bassaganyas, L., Ramsay, A.J., Bea, S., Pinyol, M., Martinez-Trillos, A., Lopez-Guerra, M., Colomer, D., Navarro, A., Baumann, T., Aymerich, M., Rozman, M., Delgado, J., Gine, E., Hernandez, J.M., Gonzalez-Diaz, M., Puente, D.A., Velasco, G., Freije, J.M., Tubio, J.M., Royo, R., Gelpi, J.L., Orozco, M., Pisano, D.G., Zamora, J., Vazquez, M., Valencia, A., Himmelbauer, H., Bayes, M., Heath, S., Gut, M., Gut, I., Estivill, X., Lopez-Guillermo, A., Puente, X.S., Campo, E. and Lopez-Otin, C. 2012. Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. *Nat Genet.* **44**(1), pp.47-52.

R Core Team. 2015. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. Available from: http://www.R-project.org/

Radlund, A., Thiede, T., Hansen, S., Carlsson, M. and Engquist, L. 1995. Incidence of myelodysplastic syndromes in a Swedish population. *Eur J Haematol.* **54**(3), pp.153-156.

Ramos, F., Fernández-Ferrero, S., Suárez, D., Barbón, M., RodrIguez, J.A., Gil, S., Megido, M., Ciudad, J., López, N., del Cañizo, C. and Orfao, A. 1999. Myelodysplastic syndrome: a search for minimal diagnostic criteria. *Leukemia Research.* **23**(3), pp.283-290.

Rawstron, A.C., Bottcher, S., Letestu, R., Villamor, N., Fazi, C., Kartsios, H., de Tute, R.M., Shingles, J., Ritgen, M., Moreno, C., Lin, K., Pettitt, A.R., Kneba, M., Montserrat, E., Cymbalista, F., Hallek, M., Hillmen, P. and Ghia, P. 2013. Improving efficiency and sensitivity: European Research Initiative in CLL (ERIC) update on the international harmonised approach for flow cytometric residual disease monitoring in CLL. *Leukemia.* **27**(1), pp.142-149.

Rawstron, A.C., Gregory, W.M., de Tute, R.M., Davies, F.E., Bell, S.E., Drayson, M.T., Cook, G., Jackson, G.H., Morgan, G.J., Child, J.A. and Owen, R.G. 2015. Minimal residual disease in myeloma by flow cytometry: independent prediction of survival benefit per log reduction. *Blood.* **125**(12), pp.1932-1935.

Rawstron, A.C., Villamor, N., Ritgen, M., Bottcher, S., Ghia, P., Zehnder, J.L., Lozanski, G., Colomer, D., Moreno, C., Geuna, M., Evans, P.A., Natkunam, Y., Coutre, S.E., Avery, E.D., Rassenti, L.Z., Kipps, T.J., Caligaris-Cappio, F., Kneba, M., Byrd, J.C., Hallek, M.J., Montserrat, E. and Hillmen, P. 2007. International standardized approach for flow cytometric residual disease monitoring in chronic lymphocytic leukaemia. *Leukemia.* **21**(5), pp.956-964.

Raza, A., Gezer, S., Mundle, S., Gao, X.Z., Alvi, S., Borok, R., Rifkin, S., Iftikhar, A., Shetty, V. and Parcharidou, A. 1995. Apoptosis in bone marrow biopsy samples involving stromal and hematopoietic cells in 50 patients with myelodysplastic syndromes. *Blood.* **86**(1), pp.268-276.

Raza, A., Ravandi, F., Rastogi, A., Bubis, J., Lim, S.H., Weitz, I., Castro-Malaspina, H., Galili, N., Jawde, R.A. and Illingworth, A. 2014. A prospective multicenter study of paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure. *Cytometry B Clin Cytom.* **86**(3), pp.175-182.

Redaelli, S., Piazza, R., Rostagno, R., Magistroni, V., Perini, P., Marega, M., Gambacorti-Passerini, C. and Boschelli, F. 2009. Activity of bosutinib, dasatinib, and nilotinib against 18 imatinib-resistant BCR/ABL mutants. *J Clin Oncol.* United States, pp.469-471.

Revised International Prognostic Scoring System (IPSS-R) for Myelodysplastic Syndromes Risk Assessment Calculator | MDS Foundation. 2016. [Online]. Available from: http://www.mds-foundation.org/ipss-r-calculator/

Richards, M.A. 2009. The size of the prize for earlier diagnosis of cancer in England. *Br J Cancer.* **101 Suppl 2**, pp.S125-129.

Rigolin, G.M., Cuneo, A., Roberti, M.G., Bardi, A. and Castoldi, G. 1997. Myelodysplastic syndromes with monocytic component: hematologic and cytogenetic characterization. *Haematologica.* **82**(1), pp.25-30.

Rollison, D.E., Howlader, N., Smith, M.T., Strom, S.S., Merritt, W.D., Ries, L.A., Edwards, B.K. and List, A.F. 2008. Epidemiology of myelodysplastic syndromes and chronic myeloproliferative disorders in the United States, 2001-2004, using data from the NAACCR and SEER programs. *Blood.* **112**(1), pp.45-52.

Rossi, D., Bruscaggin, A., Spina, V., Rasi, S., Khiabanian, H., Messina, M., Fangazio, M., Vaisitti, T., Monti, S., Chiaretti, S., Guarini, A., Del Giudice, I., Cerri, M., Cresta, S., Deambrogi, C., Gargiulo, E., Gattei, V., Forconi, F., Bertoni, F., Deaglio, S., Rabadan, R., Pasqualucci, L., Foa, R., Dalla-Favera, R. and Gaidano, G. 2011. Mutations of the SF3B1 splicing factor in chronic lymphocytic leukemia: association with progression and fludarabine-refractoriness. *Blood.* **118**(26), pp.6904-6908.

Rusten, L.S., Jacobsen, S.E., Kaalhus, O., Veiby, O.P., Funderud, S. and Smeland, E.B. 1994. Functional differences between CD38- and DR- subfractions of CD34+ bone marrow cells. *Blood.* **84**(5), pp.1473-1481.

Ríos, A., Cańtizo, M.C., Sanz, M.A., Vallespí, T., Sanz, G., Torrabadella, M., Gomis, F., Ruiz†, C. and San Miguel, J.F. 1990. Bone marrow biopsy in myelodysplastic syndromes: morphological characteristics and contribution to the study of prognostic factors. *British Journal of Haematology.* **75**(1), pp.26-33.

Saft, L., Björklund, E., Berg, E., Hellström-Lindberg, E. and Porwit, A. 2013. Bone marrow dendritic cells are reduced in patients with high-risk myelodysplastic syndromes. *Leukemia Research.* **37**(3), pp.266-273.

Sandes, A.F., Kerbauy, D.M., Matarraz, S., Chauffaille Mde, L., Lopez, A., Orfao, A. and Yamamoto, M. 2013. Combined flow cytometric assessment of CD45, HLA-DR, CD34, and CD117 expression is a useful approach for reliable quantification of blast cells in myelodysplastic syndromes. *Cytometry B Clin Cytom.* **84**(3), pp.157-166.

Sandes, A.F., Yamamoto, M., Matarraz, S., Chauffaille Mde, L., Quijano, S., Lopez, A., Oguro, T., Kimura, E.Y. and Orfao, A. 2012. Altered immunophenotypic features of peripheral blood platelets in myelodysplastic syndromes. *Haematologica.* **97**(6), pp.895-902.

Sant, M., Allemani, C., Tereanu, C., De Angelis, R., Capocaccia, R., Visser, O., Marcos-Gragera, R., Maynadié, M., Simonetti, A., Lutz, J.-M. and Berrino, F. 2010. Incidence of hematologic malignancies in Europe by morphologic subtype: results of the HAEMACARE project. *Blood.* **116**(19), pp.3724-3734.

Santacruz, R., Villamor, N., Aymerich, M., Martínez-Trillos, A., López, C., Navarro, A., Rozman, M., Beà, S., Royo, C., Cazorla, M., Colomer, D., Giné, E., Pinyol, M., Puente, X.S., López-Otín, C., Campo, E., López-Guillermo, A. and Delgado, J. 2014.

The prognostic impact of minimal residual disease in patients with chronic lymphocytic leukemia requiring first-line therapy. *Haematologica.* **99**(5), pp.873-880.

Satoh, C., Dan, K., Yamashita, T., Jo, R., Tamura, H. and Ogata, K. 2008. Flow cytometric parameters with little interexaminer variability for diagnosing low-grade myelodysplastic syndromes. *Leuk Res.* **32**(5), pp.699-707.

Schanz, J., Tuchler, H., Sole, F., Mallo, M., Luno, E., Cervera, J., Granada, I., Hildebrandt, B., Slovak, M.L., Ohyashiki, K., Steidl, C., Fonatsch, C., Pfeilstocker, M., Nosslinger, T., Valent, P., Giagounidis, A., Aul, C., Lubbert, M., Stauder, R., Krieger, O., Garcia-Manero, G., Faderl, S., Pierce, S., Le Beau, M.M., Bennett, J.M., Greenberg, P., Germing, U. and Haase, D. 2012. New comprehensive cytogenetic scoring system for primary myelodysplastic syndromes (MDS) and oligoblastic acute myeloid leukemia after MDS derived from an international database merge. *J Clin Oncol.* **30**(8), pp.820-829.

Schroeder, T., Ruf, L., Bernhardt, A., Hildebrandt, B., Aivado, M., Aul, C., Gattermann, N., Haas, R. and Germing, U. 2010. Distinguishing myelodysplastic syndromes (MDS) from idiopathic cytopenia of undetermined significance (ICUS): HUMARA unravels clonality in a subgroup of patients. *Annals of Oncology.* **21**(11), pp.2267-2271.

Scott, B.L., Wells, D.A., Loken, M.R., Myerson, D., Leisenring, W.M. and Deeg, H.J. 2008. Validation of a flow cytometric scoring system as a prognostic indicator for posttransplantation outcome in patients with myelodysplastic syndrome. *Blood.* **112**(7), pp.2681-2686.

Sekeres, M.A. and Cutler, C. 2013. How we treat higher-risk myelodysplastic syndromes. *Blood.* **123**(6), pp.829-836.

Sekeres, M.A., Swern, A.S., Fenaux, P., Greenberg, P.L., Sanz, G.F., Bennett, J.M., Dreyfus, F., List, A.F., Li, J.S. and Sugrue, M.M. 2014. Validation of the IPSS-R in lenalidomide-treated, lower-risk myelodysplastic syndrome patients with del(5q). *Blood Cancer Journal.* **4**(8), pe242.

Selimoglu-Buet, D., Wagner-Ballon, O., Saada, V., Bardet, V., Itzykson, R., Bencheikh, L., Morabito, M., Met, E., Debord, C., Benayoun, E., Nloga, A.M., Fenaux, P., Braun, T., Willekens, C., Quesnel, B., Ades, L., Fontenay, M., Rameau, P., Droin, N., Koscielny, S. and Solary, E. 2015. Characteristic repartition of monocyte subsets as a diagnostic signature of chronic myelomonocytic leukemia. *Blood.* **125**(23), pp.3618-3626.

Senent, L., Arenillas, L., Luno, E., Ruiz, J.C., Sanz, G. and Florensa, L. 2013. Reproducibility of the World Health Organization 2008 criteria for myelodysplastic syndromes. *Haematologica.* **98**(4), pp.568-575.

Sha, C., Barrans, S., Care, M.A., Cunningham, D., Tooze, R.M., Jack, A. and Westhead, D.R. 2015. Transferring genomics to the clinic: distinguishing Burkitt and diffuse large B cell lymphomas. *Genome Med.* **7**(1), p64.

Shaffer, L., McGowan-Jordan, J. and Schmid, M. 2013. *ISCN 2013: An International System for Human Cytogenetic Nomenclature.*

Shaffer, L., Slovak, M. and Campbell, L. 2009. *ISCN 2009: An International System for Human Cytogenetic Nomenclature.*

Smith, A., Howell, D., Patmore, R., Jack, A. and Roman, E. 2011. Incidence of haematological malignancy by sub-type: a report from the Haematological Malignancy Research Network. *British Journal of Cancer.* **105**(11), pp.1684-1692.

Smith, A., Roman, E., Howell, D., Jones, R., Patmore, R., Jack, A. and on behalf of the Haematological Malignancy Research, N. 2010a. The Haematological Malignancy Research Network (HMRN): a new information strategy for population based epidemiology and health service research. *British Journal of Haematology.* **148**(5), pp.739-753.

Smith, A.E., Mohamedali, A.M., Kulasekararaj, A., Lim, Z., Gaken, J., Lea, N.C., Przychodzen, B., Mian, S.A., Nasser, E.E., Shooter, C., Westwood, N.B., Strupp, C., Gattermann, N., Maciejewski, J.P., Germing, U. and Mufti, G.J. 2010b. Next-generation sequencing of the TET2 gene in 355 MDS and CMML patients reveals

low-abundance mutant clones with early origins, but indicates no definite prognostic value. *Blood.* **116**(19), pp.3923-3932.

Solary, E., Bernard, O.A., Tefferi, A., Fuks, F. and Vainchenker, W. 2014. The Ten-Eleven Translocation-2 (TET2) gene in hematopoiesis and hematopoietic diseases. *Leukemia.* **28**(3), pp.485-496.

Solé, F., Espinet, B., Sanz, G.F., Cervera, J., Calasanz, M.J., Luño, E., Prieto, F., Granada, I., Hernández, J.M., Cigudosa, J.C., Diez, J.L., Bureo, E., Marqués, M.L., Arranz, E., Ríos, R., Martínez Climent, J.A., Vallespí, T., Florensa, L. and Woessner, S. 2000. Incidence, characterization and prognostic significance of chromosomal abnormalities in 640 patients with primary myelodysplastic syndromes. Grupo Cooperativo Español de Citogenética Hematológica. *British Journal of Haematology.* **108**(2), pp.346-356.

Solé, F., Luño, E., Sanzo, C., Espinet, B., Sanz, G.F., Cervera, J., Calasanz, M.J., Cigudosa, J.C., Millà, F., Ribera, J.M., Bureo, E., Marquez, M.L., Arranz, E. and Florensa, L. 2005. Identification of novel cytogenetic markers with prognostic significance in a series of 968 patients with primary myelodysplastic syndromes. *Haematologica.* **90**(9), pp.1168-1178.

Stachurski, D., Smith, B.R., Pozdnyakova, O., Andersen, M., Xiao, Z., Raza, A., Woda, B.A. and Wang, S.A. 2008. Flow cytometric analysis of myelomonocytic cells by a pattern recognition approach is sensitive and specific in diagnosing myelodysplastic syndrome and related marrow diseases: emphasis on a global evaluation and recognition of diagnostic pitfalls. *Leuk Res.* **32**(2), pp.215-224.

Steensma, D.P., Bejar, R., Jaiswal, S., Lindsley, R.C., Sekeres, M.A., Hasserjian, R.P. and Ebert, B.L. 2015. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood.* **126**(1), pp.9-16.

Steensma, D.P. and Bennett, J.M. 2006. The Myelodysplastic Syndromes: Diagnosis and Treatment. *Mayo Clinic Proceedings.* **81**(1), pp.104-130.

Steensma, D.P., Dewald, G.W., Hodnefield, J.M., Tefferi, A. and Hanson, C.A. 2003. Clonal cytogenetic abnormalities in bone marrow specimens without clear morphologic evidence of dysplasia: a form fruste of myelodysplasia? *Leukemia Research.* **27**(3), pp.235-242.

Sternberg, A., Killick, S., Littlewood, T., Hatton, C., Peniket, A., Seidl, T., Soneji, S., Leach, J., Bowen, D., Chapman, C., Standen, G., Massey, E., Robinson, L., Vadher, B., Kaczmarski, R., Janmohammed, R., Clipsham, K., Carr, A. and Vyas, P. 2005. Evidence for reduced B-cell progenitors in early (low-risk) myelodysplastic syndrome. *Blood.* **106**(9), pp.2982-2991.

Stetler-Stevenson, M., Arthur, D.C., Jabbour, N., Xie, X.Y., Molldrem, J., Barrett, A.J., Venzon, D. and Rick, M.E. 2001. Diagnostic utility of flow cytometric immunophenotyping in myelodysplastic syndrome. *Blood.* **98**(4), pp.979-987.

Stone, M. 1974. Cross-Validatory Choice and Assessment of Statistical Predictions. *Journal of the Royal Statistical Society. Series B (Methodological).* **36**(2), pp.111-147.

Stone, R.M. 2009. How I treat patients with myelodysplastic syndromes. *Blood.* **113**(25), pp.6296-6303.

Stratton, M.R., Campbell, P.J. and Futreal, P.A. 2009. The cancer genome. *Nature.* **458**(7239), pp.719-724.

Such, E., Cervera, J., Costa, D., Solé, F., Vallespí, T., Luño, E., Collado, R., Calasanz, M.J., Hernández-Rivas, J.M., Cigudosa, J.C., Nomdedeu, B., Mallo, M., Carbonell, F., Bueno, J., Ardanaz, M.T., Ramos, F., Tormo, M., Sancho-Tello, R., Cañizo, C.d., Gómez, V., Marco, V., Xicoy, B., Bonanad, S., Pedro, C., Bernal, T. and Sanz, G.F. 2011. Cytogenetic risk stratification in chronic myelomonocytic leukemia. *Haematologica.* **96**(3), pp.375-383.

Such, E., Germing, U., Malcovati, L., Cervera, J., Kuendgen, A., Della Porta, M.G., Nomdedeu, B., Arenillas, L., Luno, E., Xicoy, B., Amigo, M.L., Valcarcel, D., Nachtkamp, K., Ambaglio, I., Hildebrandt, B., Lorenzo, I., Cazzola, M. and Sanz, G.

2013. Development and validation of a prognostic scoring system for patients with chronic myelomonocytic leukemia. *Blood.* **121**(15), pp.3005-3015.

Sugimoto, K., Hirano, N., Toyoshima, H., Chiba, S., Mano, H., Takaku, F., Yazaki, Y. and Hirai, H. 1993. Mutations of the p53 gene in myelodysplastic syndrome (MDS) and MDS- derived leukemia. *Blood.* **81**(11), pp.3022-3026.

Swerdlow, S.H., International Agency for Research on, C. and World Health, O. 2008. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.* International Agency for Research on Cancer.

Tehranchi, R., Woll, P.S., Anderson, K., Buza-Vidas, N., Mizukami, T., Mead, A.J., Astrand-Grundström, I., Strömbeck, B., Horvat, A., Ferry, H., Dhanda, R.S., Hast, R., Rydén, T., Vyas, P., Göhring, G., Schlegelberger, B., Johansson, B., Hellström-Lindberg, E., List, A., Nilsson, L. and Jacobsen, S.E.W. 2010. Persistent malignant stem cells in del(5q) myelodysplasia in remission. *The New England Journal of Medicine.* **363**(11), pp.1025-1037.

Terstappen, L.W., Huang, S., Safford, M., Lansdorp, P.M. and Loken, M.R. 1991. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. *Blood.* **77**(6), pp.1218-1227.

Terstappen, L.W. and Loken, M.R. 1990. Myeloid cell differentiation in normal bone marrow and acute myeloid leukemia assessed by multi-dimensional flow cytometry. *Analytical Cellular Pathology: The Journal of the European Society for Analytical Cellular Pathology.* **2**(4), pp.229-240.

Terstappen, L.W., Safford, M. and Loken, M.R. 1990. Flow cytometric analysis of human bone marrow. III. Neutrophil maturation. *Leukemia: Official Journal of the Leukemia Society of America, Leukemia Research Fund, U.K.* **4**(9), pp.657-663.

Thanopoulou, E., Cashman, J., Kakagianne, T., Eaves, A., Zoumbos, N. and Eaves, C. 2004. Engraftment of NOD/SCID-beta2 microglobulin null mice with multilineage neoplastic cells from patients with myelodysplastic syndrome. *Blood.* **103**(11), pp.4285-4293.

Thiede, C., Koch, S., Creutzig, E., Steudel, C., Illmer, T., Schaich, M. and Ehninger, G. 2006. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood.* **107**(10), pp.4011-4020.

Thol, F., Weissinger, E.M., Krauter, J., Wagner, K., Damm, F., Wichmann, M., Gohring, G., Schumann, C., Bug, G., Ottmann, O., Hofmann, W.K., Schlegelberger, B., Ganser, A. and Heuser, M. 2010. IDH1 mutations in patients with myelodysplastic syndromes are associated with an unfavorable prognosis. *Haematologica.* **95**(10), pp.1668-1674.

Tiu, R.V., Gondek, L.P., O'Keefe, C.L., Elson, P., Huh, J., Mohamedali, A., Kulasekararaj, A., Advani, A.S., Paquette, R., List, A.F., Sekeres, M.A., McDevitt, M.A., Mufti, G.J. and Maciejewski, J.P. 2011. Prognostic impact of SNP array karyotyping in myelodysplastic syndromes and related myeloid malignancies. *Blood.* **117**(17), pp.4552-4560.

Tricot, G., Wolf-Peeters, C.D., Hendrickx, B. and Verwilghen, R.L. 1984. Bone marrow histology in myelodysplastic syndromes. *British Journal of Haematology.* **57**(3), pp.423-430.

Truong, F., Smith, B.R., Stachurski, D., Cerny, J., Medeiros, L.J., Woda, B.A. and Wang, S.A. 2009. The utility of flow cytometric immunophenotyping in cytopenic patients with a non-diagnostic bone marrow: a prospective study. *Leuk Res.* **33**(8), pp.1039-1046.

Tuna, M., Knuutila, S. and Mills, G.B. 2009. Uniparental disomy in cancer. *Trends in Molecular Medicine.* **15**(3), pp.120-128.

Valent, P., Bain, B.J., Bennett, J.M., Wimazal, F., Sperr, W.R., Mufti, G. and Horny, H.P. 2012. Idiopathic cytopenia of undetermined significance (ICUS) and idiopathic dysplasia of uncertain significance (IDUS), and their distinction from low risk MDS. *Leuk Res.* **36**(1), pp.1-5.

Valent, P., Fonatsch, C., Stindl, R., Schwarzinger, I., Haas, O.A., Sperr, W.R., Geissler, K. and Lechner, K. 2004. Normal bone marrow function over 6 years in a patient with dysplastic hematopoiesis and a complex karyotype. *Leuk Res.* **28**(6), pp.651-655.

Valent, P., Horny, H.-P., Bennett, J.M., Fonatsch, C., Germing, U., Greenberg, P., Haferlach, T., Haase, D., Kolb, H.-J., Krieger, O., Loken, M., van de Loosdrecht, A., Ogata, K., Orfao, A., Pfeilstöcker, M., Rüter, B., Sperr, W.R., Stauder, R. and Wells, D.A. 2007. Definitions and standards in the diagnosis and treatment of the myelodysplastic syndromes: Consensus statements and report from a working conference. *Leukemia Research.* **31**(6), pp.727-736.

Valent, P., Jäger, E., Mitterbauer-Hohendanner, G., Müllauer, L., Schwarzinger, I., Sperr, W.R., Thalhammer, R. and Wimazal, F. 2011. Idiopathic bone marrow dysplasia of unknown significance (IDUS): definition, pathogenesis, follow up, and prognosis. *American Journal of Cancer Research.* **1**(4), pp.531-541.

van de Loosdrecht, A.A., Alhan, C., Béné, M.C., Della Porta, M.G., Dräger, A.M., Feuillard, J., Font, P., Germing, U., Haase, D., Homburg, C.H., Ireland, R., Jansen, J.H., Kern, W., Malcovati, L., te Marvelde, J.G., Mufti, G.J., Ogata, K., Orfao, A., Ossenkoppele, G.J., Porwit, A., Preijers, F.W., Richards, S.J., Schuurhuis, G.J., Subirá, D., Valent, P., van der Velden, V.H.J., Vyas, P., Westra, A.H., de Witte, T.M., Wells, D.A., Loken, M.R. and Westers, T.M. 2009. Standardization of flow cytometry in myelodysplastic syndromes: report from the first European LeukemiaNet working conference on flow cytometry in myelodysplastic syndromes. *Haematologica.* **94**(8), pp.1124-1134.

van de Loosdrecht, A.A., Westers, T.M., Westra, A.H., Drager, A.M., van der Velden, V.H.J. and Ossenkoppele, G.J. 2008. Identification of distinct prognostic subgroups in low- and intermediate-1-risk myelodysplastic syndromes by flow cytometry. *Blood.* **111**(3), pp.1067-1077.

Van den Berghe, H., Vermaelen, K., Mecucci, C., Barbieri, D. and Tricot, G. 1985. The 5q- anomaly. *Cancer Genetics and Cytogenetics.* **17**(3), pp.189-255.

van Kamp, H., Smit, J.W., van den Berg, E., Ruud Halie, M. and Vellenga, E. 1994. Myelodysplasia following paroxysmal nocturnal haemoglobinuria: evidence for the emergence of a separate clone. *Br J Haematol.* **87**(2), pp.399-400.

van Lochem, E.G., van der Velden, V.H.J., Wind, H.K., te Marvelde, J.G., Westerdaal, N.A.C. and van Dongen, J.J.M. 2004. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: Reference patterns for age-related changes and disease-induced shifts. *Cytometry.* **60B**(1), pp.1-13.

van Rhenen, A., van Dongen, G.A.M.S., Kelder, A., Rombouts, E.J., Feller, N., Moshaver, B., Stigter-van Walsum, M., Zweegman, S., Ossenkoppele, G.J. and Jan Schuurhuis, G. 2007. The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. *Blood.* **110**(7), pp.2659-2666.

Vardiman, J.W., Harris, N.L. and Brunning, R.D. 2002. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood.* **100**(7), pp.2292-2302.

Vardiman, J.W., Thiele, J., Arber, D.A., Brunning, R.D., Borowitz, M.J., Porwit, A., Harris, N.L., Le Beau, M.M., Hellström-Lindberg, E., Tefferi, A. and Bloomfield, C.D. 2009. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood.* **114**(5), pp.937-951.

Verburgh, E., Achten, R., Louw, V.J., Brusselmans, C., Delforge, M., Boogaerts, M., Hagemeijer, A., Vandenberghe, P. and Verhoef, G. 2007. A new disease categorization of low-grade myelodysplastic syndromes based on the expression of cytopenia and dysplasia in one versus more than one lineage improves on the WHO classification. *Leukemia.* **21**(4), pp.668-677.

Visser, O., Trama, A., Maynadie, M., Stiller, C., Marcos-Gragera, R., De Angelis, R., Mallone, S., Tereanu, C., Allemani, C., Ricardi, U. and Schouten, H.C. 2012. Incidence, survival and prevalence of myeloid malignancies in Europe. *Eur J Cancer.* **48**(17), pp.3257-3266.

Volkert, S., Haferlach, T., Holzwarth, J., Zenger, M., Kern, W., Staller, M., Nagata, Y., Yoshida, K., Ogawa, S., Schnittger, S. and Haferlach, C. 2016. Array CGH identifies copy number changes in 11% of 520 MDS patients with normal karyotype and uncovers prognostically relevant deletions. *Leukemia.* **30**(1), pp.259-261.

Vollmer, R.T. 2009. Blast Counts in Bone Marrow Aspirate Smears. *American Journal of Clinical Pathology.* **131**(2), pp.183-188.

Walter, M.J., Ding, L., Shen, D., Shao, J., Grillot, M., McLellan, M., Fulton, R., Schmidt, H., Kalicki-Veizer, J., O'Laughlin, M., Kandoth, C., Baty, J., Westervelt, P., DiPersio, J.F., Mardis, E.R., Wilson, R.K., Ley, T.J. and Graubert, T.A. 2011. Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. *Leukemia.* **25**(7), pp.1153-1158.

Wang, J.C.Y. and Dick, J.E. 2005. Cancer stem cells: lessons from leukemia. *Trends in Cell Biology.* **15**(9), pp.494-501.

Wang, L., Lawrence, M.S., Wan, Y., Stojanov, P., Sougnez, C., Stevenson, K., Werner, L., Sivachenko, A., DeLuca, D.S., Zhang, L., Zhang, W., Vartanov, A.R., Fernandes, S.M., Goldstein, N.R., Folco, E.G., Cibulskis, K., Tesar, B., Sievers, Q.L., Shefler, E., Gabriel, S., Hacohen, N., Reed, R., Meyerson, M., Golub, T.R., Lander, E.S., Neuberg, D., Brown, J.R., Getz, G. and Wu, C.J. 2011. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. *N Engl J Med.* **365**(26), pp.2497-2506.

Webb, G.I., Boughton, J.R. and Wang, Z. 2005. Not So Naive Bayes: Aggregating One-Dependence Estimators. *Machine Learning.* **58**(1), pp.5-24.

Wells, D.A., Benesch, M., Loken, M.R., Vallejo, C., Myerson, D., Leisenring, W.M. and Deeg, H.J. 2003. Myeloid and monocytic dyspoiesis as determined by flow cytometric scoring in myelodysplastic syndrome correlates with the IPSS and with outcome after hematopoietic stem cell transplantation. *Blood.* United States, pp.394-403.

Westers, T.M., Alhan, C., Chamuleau, M.E.D., van der Vorst, M.J.D.L., Eeltink, C., Ossenkoppele, G.J. and van de Loosdrecht, A.A. 2010. Aberrant immunophenotype of blasts in myelodysplastic syndromes is a clinically relevant biomarker in predicting response to growth factor treatment. *Blood.* **115**(9), pp.1779-1784.

Westers, T.M., Ireland, R., Kern, W., Alhan, C., Balleisen, J.S., Bettelheim, P., Burbury, K., Cullen, M., Cutler, J.A., Della Porta, M.G., Drager, A.M., Feuillard, J., Font, P., Germing, U., Haase, D., Johansson, U., Kordasti, S., Loken, M.R., Malcovati, L., te Marvelde, J.G., Matarraz, S., Milne, T., Moshaver, B., Mufti, G.J., Ogata, K., Orfao, A., Porwit, A., Psarra, K., Richards, S.J., Subira, D., Tindell, V., Vallespi, T., Valent, P., van der Velden, V.H., de Witte, T.M., Wells, D.A., Zettl, F., Bene, M.C. and van de Loosdrecht, A.A. 2012. Standardization of flow cytometry in myelodysplastic syndromes: a report from an international consortium and the European LeukemiaNet Working Group. *Leukemia.* **26**(7), pp.1730-1741.

Wetterstrand, K. 2016. *DNA Sequencing Costs: Data from the NHGRI Genome Sequencing Program (GSP) Available at: www.genome.gov/sequencingcosts.* [Online]. [Accessed 1st February].

Will, B., Zhou, L., Vogler, T.O., Ben-Neriah, S., Schinke, C., Tamari, R., Yu, Y., Bhagat, T.D., Bhattacharyya, S., Barreyro, L., Heuck, C., Mo, Y., Parekh, S., McMahon, C., Pellagatti, A., Boultwood, J., Montagna, C., Silverman, L., Maciejewski, J., Greally, J.M., Ye, B.H., List, A.F., Steidl, C., Steidl, U. and Verma, A. 2012. Stem and progenitor cells in myelodysplastic syndromes show aberrant stage-specific expansion and harbor genetic and epigenetic alterations. *Blood.* **120**(10), pp.2076-2086.

Wimazal, F., Fonatsch, C., Thalhammer, R., Schwarzinger, I., Müllauer, L., Sperr, W.R., Bennett, J.M. and Valent, P. 2007. Idiopathic cytopenia of undetermined significance (ICUS) versus low risk MDS: The diagnostic interface. *Leukemia Research.* **31**(11), pp.1461-1468.

Witten, I.H., Frank, E. and Hall, M.A. 2011. *Data Mining: Practical Machine Learning Tools and Techniques.* Morgan Kaufmann Publishers Inc.

Woll, P.S., Kjallquist, U., Chowdhury, O., Doolittle, H., Wedge, D.C., Thongjuea, S., Erlandsson, R., Ngara, M., Anderson, K., Deng, Q., Mead, A.J., Stenson, L., Giustacchini, A., Duarte, S., Giannoulatou, E., Taylor, S., Karimi, M., Scharenberg, C., Mortera-Blanco, T., Macaulay, I.C., Clark, S.A., Dybedal, I., Josefsen, D., Fenaux, P., Hokland, P., Holm, M.S., Cazzola, M., Malcovati, L., Tauro, S., Bowen, D., Boultwood, J., Pellagatti, A., Pimanda, J.E., Unnikrishnan, A., Vyas, P., Gohring, G., Schlegelberger, B., Tobiasson, M., Kvalheim, G., Constantinescu, S.N., Nerlov, C., Nilsson, L., Campbell, P.J., Sandberg, R., Papaemmanuil, E., Hellstrom-Lindberg, E., Linnarsson, S. and Jacobsen, S.E. 2014. Myelodysplastic syndromes are propagated by rare and distinct human cancer stem cells in vivo. *Cancer Cell.* **25**(6), pp.794-808.

Wolpert, D.H. and Macready, W.G. 1997. No free lunch theorems for optimization. *Evolutionary Computation, IEEE Transactions on.* **1**(1), pp.67-82.

Worsley, A., Oscier, D.G., Stevens, J., Darlow, S., Figes, A., Mufti, G.J. and Hamblin, T.J. 1988. Prognostic features of chronic myelomonocytic leukaemia: a modified Bournemouth score gives the best prediction of survival. *Br J Haematol.* **68**(1), pp.17-21.

Xie, W., Wang, X., Du, W., Liu, W., Qin, X. and Huang, S. 2010. Detection of molecular targets on the surface of CD34+CD38- bone marrow cells in myelodysplastic syndromes. *Cytometry. Part A: The Journal of the International Society for Analytical Cytology.* **77**(9), pp.840-848.

Xu, F., Guo, J., Wu, L.Y., He, Q., Zhang, Z., Chang, C.K. and Li, X. 2013. Diagnostic application and clinical significance of FCM progress scoring system based on immunophenotyping in CD34+ blasts in myelodysplastic syndromes. *Cytometry B Clin Cytom.* **84**(4), pp.267-278.

Xu, F., Li, X., Wu, L., He, Q., Zhang, Z. and Chang, C. 2010. Flow cytometric scoring system (FCMSS) assisted diagnosis of myelodysplastic syndromes (MDS) and the biological significance of FCMSS-based immunophenotypes. *Br J Haematol.* **149**(4), pp.587-597.

Xu, F., Wu, L., He, Q., Zhang, Z., Chang, C. and Li, X. 2012. Immunophenotypic analysis of erythroid dysplasia and its diagnostic application in myelodysplastic syndromes. *Intern Med J.* **42**(4), pp.401-411.

Xu, Y., McKenna, R.W., Karandikar, N.J., Pildain, A.J. and Kroft, S.H. 2005. Flow cytometric analysis of monocytes as a tool for distinguishing chronic myelomonocytic leukemia from reactive monocytosis. *American Journal of Clinical Pathology.* **124**(5), pp.799-806.

Yin, A.H., Miraglia, S., Zanjani, E.D., Almeida-Porada, G., Ogawa, M., Leary, A.G., Olweus, J., Kearney, J. and Buck, D.W. 1997. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood.* **90**(12), pp.5002-5012.

Yokota, S., Kiyoi, H., Nakao, M., Iwai, T., Misawa, S., Okuda, T., Sonoda, Y., Abe, T., Kahsima, K., Matsuo, Y. and Naoe, T. 1997. Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. *Leukemia.* **11**(10), pp.1605-1609.

Yoshida, K., Sanada, M., Shiraishi, Y., Nowak, D., Nagata, Y., Yamamoto, R., Sato, Y., Sato-Otsubo, A., Kon, A., Nagasaki, M., Chalkidis, G., Suzuki, Y., Shiosaka, M., Kawahata, R., Yamaguchi, T., Otsu, M., Obara, N., Sakata-Yanagimoto, M., Ishiyama, K., Mori, H., Nolte, F., Hofmann, W.K., Miyawaki, S., Sugano, S., Haferlach, C., Koeffler, H.P., Shih, L.Y., Haferlach, T., Chiba, S., Nakauchi, H., Miyano, S. and Ogawa, S. 2011. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature.* **478**(7367), pp.64-69.

Yoshizato, T., Dumitriu, B., Hosokawa, K., Makishima, H., Yoshida, K., Townsley, D., Sato-Otsubo, A., Sato, Y., Liu, D., Suzuki, H., Wu, C.O., Shiraishi, Y., Clemente, M.J., Kataoka, K., Shiozawa, Y., Okuno, Y., Chiba, K., Tanaka, H., Nagata, Y., Katagiri, T., Kon, A., Sanada, M., Scheinberg, P., Miyano, S., Maciejewski, J.P., Nakao, S.,

Young, N.S. and Ogawa, S. 2015. Somatic Mutations and Clonal Hematopoiesis in Aplastic Anemia. *New England Journal of Medicine.* **373**(1), pp.35-47.

Zaiss, M., Hirtreiter, C., Rehli, M., Rehm, A., Kunz-Schughart, L.A., Andreesen, R. and Hennemann, B. 2003. CD84 expression on human hematopoietic progenitor cells. *Exp Hematol.* **31**(9), pp.798-805.

Zhu, J. and Emerson, S.G. 2002. Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene.* **21**(21), pp.3295-3313.

8 List of abbreviations

AML - Acute myeloid

APC - Allophycocyanin

AUROC - Area under the receiver operating curve

BSA - Bovine serum albumin

CD - Cluster of differentiation

CDS - Coding sequence

CHIP - Clonal haematopoeisis of indeteminate potential

CLL-1 - C-type lectin-like molecule 1

CLL - Chronic lymphocytic leukaemia

CML - Chronic myeloid leukaemia

CMML - Chronic myelomonocytic leukaemia

CMP - Common myeloid progenitor

CN - LOH - Copy neutral loss of heterozygosity

CS&T - Cytometer Setup & Tracking beads

CV - Coefficient of variation

Cy - Cyanine

DLBCL - Diffuse large B - cell lymphoma

DNA - Deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

ELN - European Leukemia Net

FAB - French - American - British

FBC - Full blood count

FCM - Flow cytometry score

FCS - Flow cytometry standard

FCSS - Flow cytometry scoring system

FDR - False discovery rate

FITC - Fluorescein isothiocyanate

FSC - Forward scatter

G-CSF - Granulocyte colony-stimulating factor

GMP - Granulocyte/macrophage progenitor

HILIS - HMDS Integrated Laboratory Information System

HLA-DR - Human leukocytye antigen-DR

HMDS - Haematological Malignancy Diagnostic Service

HMRN - Haematological Malignancy Research Network
HSC - Haematopoietic stem cell
ICDO - International Classification of Diseases for Oncology
ICUS - Idiopathic cytopenia of undetermined significance
IDUS - Idiopathic dysplasia of uncertain significance
IPSS - International Prognostic Scoring System
IPSS-R - Revised International Prognostic Scoring System
IS - Immunophenotypic score
ITP - Idiopathic thrombocytopenia purpura
IWG - International Working Group
LOH - Loss of heterozygosity
LPD - Lymphoproliferative disorder
MBL - Monoclonal B-cell lymphocytosis
MCC - Matthews Correlation Coefficient
MDS - Myelodysplastic syndrome
MEP - Mmegakaryocytic/erythroid progenitor
MDS-U - Myelodysplastic syndrome unclassified
MFI - Median fluorescent intensity
MGUS - Monoclonal gammopathy of undetermined significance
MLP - Multilymphoid progenitor
MPN - Myeloproliferative neoplasm
MPP - Multipotent progenitor
NICE - National Institute for Clinical Excellence
NOD-SCID - Non-obese diabetic severe combined immunodeficient
OS - Overall survival
pDC - Plasmacytoid dendritic cells
PE - Phycoerythrin
PerCp - Peridinin chlorophyll protein complex
PMT - Photomultiplier tube
PNH - Paroxysmal nocturnal haemoglobinuria
Q-Q plot - Qunatile-Quantile plot
RAEB-F - Refractory anaemia with excess blasts with fibrosis
RAEB - Refractory anaemia with excess blasts
RAEB-T - Refractory anaemia with excess blasts ‘in transformation’
RA - Refractory anaemia
RARS - Refractory anaemia with ring sideroblasts

RARS-T - Refractory anaemia with ring sideroblasts with thrombocytosis

RCMD - Refractory cytopenia with multilineage dysplasia

RCMD-RS - Refractory cytopenia with multilineage dysplasia with ringed sideroblasts

RCUD - Refractory cytopenia with unilineage dysplasia

RN - Refractory Neutropenia

ROC - Receiver operating characteristics

RT - Refractory Thrombocytopenia

SCS - Progenitor cell screening tube

SD - Standard Deviation

SM-AHNMD - Systemic mastocytosis with associated clonal haematological non-mast cell lineage disease

SNP - Single nucleotide polymorphism

SQL - Structured Query Language

SSCP - Single Strand Conformational Polymorphism

SSC - Side scatter

UPD - Uniparental disomy

VAF - Variant Allelic Fraction

WHO - World Health Organisation

WPSS - WHO - based scoring system

9 Appendices

Antigen	Conjugate	Clone	Supplier	Product code	Dilution
CD2	FITC	S5.2	BD Biosciences	347404	None
CD2	APC	S5.2	BD Biosciences	341024	None
CD3	FITC	UCHT1	BD Pharmingen	555332	None
CD3	PE-Cy7	SK7	BD Biosciences	341111	1 in 2
CD4	FITC	QS4120	In-house	In-house	None
CD4	APC-Cy7	RPA-T4	BD Pharmingen	557871	None
CD5	PerCp-Cy5.5	L17F12	BD Biosciences	341109	None
CD5	APC	L17F12	BD Biosciences	345783	None
CD7	FITC	M-T701	BD Biosciences	332773	None
CD7	PE	M-T701	BD Biosciences	332774	None
CD9	FITC	M-L13	BD Biosciences	341646	None
CD10	APC	HI10A	BD Biosciences	332777	None
CD11a	FITC	G-25.2	BD Biosciences	347983	None
CD11b	APC	D12	BD Biosciences	333143	None
CD13	PE	L138	BD Biosciences	347406	None
CD14	FITC*	MDP9	BD Biosciences	333179	None
CD15	FITC	C3D-1	Dako	F0830	1 in 5
CD15	APC	HI98	BD Pharmingen	551376	None
CD16	FITC	NKP15	BD Biosciences	347523/335035	None
CD18	FITC	L130	BD Biosciences	347953	None
CD19	PE	SJ25C1	BD Biosciences	345789	None
CD19	PerCp-Cy5.5	SJ25C1	BD Biosciences	332780	1 in 2
CD19	BV421	HIB19	Biolegend	302234	1 in 5
CD22	APC	S-HCL-1	BD Biosciences	333145	None
CD24	FITC	ML5	BD Pharmingen	555427	None
CD25	APC	2A3	BD Biosciences	340907	None
CD28	APC	CD28.2	BD Pharmingen	559770	None
CD33	APC	P67,6	BD Biosciences	345800	None
CD33	APC	WM53	BD Pharmingen	551378	None
CD34	PerCp-Cy5.5	8G12	BD Biosciences	347222	1 in 5
CD34	APC	8G12	BD Biosciences	345804	1 in 10
CD36	FITC	CLB-IVC7	Sanquin	M1613	None
CD38	PerCp-Cy5.5	HIT2	BD Pharmingen	551400	1 in 10
CD38	APC-H7	HB7	BD Biosciences	Custom conjugate	1 in 5
CD42b	PE	AN51	Dako	R7014	None
CD43	PE	L10	Caltag	MHCD4304	None
CD43	APC	L10	Invitrogen	MHCD4305	None
CD45	APC-Cy7	2D1	BD Biosciences	348815	1 in 2
CD45	APC-H7	2D1	BD Biosciences	641417	1 in 2
CD45	Pacific Orange	HI30	Invitrogen	MHCD4530	1 in 5
CD45	V500	HI30	BD Horizon	560777	1 in 4
CD45RA	FITC	L48	BD Biosciences	335039	None
CD45RA	PE	ALB11	Beckman Coulter	PNIM1834U	None
CD45RO	FITC	UCHL1	eBiosciences	11-0457-41	None
CD45RO	APC	UCHL1	BD Biosciences	340438	None
CD48	FITC	TU145	BD Pharmingen	555759	None
CD49d	FITC	44H6	Serotec	MCA923F	None
CD56	PE-Cy7	335826	BD Biosciences	NCAM16.2	1 in 10
CD56	APC	B159	BD Pharmingen	555518	None

CD59	FITC	P282 (H19)	BD Pharmingen	555763	None
CD61	FITC	Y2/51	Dako	F0803	None
CD62L	PE	SK11	BD Biosciences	341012	None
CD64	FITC	10.1	BD Pharmingen	555527	None
CD64	PE*	MD22	BD Biosciences	333179	None
CD71	FITC	L01.1	BD Biosciences	333151	1 in 2
CD71	APC	M-A712	BD Pharmingen	551374	1 in 5
CD75	FITC	LN1	BD Pharmingen	555654	None
CD81	FITC	JS-81	BD Pharmingen	551108	None
CD82	PE	50F11	BD Pharmingen	555908	None
CD84	PE	2G7	BD Pharmingen	559589	None
CD84	PE	CD84.1.21	Biolegend	326007	None
CD86	PE	2331 (FUN-1)	BD Pharmingen	555658	None
CD90	FITC	Thy1/310	Immunotech	1839	None
CD95	PE	DX2	BD Pharmingen	555674	None
CD105	PE	1G2	Beckman Coulter	A07414	None
CD106	PE	51-10C9	BD Pharmingen	555647	None
CD117	PE	104D2	BD Biosciences	332785	None
CD117	PC7	104D2D1	Beckman Coulter	IM3698	1 in 10
CD122	PE	TU27	BD Biosciences	340254	None
CD123	FITC	AC145	Miltenyi- Biotec	130-090-897	None
CD123	PE	AC145	Miltenyi- Biotec	130-090-899	None
CD123	APC	AC145	Miltenyi- Biotec	130-090-901	None
CD133	PE	AC133	Miltenyi-Biotec	130-080-801	None
CD133	PE	293C3	Miltenyi-Biotec	130-090-853	None
CD150	PE	A12	BD Pharmingen	559592	None
CD154	PE	TRAP1	BD Pharmingen	555700	None
CD163	PE	GHI/61	BD Pharmingen	556018	None
CD203c	PE	97A6	Beckman Coulter	IM3575	None
CD300e/ IREM2	APC	UP-H2	Immunostep	IREM2A-100T	None
CCR1	PE	53504	R&D Systems	FAB145P	None
CXCR4	PE	12G5	BD Pharmingen	555974	None
CXCR5	APC	51505	R&D Systems	FAB190A	None
HLA-DR	APC-Cy7	L243	BD Biosciences	335831	1 in 10
HLA-DR	Pacific Blue	L243	Biolegend	307624	1 in 50

Appendix Table 2.1. Details of antibodies used in immunophenotyping studies

*CD14 and CD64 in combination as an BD Oncomark™ reagent

Reagent	**Manufacturer**	**Comments**
Ammonium Chloride	Sigma Aldrich	8.6 g/l in distilled H_2O
FACSFlow	BD Biosciences	
Bovine Serum Albumin	Sigma Aldrich	0.3% in FACSFlow
Simply Cellular anti-Mouse Silica	Bangs Laboratories, Inc.	

Appendix Table 2.2. Details of reagents used in immunophenotyping studies

	MDS MFI			Control MFI				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR *p* value**
CD34	1019	10440	4488	951	8420	4088.5	0.635486	0.85044052
CD117	2159	20867	12192	3405	7676	6131.5	0.007229	0.126758939
CD38	180	3707	1498	1471	3032	1697	0.484088	0.795873426
HLA.DR	312	18797	5939	1467	8351	3482.5	0.354723	0.688163462
CD45	660	2447	1294	998	1489	1264.5	1	1
CD13	698	24861	5518	636	3497	1957.5	0.001985	0.071917916
CD71	519	5821	1853	898	3836	1720.5	0.946011	0.983436591
CD105	309	2398	833	500	1072	776	0.874452	0.966630402
CD36	51	6512	257	138	570	203	0.770404	0.930806906
CD95	317	2682	929	620	2329	821	0.957687	0.988906502
CD33	47	10249	2436	53	978	634	0.015762	0.152895823
CD45RA	37	1130	271	149	538	222.5	0.60361	0.843851151
CD45RO	39	4086	382	53	1243	870.5	0.512642	0.797027382
CD43	9381	79905	22453	5880	23836	17021.5	0.055029	0.272526844
CD133	285	2795	621	382	1414	550.5	0.542224	0.797027382
CD62L	191	3786	594	243	4891	1001.5	0.734922	0.916573092
CD123	225	2536	1156	96	1393	645.5	0.048132	0.25236986
CD59	2305	21062	7139	5565	16807	10364	0.151999	0.453657975
CD84	178	7477	2075	1209	3097	1770	0.831933	0.954999867
CD18	256	2263	727	459	944	592	0.366534	0.703463308
CD49d	1140	3969	2035	1506	2421	1798	0.844504	0.95556308
CD11a	225	2030	690	165	1377	549	0.404519	0.716682686
CD81	2437	9042	6386	3956	6284	5384	0.404519	0.716682686

Appendix Table 4.1. Statistical analysis of the antigen expression for the MFI for $CD34^{+}$ progenitors for the MDS and the normal groups.

	MDS CV			Control CV				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR *p* value**
CD34	40.1	103.8	54.4	48.4	70.3	58	0.484088	0.795873426
CD117	46.4	154.4	76.3	86.4	129.2	108.8	0.022619	0.195027183
CD38	39	146.4	65.1	57.8	89.9	68.3	0.619442	0.843851151
HLA.DR	54.6	101.8	82.9	80.7	103.6	94.4	0.04697	0.249650989
CD45	22	52.3	41.1	33.4	43.6	37.95	0.512684	0.797027382
CD13	57.7	139.3	108.7	121.5	129.3	124.1	0.005496	0.122889817
CD71	50.4	123.8	85.1	84.4	113.3	101.6	0.009437	0.128631245
CD105	51.1	128.1	67.5	69.9	87.9	81.6	0.060842	0.282922122
CD36	49.4	188.6	92.3	91	120.7	103.2	0.426127	0.744761959
CD95	41.8	86.9	65.5	53.1	125.1	61.3	0.457614	0.768633151
CD33	38.7	305.7	113.9	115.6	274.8	128.3	0.033818	0.220462702
CD45RA	27.3	263.4	85.8	82.1	109.1	101.8	0.142321	0.446101482
CD45RO	55.4	371.7	124.9	103.7	274.8	140.2	0.231566	0.552507194
CD43	32.5	97.8	52.6	57.4	78.3	65.4	0.042182	0.23381105
CD133	49.7	150.2	81.4	97.3	126.5	110.85	0.074551	0.302330372
CD62L	72.9	142.9	103.1	102.7	138	118.1	0.015725	0.152895823
CD123	46.7	152.2	95	117.5	198.2	119.95	0.002285	0.072469761
CD59	32.9	98.5	42.6	37	48.8	39.5	0.395812	0.714800675
CD84	52.2	123.2	87	90	120.7	109.7	0.006813	0.126758939
CD18	47.4	114.7	80.6	73.1	104.4	93.05	0.028559	0.213095815
CD49d	35	87.2	56	54.5	78.2	68.1	0.069638	0.302330372
CD11a	40.3	118.5	72.7	74.3	114.8	95.8	0.062392	0.2848027
CD81	39.8	70.4	54.8	47.3	62.7	56.4	0.96087	0.988906502

Appendix Table 4.2. Statistical analysis of the antigen expression for the CV for $CD34^{+}$ progenitors for the MDS and the normal groups.

	MDS MFI			Control MFI				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR *p* value**
CD34	2793	14090	7943	1159	10766	5185.5	0.15789	0.453787863
CD117	2677	16920	8372	3597	8325	5443	0.039019	0.225959137
HLA.DR	546	10853	3372	766	3386	1946.5	0.054966	0.272526844
CD45	1080	2872	1498	1165	2039	1433	0.913467	0.981817505
CD13	1428	44104	6069	1585	7560	2994	0.11946	0.396157551
CD71	398	2794	1070	550	1483	831.5	0.447005	0.76742497
CD105	467	2752	874	438	1048	694	0.23211	0.552507194
CD95	278	1457	650	368	3270	690	1	1
CD33	231	7079	1388	121	1153	671	0.085293	0.315178383
CD45RA	88	2074	165	106	264	152.5	0.218117	0.535628254
CD45RO	58	4217	951	76	1923	1232	0.63783	0.85044052
CD43	6628	67615	14954	2366	14949	8792.5	0.039019	0.225959137
CD133	468	3296	1323	888	3589	1107	0.538175	0.797027382
CD62L	185	5117	622	278	7931	1831	0.491455	0.796003165
CD123	417	3540	1653	157	1378	869.5	0.052325	0.269193675
CD59	2202	21451	11297.5	6184	18635	13268	0.526168	0.797027382
CD84	245	2498	1047.5	389	1336	545	0.290766	0.626762163
CD18	245	1878	682	363	914	586	0.447005	0.76742497
CD49d	977	1631	1246	810	1287	930	0.004334	0.105108085
CD11a	215	1844	509	135	1429	710	0.936839	0.983436591
CD81	2218	8056	5911	3286	6360	4359	0.112999	0.384591958

Appendix Table 4.3. Statistical analysis of the antigen expression for the MFI for $CD34^{+}$ $CD38^{-}$ progenitors for the MDS and the normal groups.

	MDS CV			Control CV				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR *p* value**
CD34	31.2	82.9	37.6	30	43.4	35.45	0.799903	0.941898876
CD117	45.9	131.8	67.6	58.2	76.4	69.95	0.538175	0.797027382
HLA.DR	48.1	90.6	68.1	58.5	79.7	72.35	0.79984	0.941898876
CD45	27.1	48.1	36.4	25.9	38.2	33.9	0.346151	0.688163462
CD13	58.5	113.2	70.7	53.9	86	76.05	1	1
CD71	62.6	102	75.7	69.6	105.4	91.25	0.059626	0.282922122
CD105	40.1	81.2	56.7	52.5	66.1	54.5	0.293672	0.629529329
CD95	34.2	69.3	52	43.7	64.9	54.2	0.717153	0.912312309
CD33	53.1	130	86.4	35.8	98.3	89.8	0.785853	0.938187182
CD45RA	34.6	85	69.6	57.4	111.2	69.45	0.537646	0.797027382
CD45RO	52	235.8	98.9	86	187.9	108.75	0.799903	0.941898876
CD43	31.1	73.8	46.6	38.9	65.9	47.7	0.491455	0.796003165
CD133	45.4	123.2	65.1	36.8	64.3	51.3	0.15789	0.453787863
CD62L	58.9	123.8	92.1	73.8	116.3	94.5	0.856327	0.960193236
CD123	47.5	88.7	61.1	56.4	136	67.8	0.148454	0.446511871
CD59	25.5	84.7	33.25	22.9	41.2	25.9	0.031151	0.220462702
CD84	43.5	94.8	69.3	67.9	85.7	80.4	0.703584	0.897995931
CD18	42.5	94.3	63.4	59.2	83.4	75.95	0.180316	0.492694494
CD49d	31	66.2	48.5	40	56.5	47.3	0.842903	0.95556308
CD11a	39.6	102.1	69.6	54.9	88.4	77.1	0.153606	0.453787863
CD81	32.5	89.6	46.5	35.4	45.3	41.2	0.23458	0.554982117

Appendix Table 4.4. Statistical analysis of the antigen expression for the CV for $CD34^{+}$ $CD38^{-}$ progenitors for the MDS and the normal groups.

	MDS MFI			Control MFI				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR *p* value**
CD34	1328	9778	4415	802	7414	3640	0.538175	0.797027382
CD117	863	34444	11263	1948	8306	5906	0.076012	0.302330372
CD38	1048	3125	2049	1993	3431	2256	0.690399	0.894917926
HLA.DR	2787	19304	8560	2184	9691	5256.5	0.11946	0.396157551
CD45	710	2340	1292	907	1386	1175	0.15789	0.453787863
CD13	599	22852	3946	340	2425	1129	0.002652	0.072469761
CD71	1016	7674	2442	1047	4817	2323.5	0.913495	0.981817505
CD105	414	1565	817	533	1126	815.5	0.63783	0.85044052
CD36	173	3051	383	244	1379	298	0.61822	0.843851151
CD95	729	2333	1400	775	1287	968	0.057184	0.277343302
CD33	339	10219	4494	43	900	577	0.008628	0.126758939
CD45RA	167	1171	380	232	691	340.5	0.538175	0.797027382
CD45RO	77	3919	275	43	1005	451	0.971115	0.99417541
CD43	13585	57287	24012	7762	27661	21961.5	0.180316	0.492694494
CD133	155	1131	429	207	781	336	0.261641	0.580095836
CD62L	197	3416	631	214	2625	689	0.942248	0.983436591
CD123	305	4961	1215	86	1404	461	0.011787	0.142918672
CD59	2600	23800	7757	4939	15994	10114	0.526168	0.797027382
CD84	763	9189	4042.5	2363	5135	3072	0.582765	0.838711243
CD18	408	2435	793	385	969	599	0.076012	0.302330372
CD49d	1693	3536	2294	1847	2754	2466	0.691947	0.894917926
CD11a	235	2228	744	191	1323	559	0.302936	0.63879998
CD81	2638	11920	7717	4622	7215	6202	0.383387	0.712962488

Appendix Table 4.5. Statistical analysis of the antigen expression for the MFI for $CD34^{+}$ $CD38^{+}$ progenitors for the MDS and the normal groups.

	MDS CV			Control CV				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR *p* value**
CD34	43.6	97.7	56.7	49.5	66.1	56.6	0.663703	0.875907184
CD117	55.5	160.2	93.5	87.8	144.9	132.2	0.039019	0.225959137
CD38	37.2	55.1	46.3	34.6	47.6	39.8	0.0647	0.288933601
HLA.DR	50	102	76.8	67	110.7	87.8	0.064787	0.288933601
CD45	31.1	52.1	39.4	31.1	39.3	37.6	0.310478	0.64420099
CD13	76.6	132.8	114.9	105.2	131.1	125.45	0.23211	0.552507194
CD71	57.2	110.5	79.5	78.4	109.1	96.8	0.027186	0.213095815
CD105	53.5	118.7	67.1	79.2	101.9	93	0.088777	0.319009549
CD36	63.2	143.2	87.4	82	137.2	114	0.204822	0.529805412
CD95	38.8	81.6	51.7	54	130.7	62.2	0.147319	0.446511871
CD33	59.9	145.7	101.9	122.6	335.2	138.2	0.002802	0.072469761
CD45RA	51.6	114.8	74.9	74.9	122.6	98	0.013774	0.148453456
CD45RO	55	223.3	152.6	104.7	335.2	167.05	0.328233	0.65986739
CD43	34.3	81.5	51.5	49.5	59.4	52.3	0.468794	0.780653301
CD133	56	148.3	102	98.2	118.4	111.3	0.328233	0.65986739
CD62L	70.3	138.2	105.5	113.2	138.8	126	0.039019	0.225959137
CD123	80.8	162.8	106.2	134.7	215	144.5	0.007283	0.126758939
CD59	31.5	118.5	44.9	38.7	44.7	42	0.176107	0.492678492
CD84	45.2	107.5	71.75	58.9	104.8	84.6	0.253887	0.569411979
CD18	51	124.3	84	71.3	116.7	95.95	0.008188	0.126758939
CD49d	37.1	73.8	52	39.8	59.3	46.7	0.500266	0.797027382
CD11a	55.8	127.9	72	84.2	126.4	101.4	0.068366	0.301432187
CD81	46	92.9	53	49.4	64.4	58	0.32173	0.65986739

Appendix Table 4.6. Statistical analysis of the antigen expression for the CV for $CD34^{+}$ $CD38^{+}$ progenitors for the MDS and the normal groups.

	MDS MFI			Control MFI				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR *p* value**
CD117	2373	10115	4658	3092	5293	4037	0.231391	0.552507194
CD38	347	3427	1423.5	871	2645	1404.5	0.844737	0.95556308
HLA-DR	321	6605	674	410	919	557	0.267109	0.588853714
CD45	948	2555	1437	948	1614	1249	0.089619	0.319009549
CD16	398	961	632	482	802	603	0.777272	0.930806906
CD13	443	53846	7191.5	445	22232	4081	0.248802	0.569411979
CD14	364	883	593.5	490	731	570.5	0.844737	0.95556308
CD64	223	5482	1368	394	3826	1106.5	0.616742	0.843851151
CD71	1626	33938	3482.5	5122	17663	8285	0.009614	0.952956131
CD105	344	33070	650	510	1162	739	0.306447	0.128631245
CD36	379	29978	696.5	726	16348	1043	0.017795	0.642709972
CD95	704	6170	2222.5	365	2683	1946	0.320314	0.16439051
CD33	305	18475	6608.5	377	5728	1682	0.034427	0.220462702
CD45RA	344	1326	633.5	494	791	617.5	0.965284	0.990820931
CD45RO	139	4693	874	253	1255	577	0.144868	0.446101482
CD43	18581	91867	43930	13962	67570	45884.5	0.616742	0.843851151
CD62L	178	1644	441	309	701	472.5	0.37227	0.703463308
CD4	510	2805	938.5	670	1268	835.5	0.396088	0.714800675
CD24	448	18739	1094.5	598	1580	852.5	0.144868	0.446101482
CD59	2448	29462	8108.5	5955	19813	9065	0.74044	0.916573092
CD84	405	6397	2813.5	1224	8258	1995	0.858427	0.960193236
CD18	448	1893	798	602	784	694	0.177265	0.492678492
CD49d	532	7073	3108	2853	5431	4393	0.247014	0.569411979
CD11a	474	5597	783.5	543	883	674	0.193812	0.515062625
CD81	526	10292	3754.5	3043	5641	4526	0.943503	0.983436591
CD15	88	1303	529	101	713	219	0.034991	0.220462702

Appendix Table 4.7. Statistical analysis of the antigen expression for the MFI for total $CD34^{-}CD117^{+}$ progenitors for the MDS and the normal groups.

	MDS CV			Control CV				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
CD117	63.3	108.5	82.3	68	89	82.2	0.744125	0.916573092
CD38	34.5	119.5	54.95	46.5	121.4	97.4	0.034799	0.220462702
HLA-DR	35.8	116.5	75.4	58.5	107.7	78.15	0.542307	0.797027382
CD45	21.5	99.3	38.05	34.8	84.2	55.95	0.017701	0.16439051
CD16	31.6	69.7	43.3	37.7	68.2	46.1	0.616742	0.843851151
CD13	85.3	146.3	109.8	126.2	160.7	141.4	0.001604	0.06915518
CD14	30.3	62.7	39.9	33.3	65.9	43.5	0.586378	0.838711243
CD64	59.9	140.7	92.75	119.6	153.2	133.05	0.001017	0.056615741
CD71	41.5	141.7	67.65	49.2	118.4	76.5	0.396001	0.783681667
CD105	34.2	142.7	47.05	45.1	105.7	78.3	0.077951	0.714800675
CD36	31.8	145.6	50.9	52.4	143.3	79.4	0.044103	0.302330372
CD95	29.4	89.5	39	33.7	59.3	40.7	0.683444	0.240614239
CD33	34.3	139.9	67.95	64.7	169.2	130.8	0.020385	0.17976092
CD45RA	30.9	70	45.25	36.7	72	42.25	0.810795	0.941898876
CD45RO	64.4	153.7	97	103	131.5	111.45	0.081695	0.306663064
CD43	32.3	84.5	42.25	38	56.9	43.85	0.777232	0.930806906
CD62L	34.9	103.7	59.8	72.5	123.8	80.65	0.005701	0.122889817
CD4	36.8	85.6	58.45	43.3	64.1	55.95	0.61671	0.843851151
CD24	38	144.7	71.8	46.9	76.7	63.7	0.127679	0.409416612
CD59	38.4	118.4	62.45	60.9	109.4	82.7	0.034427	0.220462702
CD84	29.4	115.5	71.25	69.2	118.6	106.7	0.011644	0.142918672
CD18	29.5	83.7	44.75	33.3	66.8	44.95	0.947952	0.983436591
CD49d	32.8	87.1	45.9	43.9	77.7	53.9	0.088941	0.319009549
CD11a	29.4	87.5	47	37.9	55.8	45.7	0.813237	0.941898876
CD81	38.1	93.8	70.25	57	72.4	60.1	0.080388	0.305790305
CD15	61.4	169.1	101.7	106.2	183.7	152.4	0.014218	0.149101301

Appendix Table 4.8 Statistical analysis of the antigen expression for the CV for total $CD34^{-}CD117^{+}$ progenitors for the MDS and the normal groups.

	MDS MFI			Control MFI				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
CD117	1413	5910	2591.5	2044	4012	2491.5	0.373488	0.703463308
CD38	570	5179	2177.5	1835	3074	2030	0.628963	0.850305938
HLADR	3638	52098	16101	6516	31696	15611.5	0.85873	0.960193236
CD45	1502	7049	1972	1748	2561	1999.5	0.959436	0.988906502
CD16	287	1219	538	427	859	494	1	1
CD13	234	89646	4897	885	12580	5114.5	0.85873	0.960193236
CD14	336	958	513.5	398	627	500.5	0.628916	0.850305938
CD64	985	43989	10016	8533	21967	13842	0.212786	0.530999582
CD71	770	5483	1651.5	1411	5030	1907	0.492373	0.796003165
CD105	355	2110	661	477	1071	605.5	0.838786	0.95556308
CD36	423	9346	807	634	1872	739	0.907958	0.981817505
CD95	1409	5696	2442	239	2117	1816	0.007834	0.126758939
CD33	1628	37027	11578	2060	14135	10089	0.073129	0.302330372
CD45RA	271	2133	670	846	1102	990.5	0.297169	0.630064159
CD43	8600	55946	23706	7898	38816	24202.5	0.74098	0.916573092
CD62L	213	5482	912	623	5229	1710.5	0.177771	0.492678492
CD123	291	5839	1662	931	3011	1883	0.676922	0.890324843
CD4	431	2393	1115	840	1278	1054.5	0.818988	0.945736462
CD24	460	5133	707	509	1380	713.5	0.740946	0.916573092
CD59	674	22792	8069.5	3495	7463	6118	0.237923	0.559256373
CD84	278	14299	5423	4782	19370	6666	0.193177	0.515062625
CD18	926	12255	3840.5	3325	4961	4059.5	0.939195	0.983436591
CD49d	2348	15844	3407	2774	4204	3418	0.933858	0.983436591
CD86	340	8759	1202	799	2617	1046.5	0.939195	0.983436591
CD11a	905	8267	3711	881	6794	5514	0.599153	0.843851151
CD48	362	1304	617.5	532	691	567	0.933858	0.983436591
CD81	780	16358	7531	6081	10308	9010	0.391119	0.714800675
CD15	109	2472	511	399	1578	841	0.203458	0.529805412

Appendix Table 4.9. Statistical analysis of the antigen expression for the MFI for $CD34^{-}CD117^{+}$ monocytic differentiating progenitors for the MDS and the normal groups.

	MDS CV			Control CV				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
CD117	40.2	90.3	58.7	52.7	74.8	66.1	0.161961	0.462065717
CD38	33.2	109.6	38.9	31.6	50.5	38.6	0.524812	0.797027382
HLADR	41.4	79.2	61.8	46	71.3	52.7	0.070982	0.302330372
CD45	28.4	63.4	40.3	31.3	51.4	42.45	0.74098	0.916573092
CD16	39.7	98.9	57.8	40.3	87.5	59.15	0.898832	0.981817505
CD13	51.6	139.5	82.2	69.5	120.9	74.1	0.430551	0.749120432
CD14	35.9	74.1	47.55	31.8	54.2	41.25	0.147126	0.446511871
CD64	54.7	142.2	83	74	134.2	105.6	0.120885	0.397485765
CD71	44	93.9	57.6	52.5	81.6	67.4	0.088362	0.319009549
CD105	36.4	97	47.3	39.1	51.7	43.9	0.093262	0.326828787
CD36	43.7	119.2	79.3	57.8	79.1	67.7	0.385882	0.712962488
CD95	35.9	62.2	45.7	38.1	66	42.2	0.728664	0.916573092
CD33	29.5	98.7	46.1	40.3	63.6	45.5	0.772516	0.930806906
CD45RA	39.3	92.2	52.7	41.3	57.9	51.5	0.647128	0.857710706
CD43	32.2	102.6	54	40	57.8	47.65	0.252527	0.569411979
CD62L	52.1	128.9	78.75	69.9	120.1	92.65	0.252462	0.569411979
CD123	49.6	124.5	82.45	71.6	96.6	79.45	0.911483	0.981817505
CD4	49.2	99.1	57.1	50.3	58.5	54.25	0.297037	0.630064159
CD24	41.1	136.8	56.7	48.2	81.4	61.65	0.898832	0.981817505
CD59	36.8	87.4	59.25	56.4	81.7	67.9	0.250081	0.569411979
CD84	32.2	86.3	51.35	36	68.7	48.5	0.585885	0.838711243
CD18	35.5	92.8	48.35	42.9	66.7	52.85	0.72178	0.915198238
CD49d	32.2	71.5	39.5	31.3	42.2	38.4	0.280264	0.610912239
CD86	49.3	128.9	98.25	88	121.6	107.7	0.127047	0.409416612
CD11a	35.5	93.3	48.9	32.4	64.8	45.1	0.391119	0.714800675
CD48	40.9	111.5	51.4	46.8	62.6	51.9	0.933858	0.983436591
CD81	42.4	72	60.9	48.9	76	59.7	0.506664	0.797027382
CD15	60.4	152.1	103.6	79.2	137.4	122.5	0.385882	0.712962488

Appendix Table 4.10. Statistical analysis of the antigen expression for the CV for $CD34^{-}CD117^{+}$ monocytic differentiating progenitors for the MDS and the normal groups.

	MDS CV			Control CV				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
CD117	1780	8970	4441	3397	5688	4469	0.874452	0.966630402
CD45	345	1102	687	485	752	654.5	0.527328	0.797027382
CD71	2628	235219	34582	4192	99104	47490	0.874452	0.966630402
CD105	613	49527	21472	18446	31070	24336.5	0.378685	0.709805965
CD36	580	115753	29623	24778	41011	36597	0.557725	0.813523862
CD95	492	6438	3069.5	452	3229	2664	0.078699	0.302330372
CD43	5685	100129	50132	12687	66281	50317.5	0.512727	0.797027382
CD59	9257	46471	22965	13323	33060	26672	0.595725	0.843581431
CD49d	404	10724	6312	5339	7895	7180	0.239269	0.559256373
CD81	1372	12237	5056	3070	7475	4523	0.59256	0.84217358

Appendix Table 4.11. Statistical analysis of the antigen expression for the MFI for $CD34^{-}CD117^{+}$ erythroid differentiating progenitors for the MDS and the normal groups.

	MDS CV			Control CV				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
CD117	60.4	100	79.7	59	83.1	75.45	0.268707	0.589030104
CD45	23.6	44.7	33.7	25.8	35.4	31.45	0.060894	0.282922122
CD71	16.3	136.1	89.9	36.5	113.1	67.75	0.354723	0.688163462
CD105	33.7	128.3	50.5	30.7	38	33.55	0.000196	0.056615741
CD36	38.9	145.2	67.35	34.9	63.4	44.3	0.010061	0.130125047
CD95	38.2	88.3	57.6	40.8	75.6	46.4	0.052729	0.269193675
CD43	32.1	103	43.8	33	47.9	39.25	0.078239	0.302330372
CD59	30.3	109.4	47.4	28.8	37.4	31.2	0.000563	0.056615741
CD49d	28.5	99.1	41.1	26.8	33.1	29.1	0.000498	0.056615741
CD81	39.3	134.7	54.6	33	45.4	41.5	0.000654	0.056615741

Appendix Table 4.12. Statistical analysis of the antigen expression for the CV for $CD34^{-}CD117^{+}$ erythroid differentiating progenitors for the MDS and the normal groups.

	MDS MFI			Control MFI				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
CD117	2501	13660	4954	3041	5208	4382	0.199204	0.5222375
CD38	617	3513	1564.5	1530	3107	1905.5	0.214863	0.530999582
HLA-DR	314	6078	720.5	503	1539	694	0.947952	0.983436591
CD45	1083	2902	1600.5	1285	1814	1650.5	0.542307	0.797027382
CD13	4766	70643	10249	14215	44533	23129	0.349444	0.688163462
CD64	382	7023	1632.5	1995	6293	2862.5	0.112183	0.384591958
CD71	1228	9785	3085	3709	17341	7284	0.001017	0.056615741
CD95	915	6114	2172.5	347	2439	1897	0.211798	0.530999582
CD33	1715	18955	8208.5	963	7617	5603	0.078699	0.302330372
CD45RO	222	5349	1253.5	491	2926	1602.5	0.810829	0.941898876
CD43	14175	93664	44584	16640	69907	45477.5	0.647704	0.857710706
CD4	565	2880	1215.5	938	1503	1099.5	1	1
CD24	544	22052	1528.5	918	2216	1337.5	0.327488	0.65986739
CD59	2032	26696	7219	4231	8852	5800	0.211798	0.530999582
CD84	417	7726	3120.5	2482	11410	4027	0.211798	0.530999582
CD18	437	2274	900.5	669	836	794.5	0.144831	0.446101482
CD49d	1670	5635	3034.5	2429	3966	2878	0.868661	0.966630402
CD11a	456	2237	819	657	927	760	0.344656	0.688163462
CD81	514	13063	3331.5	2905	4115	3858	0.75877	0.930806906
CD15	199	1591	833	506	1440	878	0.883052	0.973363741

Appendix Table 4.13. Statistical analysis of the antigen expression for the MFI for $CD34^{-}CD117^{+}$ granulocytic differentiating progenitors for the MDS and the normal groups.

	MDS CV			Control CV				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
CD117	65.3	107.8	80.15	68.2	92.7	83.65	0.744125	0.916573092
CD38	30.5	96.9	38.65	29.2	49.6	36.55	0.102558	0.355290488
HLA-DR	31.8	104.2	61.75	41.7	80.1	57.75	0.947947	0.983436591
CD45	21.1	40.6	26.9	22	31.7	26.5	0.810829	0.941898876
CD13	47.2	139.8	89.15	63	100.8	87.05	0.420714	0.738704055
CD64	54.5	101	68.9	64.1	89	74.05	0.446305	0.76742497
CD71	39.9	73.7	52.7	38.8	57.6	43.85	0.02501	0.205636128
CD95	28.8	60	31.85	28.6	49.3	29.9	0.358774	0.692558421
CD33	31.7	102.2	42.9	38.6	59.1	42.5	0.740409	0.916573092
CD45RO	43.4	152.2	75.15	63.9	116.2	98.2	0.157212	0.453787863
CD43	30	77.1	38.55	35.9	51.1	38.95	0.513852	0.797027382
CD4	36.4	74.9	52.75	52.6	57.3	54.7	0.486154	0.795898336
CD24	40.3	144.1	78.2	57.2	75.9	64.6	0.184355	0.500208819
CD59	35.7	99.5	49.3	42.4	55.2	47.3	0.798811	0.941898876
CD84	29	97.2	50.45	37.5	56.5	47.2	0.574968	0.832416281
CD18	26.7	89.4	37.45	30.5	40.1	34.7	0.19145	0.515062625
CD49d	30.6	95.2	36.1	31.1	47.8	38.8	0.88722	0.975188236
CD11a	27.1	76.5	38.05	31	40.7	34.5	0.142785	0.446101482
CD81	33.3	81.4	67.25	59	78.1	74.8	0.04465	0.240614239
CD15	48.7	132	79.6	61	74.8	68	0.169768	0.480802084

Appendix Table 4.14. Statistical analysis of the antigen expression for the CV for $CD34^{-}CD117^{+}$ granulocytic differentiating progenitors for the MDS and the normal groups.

	MDS MFI			Control MFI				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
CD16	271	9290	606	625	2591	803	0.024983	0.205636128
CD13	1157	22360	3353.5	972	4788	2598.5	0.327488	0.65986739
CD11b	415	9089	3913.5	1136	8939	4180.5	0.98264	0.998073766
CD64	386	16149	2371	1014	5138	3371	0.810829	0.941898876
CD71	225	1016	563	319	580	455.5	0.257799	0.57486231
CD95	1436	6158	2795.5	2442	3560	2607	0.702287	0.897995931
CD33	1128	8418	3039.5	556	3222	2183	0.013444	0.148453456
CD45RO	1619	16629	7394.5	1080	10704	5888	0.372314	0.703463308
CD43	9491	73639	36578.5	13954	47245	32632	0.372314	0.703463308
CD62L	198	22502	782.5	415	3696	1424	0.420757	0.738704055
CD24	7580	40520	15055.5	7929	28526	13504	0.52802	0.797027382
CD10	139	1377	322	220	473	291.5	0.777272	0.930806906
CD59	5023	24366	10557.5	6542	12599	9262	0.459914	0.769166557
CD18	657	3568	1729.5	1056	2671	1726	0.810829	0.941898876
CD49d	340	1731	556.5	294	656	366	0.042157	0.23381105
CD11a	472	2546	935	346	1202	959	0.619839	0.843851151
CD81	229	1374	401.5	247	392	284	0.008145	0.126758939
CD15	694	6969	3455	2496	7777	3842	0.404519	0.716682686

Appendix Table 4.15. Statistical analysis of the antigen expression for the MFI for $CD34^{-}CD117^{-}$ mature granulocyte compartment for the MDS and the normal groups.

	MDS CV			Control CV				
Antigen	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
CD16	41.1	142.3	93.3	92.5	133	114.85	0.055489	0.272526844
CD13	63.2	137.6	115.65	102.3	134.5	122.8	0.061251	0.282922122
CD11b	58.3	136.4	109.8	71.2	131	93.1	0.286101	0.62015137
CD64	50.6	133.7	103.5	89.6	132.7	124.6	0.019897	0.179533754
CD71	35.8	132.2	70.3	51.8	91.8	66.55	0.55686	0.813523862
CD95	32.4	65.7	40.7	32.5	40	36.7	0.070378	0.302330372
CD33	41	104	60.8	46.7	67	53.6	0.241063	0.560075486
CD45RO	34.8	83.4	56.7	38.7	57.8	46.55	0.034799	0.220462702
CD43	31.5	86.3	49.65	29.6	49.7	38.6	0.006529	0.126758939
CD62L	52.5	144.9	110.6	91.9	140.7	125.75	0.349444	0.688163462
CD24	42.7	101.2	66.25	47.4	81.5	66.1	0.571492	0.830482181
CD10	54	135.3	92.45	74.6	112.3	85.65	0.52802	0.797027382
CD59	31.9	58.1	43.2	29.4	42.5	32.1	0.002039	0.071917916
CD18	41.6	96.5	70.95	47.2	89.3	73.65	0.777272	0.930806906
CD49d	37.9	96.6	66.1	49.1	67.5	56.4	0.0935	0.326828787
CD11a	32.5	98.2	39	29.7	39.9	36.4	0.072543	0.302330372
CD81	44	115.6	63.1	39	54.4	44.4	0.001021	0.056615741
CD15	43.9	100.1	58.6	58.3	68	60.7	0.211014	0.530999582

Appendix Table 4.16. Statistical analysis of the antigen expression for the CV for $CD34^{-}CD117^{-}$ mature granulocyte compartment for the MDS and the normal groups.

		MDS MFI			Control MFI				
Population	**Antigen**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
Total $CD64^+$ Monocytes	CD14	374	35937	1229	3570	16386	9738.5	0.012226783	0.143757327
	CD64	13792	59797	27581	18297	50520	29429	0.512726563	0.797027382
	CD300e	125	4545	341	317	858	500	0.028543211	0.213095815
	CD38	373	2180	1492	1104	1463	1297	0.456339753	0.768633151
	HLA-DR	1006	11721	3719	1684	5923	2731	0.701178644	0.897995931
	CD45	2241	5756	2995	2436	4495	3619	0.456339753	0.768633151
$CD64^+CD14^-$ Monocytes	CD14	181	1008	379	347	535	399.5	0.354723434	0.688163462
	CD64	15269	55340	26869	20963	53533	29655	0.198225442	0.5222375
	CD300e	79	367	176	103	212	164.5	0.587962521	0.838711243
	CD38	563	3062	1844	1632	2406	1935.5	0.231566084	0.552507194
	HLA-DR	1102	27959	8812	5481	22224	11223	0.456339753	0.768633151
	CD45	1625	4617	2228	1567	3155	2399	0.981991402	0.998073766
$CD64^+CD14^+$ Monocytes	CD14	2840	38111	9878	9396	28076	19227.5	0.032003011	0.220462702
	CD64	12494	62100	28534	17156	49691	29432	0.603646387	0.843851151
	CD300e	215	5281	718	853	2669	1262	0.082198347	0.306663064
	CD38	326	1910	1083	984	1191	1129	0.769162849	0.930806906
	HLA-DR	754	6385	2053	1143	4053	2113	0.701178644	0.897995931
	CD45	2809	7146	3833	2701	5299	4092	0.981991402	0.998073766
$CD64^+CD14^+$ $CD300e^+$ Monocytes	CD14	5119	50961	21163	15216	43013	28494.5	0.214428811	0.530999582
	CD64	11770	62467	27189	16106	48004	28286.5	0.910140172	0.981817505
	CD300e	1589	7796	3857	3750	8331	5234.5	0.015724794	0.152895823
	CD38	241	1619	938	769	1050	933	1	1
	HLA-DR	1275	12482	2671	1603	5131	2428	0.76918546	0.930806906
	CD45	3011	7967	4902	3325	6475	4730.5	0.910140172	0.981817505

Appendix Table 4.17. Statistical analysis of the antigen expression for the MFI for the different $CD64^+$ monocytic populations for the MDS and the normal groups.

		MDS CV			Control CV				
Population	**Antigen**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**P value**	**FDR p value**
Total $CD64^+$ Monocytes	CD14	45.5	145.3	124.4	126.4	143.8	137.85	0.02544	0.205636128
	CD64	29.3	51.9	37.9	29.1	40.1	35.35	0.114019	0.384690822
	CD300e	93.4	160.1	111.9	123.4	151.7	135	0.002486	0.072469761
	CD38	33	90.2	51.5	42.1	55.8	50.65	0.68446	0.891176538
	HLA-DR	72.9	119.8	104.1	88	101.1	93.55	0.074551	0.302330372
	CD45	28.2	61.9	42.3	32	53.9	45.25	0.635418	0.85044052
$CD64^+CD14^-$ Monocytes	CD14	37.9	94.6	52.5	38.6	65	47.1	0.039968	0.228050418
	CD64	30	53.2	36.9	33.7	39.4	36.4	0.54198	0.797027382
	CD300e	83.9	171.6	112.7	95.4	146.1	111.9	0.946005	0.983436591
	CD38	32.7	92.1	40.5	31.4	37.2	35.1	0.013002	0.148377864
	HLA-DR	56.4	126	96.3	69.9	97.6	78.7	0.035797	0.220462702
	CD45	22.1	40.7	33.3	26.9	37.3	33.25	0.684189	0.891176538
$CD64^+CD14^+$ Monocytes	CD14	51.4	118.4	97.4	70.3	103.1	93.45	0.403618	0.716682686
	CD64	26.9	56.3	39.5	28	41.2	33.55	0.035759	0.220462702
	CD300e	81.9	136.4	118.7	125.1	139.8	132.75	0.001244	0.06035471
	CD38	32.4	80.8	46.5	36.8	48	45.25	0.309746	0.64420099
	HLA-DR	44.9	100.4	76.1	69.2	88	74.9	0.512684	0.797027382
	CD45	23.8	41.9	32	25.8	40.7	35.95	0.124766	0.406798629
$CD64^+CD14^+$ $CD300e^+$ Monocytes	CD14	34.5	94.8	51.3	29.1	56.1	42.1	0.028559	0.213095815
	CD64	29.3	55.9	38.9	28.5	46.6	33.9	0.035797	0.220462702
	CD300e	45.7	101.2	73.1	60.9	108.2	71.35	0.512727	0.797027382
	CD38	36.5	78.2	44.9	38	49.7	39.85	0.008821	0.126758939
	HLA-DR	61.6	111.5	82.7	70.5	95.1	75.4	0.619442	0.843851151
	CD45	17.8	38.7	29	21.6	38.6	31.55	0.456294	0.768633151

Appendix Table 4.18 Statistical analysis of the antigen expression for the CV for the different $CD64^+$ monocytic populations for the MDS and the normal groups.

Attribute	*P* value	FDR *P* value	Bonferroni correction	Attribute	*P* value	FDR *P* value	Bonferroni correction
CD34	<0.00001	0	0	Percentage CD64posMonocytes	0.5407	0.628	1
Percentage $CD19^+$ B-Progenitors Of $CD34^+$ cells	<0.000001	0	0	Percentage Basophils	0.3417	0.444	1
Percentage $CD34^-CD117^+$	<0.000001	0	0	Percentage pDCs	0.8714	0.885	1
$CD34^-CD117^+$DRPosMonoOf$CD34^-CD117^+$	0.020148	0.05	1	$CD34^+$ CD64 CV	0.0652	0.137	1
CD34-CD117+DRnegErythroidOfCD34-CD117+	0.000419	0.002	0.027	$CD34^+$ CD123 MFI	0.4040	0.505	1
CD34-CD117+DRnegGranOfCD34-CD117+	0.000436	0.002	0.028	$CD34^+$ CD38 CV	0.1594	0.256	1
Percentage Granulocytes	0.000112	0.001	0.007	$CD34^+$ CD34 MFI	0.3617	0.461	1
Percentage Mast Cells	0.019625	0.05	1	$CD34^+$ CD34 CV	0.0956	0.173	1
$CD34^+$ CD64 MFI	0.048011	0.108	1	$CD34^+$ HLA-DR MFI	0.1154	0.203	1
$CD34^+$ CD123 CV	0.000014	0	0.001	$CD34^+$ CD45 MFI	0.0719	0.142	1
$CD34^+$CD38 MFI	0.000004	0	0	$CD34^+$ CD45 CV	0.4787	0.566	1
$CD34^+$ CD117 MFI	0.000006	0	0	$CD34^+$ CD13 CV	0.1616	0.256	1
$CD34^+$ CD117 CV	0.000383	0.002	0.025	$CD34^-CD117^+$ CD64 MFI	0.1455	0.249	1
$CD34^+$ HLADR CV	0.002239	0.01	0.146	$CD34^-CD117^+$ CD123 MFI	0.2880	0.398	1
$CD34^+$ CD13 MFI	0.004468	0.018	0.29	$CD34^-CD117^+$ CD117 MFI	0.6518	0.731	1
$CD34^-CD117^+$ CD64 CV	0.006212	0.02	0.404	$CD34^-CD117^+$ HLA-DR MFI	0.1594	0.256	1
$CD34^-CD117^+$ CD123 CV	0.009441	0.028	0.614	$CD34^-CD117^+$ CD45 MFI	0.4178	0.512	1
$CD34^-CD117^+$ CD38 MFI	<0.000001	0	0	$CD34^-CD117^+$ CD16 MFI	0.7015	0.76	1

$CD34^{-}CD117^{+}$ CD38 CV	0.005943	0.02	0.386	$CD34^{-}CD117^{+}$CD13 MFI	0.3392	0.444	1
$CD34^{-}CD117^{+}$ CD117 CV	0.004782	0.018	0.311	Granulocytes CD64 MFI	0.9362	0.936	1
$CD34^{-}CD117^{+}$ HLA-DR CV	0.019361	0.05	1	Granulocytes CD64 CV	0.2178	0.315	1
$CD34^{-}CD117^{+}$ CD45 CV	0.008608	0.027	0.56	Granulocytes CD123 MFI	0.5769	0.658	1
$CD34^{-}CD117^{+}$ CD16 CV	0.041327	0.096	1	Granulocytes CD123 CV	0.7159	0.763	1
$CD34^{-}CD117^{+}$ CD13 CV	0.000004	0	0	Granulocytes CD38 MFI	0.8518	0.879	1
Granulocytes CD45 CV	0.006120	0.02	0.398	Granulocytes CD38 CV	0.2054	0.31	1
Monocytes CD64 CV	0.000103	0.001	0.007	Granulocytes CD45 MFI	0.2624	0.371	1
Monocytes CD38 MFI	0.017181	0.049	1	Granulocytes CD16 MFI	0.2133	0.315	1
Monocytes HLA-DR CV	0.033783	0.081	1	Granulocytes CD16 CV	0.0726	0.142	1
Monocytes CD45 MFI	0.002221	0.01	0.144	Granulocytes CD13 MFI	0.4653	0.56	1
				Granulocytes CD13 CV	0.6872	0.757	1
				Monocytes CD64 MFI	0.1682	0.26	1
				Monocytes CD123 MFI	0.8196	0.859	1
				Monocytes CD123 CV	0.0625	0.135	1
				Monocytes CD38 CV	0.3270	0.443	1
				Monocytes HLA-DR MFI	0.0742	0.142	1
				Monocytes CD45 CV	0.0794	0.148	1

Appendix Table 5.1. Statistical comparison between the MDS and Reactive groups for numerical and phenotypic attributes. The attributes on the left hand side of the table were all statistical significant at the $p<0.05$ level (Wilcoxon signed ranks). Benjamini-Hochberg false discovery rate adjusted *p* values and Bonferroni correction *p* values are also quoted. All attributes on the right hand side of the table did not show significant differences between the MDS and Reactive groups.

Classifier Family	Classifier	Description
Bayes	A1DE	A1DE is an Averaged one-dependence estimator which achieves highly accurate classification by averaging over all of a small space of alternative naive-Bayes-like models that have weaker (and hence less detrimental) independence assumptions than naive Bayes.
	A2DE	A2DE is an Averaged one-dependence estimator which achieves highly accurate classification by averaging over all of a small space of alternative naive-Bayes-like models that have weaker (and hence less detrimental) independence assumptions than naive Bayes.
	Bayesian Logistic Regression	Bayesian approach to learning a linear logistic regression model. Implements Bayesian Logistic Regression for both Gaussian and Laplace Priors.
	Bayes Net	Bayes Network learning using various search algorithms and quality measures.
	Naive Bayes	Standard probabilistic Naive Bayes classifier using estimator classes.
Functions	KernelLogisticRegression	This classifier generates a two-class kernel logistic regression model.
	Logistic	Class for building and using a multinomial logistic regression model with a ridge estimator.
	MLPClassifier	Trains a multilayer perceptron with one hidden layer using WEKA's Optimization class by minimizing the squared error plus a quadratic penalty with the BFGS method.
	MultilayerPerceptron	A classifier that uses a backpropagation neural network to classify instances.
	RBFClassifier	Class implementing radial basis function networks for classification, trained in a fully supervised manner using WEKA's Optimization class by minimizing squared error with the BFGS method.
	RBFNetwork	Implements a normalized Gaussian radial basis function network
	SGD	Implements stochastic gradient descent for learning various linear models (binary class SVM, binary class logistic regression, squared loss, Huber loss and epsilon-insensitive loss linear regression).
	SimpleLogistic	Builds linear logistic regression models with built-in attribute selection

	SMO	Sequential minimal optimization algorithm for support vector classification
	SPegasos	Implements the stochastic variant of the Pegasos (Primal Estimated sub-GrAdient SOlver for SVM) method of Shalev-Shwartz *et al*.
	VotedPerceptron	Implementation of the voted perceptron algorithm by Freund and Schapire.
Miscellaneous	CHIRP	CHIRP is an iterative sequence of three stages (projecting, binning, and covering) that are designed to deal with the curse of dimensionality, computational complexity, and nonlinear separability.
	VFI	Classification by voting feature intervals methods, simple and fast
Rules	ConjunctiveRule	This class implements a single conjunctive rule learner that can predict for numeric and nominal class labels.
	FURIA	FURIA: Fuzzy Unordered Rule Induction Algorithm
	JRip	This class implements a propositional rule learner, Repeated Incremental Pruning to Produce Error Reduction (RIPPER), which was proposed by William W Cohen as an optimized version of IREP.
	Ridor	An implementation of a RIpple-DOwn Rule learner. It generates a default rule first and then the exceptions for the default rule with the least (weighted) error rate. Then it generates the "best" exceptions for each exception and iterates until pure. Thus it performs a tree-like expansion of exceptions.The exceptions are a set of rules that predict classes other than the default. IREP is used to generate the exceptions.
Trees	ADTree	Classifier for generating an alternating decision tree.
	BFTree	Classifier for building a best-first decision tree classifier. This class uses binary split for both nominal and numeric attributes. For missing values, the method of 'fractional' instances is used.
	DecisionStump	Classifier for building and using a decision one-level decision trees. Usually used in conjunction with a boosting algorithm. Does regression (based on mean-squared error) or classification (based on entropy).
	FT	Classifier for building 'Functional trees', which are classification trees with oblique splits that could have logistic regression functions at the inner nodes and/or leaves.

	HoeffdingTree	A Hoeffding tree (VFDT) is an incremental, anytime decision tree induction algorithm that is capable of learning from massive data streams, assuming that the distribution generating examples does not change over time.
	J48	Classifier for generating a pruned or unpruned C4.
	J48graft	Classifier for generating a grafted (pruned or unpruned) C4.
	LMT	Classifier for building 'logistic model trees', which are classification trees with logistic regression functions at the leaves.
	NBTree	Classifier for generating a decision tree with naive Bayes classifiers at the leaves.
	RandomForest	Classifier for constructing a forest of random trees.
	REPTree	Fast decision tree learner. Builds a decision/regression tree using information gain/variance and prunes it using reduced-error pruning (with backfitting). Only sorts values for numeric attributes once.
	SimpleCart	Classifier implementing minimal cost-complexity pruning.
	OneR	Classifier for building and using a 1R classifier; in other words, uses the minimum-error attribute for prediction, discretizing numeric attributes.
	ZeroR	Class for building and using a 0-R classifier. Predicts the majority class.

Appendix Table 5.2. Synopsis of classifier features. All descriptions reproduced from the either the *weka.gui.GenericObjectEditor* in Weka software alone or as an amalgamation with Chapter 11 “The Explorer”, section 11.4 “Learning Algorithms”, Table 11.5 “Classifier Algorithms in Weka” in “Data Mining – Practical Machine Learning Tools and Techniques” (Hall et al., 2009; Witten et al., 2011)

(A)

	Predicted MDS	**Predicted Reactive**	**Total**
Actual MDS	True Positive (TP)	False Negative (FN)	TP + FN
Actual Reactive	False Positive (FP)	True Negative (TN)	FP + TN
Total	TP + FP	FN + TN	TP + FP + FN + TN

(B)

Metric	**Description**	**Formula**
Accuracy	Degree of closeness to the actual results from the predicted results	= (TP + TN)/(TP+FP+FN+TN)
Sensitivity	Proportion of Actual MDS predicted to be MDS	= TP/(TP + FN)
Specificity	Proportion of Actual Reactive predicted to be Reactive	= TN/(FP + TN)
Kappa Statistic	Compares the accuracy of the classifier compared to the random accuracy of the classifier	See (Cohen, 1960)
Precision	True MDS as a proportion of True MDS plus Reactive cases predicted as MDS. Also known as the Positive Predictive Value	= TP/(TP + FP)
F-measure	A measure of the test's accuracy taking into account sensitivity and precision	= 2 x TP/(2 x TP + FP + FN)
Matthews Correlation Coefficient (MCC)	Generalises the confusion matrix as a single number taking into account both actual and predicted MDS and Reactive cases and imbalanced datasets.	= TP x TN – FP x FN/ √(TP + FP)(TP + FN)(TN + FP)(TN + FN)
Area under the Receiver Operating Characteristic Curve (AUROC)	Reduces the ROC performance (FN and FP for every cut-off) to a single value thereby allowing classifier comparison	See (Fawcett, 2006)

Appendix Table 5.3. Confusion matrix (A) and (B) descriptions and formulas of the metrics evaluated. The formula is shown for metrics which are a function of the confusion matrix.

Classifier	Accuracy	Sensitivity	Specificity	Kappa Statistic	Precision	F-Measure	MCC	ROC Area	True Positives	False Negative	False Positive	True Negative
A1DE	0.953	0.904	0.987	0.9016	0.979	0.94	0.904	0.985	47	5	1	75
A2DE	0.969	0.923	1	0.9344	1	0.96	0.936	0.988	48	4	**0**	**76**
Bayesian Logistic Regression	0.977	0.962	0.987	0.9513	0.98	0.971	0.95	0.974	50	2	1	75
Bayes Net	0.945	0.904	0.974	0.8856	0.959	0.931	0.887	0.977	47	5	2	74
Naive Bayes	0.883	0.808	0.934	0.7533	0.894	0.848	0.759	0.961	42	10	5	71
KernelLogisticRegression	**1.000**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**52**	**0**	**0**	**76**
Logistic	**1.000**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**52**	**0**	**0**	**76**
MLPClassifier	0.992	**1**	0.987	0.9839	0.981	0.99	0.984	0.99	**52**	**0**	1	75
MultilayerPerceptron	0.984	0.962	**1**	0.9674	**1**	0.98	0.968	0.982	50	2	**0**	**76**
RBFClassifier	0.930	0.904	0.947	0.8538	0.922	0.913	0.854	0.97	47	5	4	72
RBFNetwork	0.891	0.788	0.961	0.7676	0.932	0.854	0.774	0.974	41	11	3	73
SGD	0.977	0.962	0.987	0.9513	0.98	0.971	0.951	0.974	50	2	1	75
SimpleLogistic	0.914	0.885	0.934	0.8213	0.902	0.893	0.821	0.98	46	6	5	71
SMO	0.938	0.904	0.961	0.8697	0.94	0.922	0.87	0.932	47	5	3	73
SPegasos	0.875	0.692	**1**	0.7277	**1**	0.818	0.756	0.846	36	16	**0**	**76**
VotedPerceptron	0.875	0.865	0.882	0.7425	0.833	0.849	0.743	0.952	45	7	9	67
CHIRP	0.953	0.942	0.961	0.9028	0.942	0.942	0.903	0.951	49	3	3	73
VFI	0.953	0.885	**1**	0.901	**1**	0.939	0.905	0.995	46	6	0	76
ConjunctiveRule	0.852	0.769	0.908	0.6876	0.851	0.808	0.69	0.839	40	12	7	69
FURIA	0.992	0.981	**1**	0.9838	**1**	0.99	0.984	**1**	51	1	**0**	**76**

JRip	0.961	0.962	0.961	0.9193	0.943	0.952	0.919	0.969	50	2	3	73
Ridor	0.852	0.635	**1**	0.6735	**1**	0.776	0.713	0.817	33	19	0	76
ADTree	**1.000**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**52**	**0**	**0**	**76**
BFTree	0.977	**1**	0.961	0.9519	0.945	0.972	0.953	0.994	**52**	**0**	3	73
DecisionStump	0.852	0.654	0.987	0.6756	0.971	0.782	0.706	0.82	34	18	1	75
FT	**1.000**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**52**	**0**	**0**	**76**
HoeffdingTree	0.891	0.788	0.961	0.7676	0.932	0.854	0.774	0.962	41	11	3	73
J48	0.984	0.981	0.987	0.9676	0.981	0.981	0.968	0.993	51	1	1	75
J48graft	0.984	0.981	0.987	0.9676	0.981	0.981	0.968	0.993	51	1	1	75
LMT	0.914	0.885	0.934	0.8213	0.902	0.893	0.821	0.98	46	6	5	71
NBTree	**1.000**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**52**	**0**	**0**	**76**
RandomForest	**1.000**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**52**	**0**	**0**	**76**
REPTree	0.852	0.654	0.987	0.6756	0.971	0.782	0.706	0.82	34	18	1	75
SimpleCart	0.852	0.654	0.987	0.6756	0.971	0.782	0.706	0.82	34	18	1	75
OneR	0.859	0.712	0.961	0.6975	0.925	0.804	0.712	0.836	37	15	3	73
ZeroR	0.594	0	1	0	0	0	0	0.5	0	52	0	76

Appendix Table 5.4. Performance metrics for the different classifiers used in regular classification of the Training set for the MDS and Reactive groups. The top score for each metric is indicated in bold (Zero R classifier excluded).

Classifier	Accuracy	Sensitivity	Specificity	Kappa Statistic	Precision	F-Measure	MCC	ROC Area	True Positives	False Negative	False Positive	True Negative
A1DE	**0.883**	**0.865**	0.895	**0.7578**	0.849	**0.857**	**0.758**	0.919	**45**	**7**	8	68
A2DE	0.867	**0.865**	0.868	0.7272	0.818	0.841	0.728	**0.92**	**45**	**7**	10	66
Bayesian Logistic Regression	0.813	0.75	0.855	0.609	0.78	0.765	0.609	0.803	39	13	11	65
Bayes Net	0.875	**0.865**	0.882	0.7425	0.833	0.849	0.743	0.919	45	7	9	67
Naive Bayes	0.867	0.788	0.921	0.7205	0.872	0.828	0.723	0.913	41	11	6	70
KernelLogisticRegression	0.781	0.692	0.842	0.541	0.75	0.72	0.542	0.863	36	16	12	64
Logistic	0.719	0.615	0.789	0.4098	0.667	0.64	0.411	0.747	32	20	16	60
MLPClassifier	0.781	0.692	0.842	0.541	0.75	0.72	0.542	0.871	36	16	12	64
MultilayerPerceptron	0.805	0.731	0.855	0.5914	0.776	0.752	0.592	0.811	38	14	11	65
RBFClassifier	0.844	0.788	0.882	0.6741	0.82	0.804	0.674	0.905	41	11	9	67
RBFNetwork	0.859	0.827	0.882	0.7085	0.827	0.827	0.709	0.856	43	9	9	67
SGD	0.805	0.731	0.855	0.5914	0.776	0.752	0.592	0.793	38	14	11	65
SimpleLogistic	0.820	0.712	0.895	0.6194	0.822	0.763	0.624	0.901	37	15	8	68
SMO	0.836	0.769	0.882	0.6568	0.816	0.792	0.658	0.825	40	12	9	67
SPegasos	0.789	0.75	0.816	0.5641	0.736	0.743	0.564	0.783	39	13	14	62
VotedPerceptron	0.828	0.808	0.842	0.6459	0.778	0.792	0.646	0.858	42	10	12	64
CHIRP	0.805	0.654	0.908	0.5812	0.829	0.731	0.591	0.781	34	18	7	69
VFI	0.805	0.827	0.789	0.6036	0.729	0.775	0.607	0.798	43	9	16	60
ConjunctiveRule	0.836	0.635	**0.974**	0.6414	**0.943**	0.759	0.67	0.785	33	19	**2**	**74**
FURIA	0.805	0.712	0.868	0.5889	0.787	0.747	0.591	0.867	37	15	10	66

JRip	0.844	0.808	0.868	0.6761	0.808	0.808	0.676	0.831	42	10	10	66
Ridor	0.758	0.712	0.789	0.4995	0.698	0.705	0.5	0.751	37	15	16	60
ADTree	0.828	0.712	0.908	0.6349	0.841	0.771	0.641	0.897	37	15	7	59
BFTree	0.781	0.692	0.842	0.541	0.75	0.72	0.542	0.757	36	16	12	64
DecisionStump	0.805	0.596	0.947	0.5731	0.886	0.713	0.599	0.759	31	21	4	72
FT	0.836	0.788	0.868	0.6589	0.804	0.796	0.659	0.889	41	11	10	66
HoeffdingTree	0.867	0.788	0.921	0.7205	0.872	0.828	0.723	0.916	41	11	6	70
J48	0.836	0.769	0.882	0.6568	0.816	0.792	0.658	0.83	40	12	9	67
J48graft	0.836	0.712	0.921	0.6504	0.86	0.779	0.658	0.807	37	15	6	70
LMT	0.852	0.75	0.921	0.6856	0.867	0.804	0.69	0.881	39	13	6	70
NBTree	0.789	0.712	0.842	0.5587	0.755	0.733	0.559	0.85	37	15	12	64
RandomForest	0.836	0.75	0.895	0.6547	0.83	0.788	0.657	0.883	39	13	8	68
REPTree	0.781	0.692	0.842	0.541	0.75	0.72	0.542	0.793	36	16	12	64
SimpleCart	0.805	0.673	0.895	0.5838	0.814	0.737	0.59	0.784	35	17	8	68
OneR	0.820	0.75	0.868	0.6241	0.796	0.772	0.625	0.809	39	13	10	66
ZeroR	0.594	0	1	0	0	0	0	0.5	0	52	0	76

Appendix Table 5.5. Performance metrics for the different classifiers used in the 10-fold cross validation set for the MDS and Reactive groups. The top score for each metric is indicated in bold (Zero R classifier excluded).

Classifier	Accuracy	Sensitivity	Specificity	Kappa Statistic	Precision	F-Measure	MCC	ROC Area	True Positives	False Negative	False Positive	True Negative
A1DE	0.844	0.865	0.829	0.6819	0.776	0.818	0.685	0.847	45	7	13	63
A2DE	0.844	0.865	0.829	0.6819	0.776	0.818	0.685	0.847	45	7	13	63
Bayesian Logistic Regression	0.813	0.75	0.855	0.609	0.78	0.765	0.609	0.803	39	13	11	65
Bayes Net	0.836	0.865	0.816	0.667	0.763	0.811	0.671	0.841	45	7	14	62
Naive Bayes	0.859	0.788	0.908	0.7049	0.854	0.82	0.706	0.848	41	11	7	69
KernelLogisticRegression	0.789	0.769	0.803	0.5667	0.727	0.748	0.567	0.786	40	12	15	61
Logistic	0.719	0.615	0.789	0.4098	0.667	0.64	0.411	0.702	32	20	16	60
MLPClassifier	0.805	0.808	0.803	0.6012	0.737	0.771	0.603	0.805	42	10	15	61
MultilayerPerceptron	0.781	0.75	0.803	0.5493	0.722	0.736	0.55	0.776	39	13	15	61
RBFClassifier	0.688	**0.981**	0.487	0.4192	0.567	0.718	0.503	0.734	**51**	**1**	39	37
RBFNetwork	**0.867**	0.846	0.882	**0.7255**	0.83	**0.838**	**0.726**	**0.864**	44	8	9	67
SGD	0.805	0.731	0.855	0.5914	0.776	0.752	0.592	0.793	38	14	11	65
SimpleLogistic	0.789	0.904	0.711	0.5842	0.681	0.777	0.605	0.807	47	5	22	54
SMO	0.836	0.769	0.882	0.6568	0.816	0.792	0.658	0.825	40	12	9	67
SPegasos	0.789	0.75	0.816	0.5641	0.736	0.743	0.564	0.783	39	13	14	62
VotedPerceptron	0.828	0.808	0.842	0.6459	0.778	0.792	0.646	0.825	42	10	12	64
CHIRP	0.805	0.654	0.908	0.5812	0.829	0.731	0.591	0.781	34	18	7	69
VFI	0.438	0.904	0.118	0.0187	0.412	0.566	0.035	0.511	47	5	67	9
ConjunctiveRule	0.492	0.942	0.184	0.1073	0.441	0.601	0.183	0.563	49	3	62	14
FURIA	0.805	0.827	0.789	0.6036	0.729	0.775	0.607	0.808	43	9	16	60

JRip	0.844	0.808	0.868	0.6761	0.808	0.808	0.676	0.838	42	10	10	66
Ridor	0.758	0.712	0.789	0.4995	0.688	0.705	0.5	0.751	37	15	16	60
ADTree	0.797	0.923	0.711	0.6008	0.686	0.787	0.625	0.817	48	4	22	54
BFTree	0.695	0.769	0.645	0.3959	0.597	0.672	0.407	0.7017	40	12	27	49
DecisionStump	0.430	0.962	0.066	0.0226	0.413	0.578	0.059	0.514	50	2	71	5
FT	0.844	0.808	0.868	0.6761	0.808	0.808	0.676	0.838	42	10	10	66
HoeffdingTree	0.859	0.808	0.895	0.7067	0.84	0.824	0.707	0.851	42	10	8	68
J48	0.844	0.788	0.882	0.6741	0.82	0.804	0.674	0.835	41	11	9	67
J48graft	0.836	0.712	**0.921**	0.6504	**0.86**	0.779	0.658	0.816	37	15	**6**	**70**
LMT	0.836	0.712	**0.921**	0.6504	**0.86**	0.779	0.658	0.816	37	15	**6**	**70**
NBTree	0.781	0.904	0.697	0.5701	0.671	0.77	0.593	0.801	47	5	23	53
RandomForest	0.641	0.962	0.421	0.3393	0.532	0.685	0.425	0.691	50	2	44	32
REPTree	0.703	0.788	0.645	0.4131	0.603	0.683	0.426	0.717	41	11	27	49
SimpleCart	0.633	0.808	0.513	0.2965	0.532	0.641	0.324	0.66	42	10	37	39
OneR	0.820	0.75	0.868	0.6241	0.796	0.772	0.625	0.809	39	13	10	66
ZeroR	0.406	1	0	0	0.406	0.579	0	0.5	52	0	76	0

Appendix Table 5.6. Performance metrics for different classifiers used for the *cost sensitive classification* analysis of the MDS and Reactive groups. The top score for each metric is indicated in bold (Zero R classifier excluded).

Classifier	Accuracy	Sensitivity	Specificity	Kappa Statistic	Precision	F-Measure	MCC	ROC Area	True Positives	False Negative	False Positive	True Negative
A1DE	**0.875**	**0.865**	0.882	**0.7425**	0.833	**0.849**	**0.743**	0.914	**45**	**7**	9	67
A2DE	0.859	0.846	0.868	0.7103	0.815	0.83	0.711	0.915	44	8	10	66
Bayesian Logistic Regression	0.836	0.788	0.868	0.6589	0.804	0.796	0.659	0.828	41	11	10	66
Bayes Net	0.867	**0.865**	0.868	0.7272	0.818	0.841	0.728	0.912	**45**	**7**	10	66
Naive Bayes	0.859	0.788	0.908	0.7049	0.854	0.82	0.706	0.913	41	11	7	69
KernelLogisticRegression	0.773	0.654	0.855	0.5202	0.756	0.701	0.524	0.844	34	18	11	65
Logistic	0.711	0.596	0.789	0.3916	0.66	0.626	0.393	0.738	31	21	16	60
MLPClassifier	0.773	0.654	0.855	0.5202	0.756	0.701	0.524	0.854	34	18	11	65
MultilayerPerceptron	0.797	0.731	0.842	0.5764	0.76	0.745	0.577	0.821	38	14	12	64
RBFClassifier	0.852	**0.865**	0.842	0.6969	0.789	0.826	0.699	0.903	**45**	**7**	12	64
RBFNetwork	0.859	0.846	0.868	0.7103	0.815	0.83	0.711	0.854	44	8	10	66
SGD	0.805	0.712	0.868	0.5889	0.787	0.747	0.591	0.79	37	15	10	66
SimpleLogistic	0.844	0.75	0.908	0.6701	0.848	0.796	0.673	0.912	39	13	7	69
SMO	0.852	0.827	0.868	0.6932	0.811	0.819	0.693	0.848	43	9	10	66
SPegasos	0.758	0.654	0.829	0.4902	0.723	0.687	0.492	0.741	34	18	13	63
VotedPerceptron	0.836	0.808	0.855	0.6609	0.792	0.8	0.661	0.836	42	10	11	65
CHIRP	0.742	0.615	0.829	0.545	0.711	0.66	0.457	0.722	32	20	13	63
VFI	0.805	0.827	0.789	0.6036	0.729	0.775	0.607	0.802	43	9	16	60
ConjunctiveRule	0.805	0.615	0.934	0.5758	0.865	0.719	0.595	0.773	32	20	5	71
FURIA	0.508	0.385	0.592	-0.0234	0.392	0.388	-0.023	0.488	20	32	31	45

JRip	0.781	0.731	0.816	0.5466	0.731	0.731	0.547	0.809	38	14	14	62
Ridor	0.781	0.769	0.789	0.552	0.714	0.741	0.553	0.779	40	12	16	60
ADTree	0.813	0.692	0.895	0.6017	0.818	0.75	0.607	0.885	36	16	8	68
BFTree	0.797	0.75	0.829	0.5789	0.75	0.75	0.579	0.766	39	13	13	63
DecisionStump	0.805	0.596	**0.947**	0.5713	**0.886**	0.713	0.599	0.759	31	21	**4**	**72**
FT	0.797	0.692	0.868	0.5711	0.783	0.735	0.574	0.835	36	16	10	66
HoeffdingTree	0.859	0.788	0.908	0.7049	0.854	0.82	0.706	**0.916**	41	11	7	69
J48	0.828	0.769	0.868	0.6415	0.8	0.784	0.642	0.815	40	12	10	66
J48graft	0.836	0.731	0.908	0.6525	0.844	0.784	0.657	0.813	38	14	7	69
LMT	0.813	0.712	0.882	0.6041	0.804	0.755	0.607	0.875	37	15	9	67
NBTree	0.750	0.712	0.776	0.4849	0.685	0.698	0.485	0.799	37	15	17	59
RandomForest	0.859	0.846	0.868	0.7103	0.815	0.83	0.711	0.909	44	8	10	66
REPTree	0.789	0.731	0.829	0.5614	0.745	0.738	0.561	0.835	38	14	13	63
SimpleCart	0.789	0.75	0.816	0.5614	0.736	0.743	0.564	0.77	39	13	14	62
OneR	0.711	0.673	0.737	0.4062	0.636	0.654	0.407	0.705	35	17	20	56
ZeroR	0.453	0.577	0.368	-0.0507	0.385	0.462	-0.055	0.469	30	22	48	28

Appendix Table 5.7. Performance metrics for the different classifiers used for the *cost sensitive learning* of the MDS and Reactive groups. The top score for each metric is indicated in bold (Zero R classifier excluded).

UPN	Exon / Allele/ Variant	Consequence	Coding sequence	Protein	Co-located variants	Cytogenetic karyotypic analysis
UPN1	SRSF2_17_74732959_G/T	missense_variant	c.284C>A	p.Pro95His	COSM146290,COSM146288,COSM2...	Not done
UPN1	ASXL1_20_31022534_-/C	frameshift_variant,feature_elongation	c.2018_2019insC	p.His674ProfsTer3	-	
UPN1	ASXL1_20_31022536_A/C	missense_variant	c.2021A>C	p.His674Pro	-	
UPN1	TET2_4_106157527_C/T	stop_gained	c.2428C>T	p.Gln810Ter	COSM43446	
UPN1	TET2_4_106158168_C/-	frameshift_variant,feature_truncation	c.3069delC	p.Ser1023ArgfsTer10	-	
UPN3	SETBP1_18_42531917_T/C	missense_variant	c.2612T>C	p.Ile871Thr	rs267607038,COSM1685361,COS...	Not done
UPN3	CSF3R_1_36933434_G/A	missense_variant	c.1853C>T	p.Thr618Ile	-	
UPN3	RUNX1_21_36231791_T/C	missense_variant	c.593A>G	p.Asp198Gly	COSM36059,COSM24799	
UPN3	U2AF1_21_44514777_T/G	missense_variant	c.470A>C	p.Gln157Pro	rs371246226,COSM211534,COSM...	
UPN4	NONE					Not done
UPN5	SF3B1_2_198266834_T/C	missense_variant	c.2098A>G	p.Lys700Glu	COSM84677	Normal Karyotype
UPN5	SF3B1_2_198267359_C/G	missense_variant	c.1998G>C	p.Lys666Asn	rs377023736,COSM131557,COSM...	
UPN5	TET2_4_106180816_G/-	frameshift_variant,feature_truncation	c.3844delG	p.Gly1282ValfsTer81	-	
UPN6	ASXL1_20_31023596_T/-	frameshift_variant,feature_truncation	c.3081delT	p.Ser1028ArgfsTer19	-	Not done
UPN9	NONE					Not done
UPN11	U2AF1_21_44524456_G/A	missense_variant	c.101C>T	p.Ser34Phe	rs371769427,COSM146287,COSM...	Normal Karyotype
UPN11	TET2_4_106180865_G/A	missense_variant	c.3893G>A	p.Cys1298Tyr	COSM87138,COSM43474	
UPN13	NONE					Missing Y as the sole abnormality
UPN14	TET2_4_106155764_AT/-	frameshift_variant,feature_truncation	c.665_666delAT	p.His222ArgfsTer2		Normal Karyotype
UPN14	TET2_4_106197210_C/G	stop_gained	c.5543C>G	p.Ser1848Ter		
UPN14	ZRSR2_X_15838340_T/C	missense_variant	c.838T>C	p.Cys280Arg		
UPN15	NONE					Not done
UPN17	NONE					Normal Karyotype
UPN18	NONE					Normal Karyotype
UPN19	SRSF2_17_74732959_G/T	missense_variant	c.284C>A	p.Pro95His	COSM146290,COSM146288,COSM2...	Normal Karyotype
UPN19	TET2_4_106156759_-/C	frameshift_variant,feature_elongation	c.1659_1660insC	p.Thr556AsnfsTer11	-	
UPN21	U2AF1_21_44514780_C/T	missense_variant	c.467G>A	p.Arg156His	COSM1235014,COSM1235015	Monosomy 7
UPN21	EZH2_7_148507475_C/T	missense_variant	c.1979G>A	p.Gly660Glu	-	
UPN22	ZRSR2_X_15838370_C/T	stop_gained	c.868C>T	p.Arg290Ter	-	Missing Y as the sole

						abnormality
UPN23	SF3B1_2_198266834_T/C	missense_variant	c.2098A>G	p.Lys700Glu	COSM84677	Normal Karyotype
UPN23	DNMT3A_2_25457242_C/T	missense_variant	c.2645G>A	p.Arg882His	rs147001633,COSM442676,COSM...	
UPN24	NONE					Not done
UPN26	SF3B1_2_198266834_T/C	missense_variant	c.2098A>G	p.Lys700Glu	COSM84677	G-banding failed. FISH found no evidence of chromosome 7 abnormalities but did detect deleted 5q.
UPN28	NONE					Not done
UPN29	NONE					Not done
UPN30	U2AF1_21_44524456_G/T	missense_variant	c.101C>A	p.Ser34Tyr	rs371769427,COSM146287,COSM...	Missing Y as the sole abnormality
UPN30	TET2_4_106156336_C/-	frameshift_variant,feature_truncation	c.1237delC	p.Pro413HisfsTer14	-	
UPN31	SRSF2_17_74732959_G/A	missense_variant	c.284C>T	p.Pro95Leu	COSM146290,COSM146288,COSM2...	Missing Y as the sole abnormality
UPN32	ASXL1_20_31024579_A/G	missense_variant	c.4064A>G	p.Asp1355Gly	-	Not done
UPN33	NONE					Not done
UPN34	NONE					Not done
UPN35	NONE					Not done
UPN36	NONE					Not done
UPN37	NONE					Normal Karyotype
UPN38	TET2_4_106180874_C/-	frameshift_variant,feature_truncation	c.3902delC	p.Arg1302GlufsTer61	-	Deleted 20q
UPN38	TET2_4_106180876_A/-	frameshift_variant,feature_truncation	c.3904delA	p.Arg1302GlufsTer61	-	
UPN39	NONE					Not done
UPN40	SRSF2_17_74732959_G/A	missense_variant	c.284C>T	p.Pro95Leu	COSM146290,COSM146288,COSM2...	Normal Karyotype
UPN40	TET2_4_106156082_-/T	frameshift_variant,feature_elongation	c.982_983insT	p.Glu330Ter	-	
UPN40	TET2_4_106164071_C/G	missense_variant	c.3581C>G	p.Pro1194Arg	-	
UPN41	NONE					Not done
UPN42	TET2_4_106164005_-/GT	frameshift_variant,feature_elongation	c.3514_3515insGT	p.Lys1173ValfsTer54	-	Not done
UPN42	TET2_4_106197080_A/-	frameshift_variant,feature_truncation	c.5413delA	p.Asn1805ThrfsTer15	-	
UPN43	ASXL1_20_31024704_G/A	missense_variant	c.4189G>A	p.Gly1397Ser	rs146464648,COSM133033	Not done
UPN44	TET2_4_106157385_-/C	frameshift_variant,feature_elongation	c.2285_2286insC	p.Gln764ProfsTer5	-	Not done
UPN44	TET2_4_106197255_C/T	missense_variant	c.5588C>T	p.Ala1863Val	COSM166836	
UPN45	NONE					Not done
UPN46	SRSF2_17_74732959_G/C	missense_variant	c.284C>G	p.Pro95Arg	COSM146290,COSM146288,COSM2...	Not done
UPN46	ASXL1_20_31023000_C/T	stop_gained	c.2485C>T	p.Gln829Ter	COSM97040	

UPN46	RUNX1_21_36252856_-/C	frameshift_variant,splice_region_variant,feature_elongation	c.506dupG	p.Gly170ArgfsTer43	COSM36060	
UPN46	STAG2_X_123181311_C/T	stop_gained	c.775C>T	p.Arg259Ter	COSM216178,COSM1598816	
UPN46	STAG2_X_123195108_T/G	missense_variant	c.1451T>G	p.Met484Arg	-	
UPN46	STAG2_X_123195109_G/A	missense_variant	c.1452G>A	p.Met484Ile	COSM372362	
UPN47	KRAS_12_25380279_C/T	missense_variant	c.179G>A	p.Gly60Asp	COSM87290,COSM1667041,COSM548	Trisomy 8
UPN47	BCOR_X_39932085_-/G	frameshift_variant,feature_elongation	c.2514dupC	p.Lys839GlnfsTer5	COSM1319442	
UPN48	SF3B1_2_198266834_T/C	missense_variant	c.2098A>G	p.Lys700Glu	COSM84677	Normal Karyotype
UPN49	SRSF2_17_74732959_G/T	missense_variant	c.284C>A	p.Pro95His	COSM146290,COSM146288,COSM2...	G-banding failed. FISH found no 5,7 or 17p abnormalities but did show trisomy 8
UPN49	RUNX1_21_36231782_C/T	missense_variant	c.602G>A	p.Arg201Gln	rs74315450,CM992140,COSM24805	
UPN49	TET2_4_106196738_T/G	missense_variant	c.5071T>G	p.Ser1691Ala	COSM1426220	
UPN49	STAG2_X_123179156_C/G	stop_gained	c.605C>G	p.Ser202Ter	-	
UPN49	STAG2_X_123179197_C/T	stop_gained	c.646C>T	p.Arg216Ter	COSM1315170,COSM1315169	
UPN49	STAG2_X_123197044_C/T	stop_gained	c.1810C>T	p.Arg604Ter	COSM487905,COSM1138070	
UPN49	BCOR_X_39933978_G/-	frameshift_variant,feature_truncation	c.621delC	p.Asp208ThrfsTer8	-	
UPN50	ASXL1_20_31022658_AG/-	frameshift_variant,feature_truncation	c.2143_2144delAG	p.Arg715SerfsTer2	-	Normal Karyotype
UPN50	ASXL1_20_31022661_G/T	missense_variant	c.2146G>T	p.Ala716Ser	-	
UPN50	TET2_4_106157341_T/-	frameshift_variant,feature_truncation	c.2242delT	p.Leu748TyrfsTer3	-	
UPN50	TET2_4_106157718_C/-	frameshift_variant,feature_truncation	c.2619delC	p.Pro874GlnfsTer47	-	
UPN50	TET2_4_106197348_C/G	missense_variant	c.5681C>G	p.Pro1894Arg	COSM87189	
UPN50	ZRSR2_X_15836741_G/T	missense_variant	c.803G>T	p.Gly268Val	-	

Appendix Table 6.1. Annotated driver mutations for 41 patients in the validation cohort.