Cytogenetically Normal Acute Myeloid Leukaemia at a single centre

in South Africa

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“Leopards? – Oom Schalk Lourens said – Oh yes, there are two varieties on this side of the Limpopo. The chief difference between them is that one kind of a leopard has a few more spots on it than the other kind. But when you meet a leopard in the veld, unexpectedly, you seldom trouble to count his spots to find out what kind he belongs to. That is unnecessary. Because whatever leopard it is that you come across in this way, you only do one kind of running. And that is the fastest kind.” – Oom Schalk Lourens (Herman Charles Bosman)

Dedication

To my wife and family, thank you for your unwavering support, immeasurable love and close counsel which guides my run through life.
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Date: 18/11/2020
Abstract

This abstract was compiled in accordance with the author guidelines stipulated by the American Journal of Hematology (Appendix D).

Introduction

The heterogeneous molecular landscape of cytogenetically normal acute myeloid leukaemia (CN-AML) renders it an ongoing therapeutic challenge worldwide. The latest European LeukaemiaNet (ELN) 2017 guidelines attempt to address this by guiding post-remission therapy according to six prognostically informative mutations. However, its applicability in a South African setting remains elusive due to limited local data. This retrospective study aimed to describe a South African CN-AML cohort according to clinico-pathological, molecular and treatment outcomes and consequently investigate the local applicability of a triple mutation testing approach for nucleophosmin (NPM1), fms-like tyrosine kinase internal tandem duplication (FLT3-ITD) and CCAAT/enhancer binding protein alpha (CEBPα) mutations in accordance with the ELN 2017 guidelines.

Methods

A review of cytogenetic results for all adult de novo AML cases diagnosed at Groote Schuur Hospital between 2005 and 2018 was performed. CN-AML cases were further characterized via molecular testing and review of clinical and laboratory data.

Results

In total, 218 patients with AML were identified of which fifty-six (33%) were cytogenetically normal. NPM1, FLT3-ITD and CEBPα mutations were found in 39%, 34% and 9% of CN-AML cases respectively, and allowed for definitive prognostication of 50% of cases. The 2-year overall survival rate for the entire CN-AML cohort was 16%.

Conclusion

Local rates of CN-AML and associated NPM1 and FLT3-ITD mutations were comparable to European cohorts. In contrast, local survival outcomes were notably inferior. Triple testing proved a resource effective prognostication approach for CN-AML. High throughput sequencing for adverse risk mutations should be considered for CN-AML patients inconclusively stratified via triple testing.
Acknowledgements and Contributions

Supervisor

› Associate Professor Karen Shires¹

Associate Professor Karen Shires acted as supervisor for the project, supervised and performed molecular work and also assisted with writing up of the manuscript.

Co-supervisor

› Dr Lee-Ann Phillips²

Dr Lee-Ann Phillips acted as co-supervisor for the project and assisted with writing up of the manuscript.

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Ms Marion Stone performed much of the molecular work.

› Associate Professor Estelle Verburgh³

Associate Professor Estelle Verburgh assisted with writing up of the manuscript.

› Dr Rory Shein⁴

Dr Rory Shein assisted with data collection via review of clinical folders.

Acknowledgements

› Ms Jenna Oosthuizen³

Ms Jenna Oosthuizen assisted with acquisition of clinical folders and also performed the statistical data analysis.

› Mrs Daphne Taylor and staff at the Cytogenetics laboratory⁵

Mrs Daphne Taylor and staff at the Cytogenetics laboratory performed and supervised the performance of cytogenetic testing (karyotyping and fluorescence in situ hybridization).

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List of Abbreviations

**AA**: Amino Acid/s

**ACC**: Association for Clinical Cytogenetics

**Allo**: Allogeneic

**AML**: Acute Myeloid Leukaemia

**AR**: Allelic ratio

**Arf**: Adenosine diphosphate ribosylation factor

**ASXL1**: Additional sex combs like 1 gene

**Auto**: Autologous

**BCR/ABL1**: Breakpoint cluster region/v-abl Abelson murine leukaemia viral oncogene homolog 1 (fusion gene/transcript)

**BM**: Bone marrow

**Bp**: Base pairs

**CBFB/MYH11**: Core binding factor sub-unit Beta/Myosin heavy chain 11 (fusion gene/transcript)

**CEBPα**: CCAAT/enhancer binding protein alpha gene

**CN-AML**: Cytogenetically normal acute myeloid leukaemia

**CR1**: First complete remission

**Dm**: Double mutant

**DNA**: Deoxyribonucleic Acid

**EDTA**: Ethylenediaminetetraacetic Acid

**EFS**: Event free survival

**ELN**: European LeukaemiaNet

**FAB**: French-American-British

**FIP1L1/PDGFRα**: Factor interacting with PAPOLA and CPSF1/Platelet derived growth factor receptor alpha polypeptide (fusion gene/transcript)

**FISH**: Fluorescence *in situ* hybridization

**FLAM**: Fludarabine, Mitoxantrone, Cytarabine

**FLT3-ITD**: Fms-like tyrosine kinase internal tandem duplication mutation

**FLT3-TKD**: Fms-like tyrosine kinase tyrosine kinase domain mutation

**GSH**: Groote Schuur Hospital

**GvHD**: Graft versus host disease

**HLA**: Human leukocyte antigen

**HREC**: Human research ethics committee

**HSC**: Haemopoietic stem cell/s

**HSCT**: Haemopoietic stem cell transplantation

**ISCN**: International system for human cytogenetic nomenclature

**KMT2A**: Histone-lysine N-methyltransferase 2A gene

**LTFU**: Lost to follow up

**MLFS**: Morphological leukaemia free state

**MRC**: Medical research council

**NCBI**: National Centre for Biotechnology information

**NES**: Nuclear export signal

**NGS**: Next generation sequencing

**NHLS**: National Health Laboratory Service

**NLS**: Nuclear localization signal

**NPM1**: Nucleophosmin 1 gene

**NRM/M**: Non-relapse related morbidity/mortality

**OS**: Overall survival

**PB**: Peripheral blood

**PCR**: Polymerase chain reaction
**PML/RARA:** Promyelocytic leukaemia/Retinoic acid receptor alpha (fusion gene/transcript)

**RFS:** Relapse free survival

**RHD:** Runt homology domain

**RNA:** Ribonucleic acid

**RT-PCR:** Reverse transcription polymerase chain reaction

**RUNX1:** Runt-Related Transcription Factor 1 gene

**RUNX1/RUNX1T1:** Runt-related transcription factor 1/Runt-related transcription factor 1; translocated, 1 (Cyclin D-related) (fusion gene/transcript)

**SA:** South Africa

**SANAS:** South African National Accreditation System

**SCT:** Stem cell transplantation

**Sm:** Single mutant

**TP53:** Tumour protein P53

**UCT:** University of Cape Town

**WHO:** World Health Organization

**WT:** Wild type
Part A: Literature Review

1 CLINICAL SIGNIFICANCE OF GENETIC TESTING IN CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKAEMIA – A BRIEF REVIEW

1.1 ACUTE MYELOID LEUKAEMIA

Acute myeloid leukaemia (AML) is a malignant clonal disorder characterized by impaired differentiation and enhanced proliferation of myeloid progenitors, with subsequent accumulation of these immature blasts in the peripheral blood and bone marrow. It is broadly accepted that without intensive therapy the disease follows an inevitably progressive and fatal course.

The World Health Organization (WHO) and European Leukaemia Net (ELN) guidelines are widely used in the diagnosis of AML. They stipulate the use of a combination of the following laboratory techniques for this purpose: peripheral blood and bone marrow morphological analysis, cytochemistry, immunophenotyping (multi-parameter flow cytometry and immunohistochemistry), cytogenetic analysis and molecular techniques.

AML is genetically heterogeneous in nature and its similarly diverse treatment responses have been shown to correlate with certain genetic findings. Definitive AML treatment modalities include chemotherapy, autologous stem cell transplantation (auto-SCT) and allogeneic stem cell transplantation (allo-SCT). Each modality varies with regards to its anti-leukemic potency, risk of non-relapse related morbidity/mortality (NRM/M), financial expense and resource dependence. This variation between treatment modalities necessitates their selection using a risk-adapted approach in order to optimize clinical outcomes whilst mitigating NRM/M in a resource effective manner.

The most recent ELN guidelines (2017) highlight the use of cytogenetic and molecular techniques in order to risk stratify patients into favourable, intermediate and adverse prognostic groups. As mentioned above, this stratification is essential in order to inform the choice of an appropriate consolidation treatment strategy for both the individual and the institution.

1.2 HISTORY OF CYTOGENETIC TESTING TECHNIQUES IN AML

In 1960 the first link between genetic abnormalities and leukemogenesis was shown by Peter Nowell when he discovered the Philadelphia chromosome, the hallmark of chronic myelogenous leukaemia. Since then, extensive research has been undertaken into identifying specific genetic abnormalities in different myeloid neoplasms, and determining their role in leukemogenesis, treatment and follow-up.

The most common genetic techniques used in haematological diagnostics can broadly be divided into two main groups: cytogenetic techniques and molecular techniques involving polymerase chain reaction (PCR).

The field of cytogenetics was developed in the first half of the 20th century and encompasses the analysis of the genetic material of a cell at a chromosomal level looking for large-scale mutations such as chromosomal number changes, re-arrangements, large insertions and large deletions.
most widely used cytogenetic techniques include conventional karyotyping (G-banding) and fluorescence in situ hybridization (FISH).

Conventional karyotyping involves culturing live cells of interest, chemically halting their mitotic division during metaphase, staining their heterochromatin with a Giemsa stain (which stains the AT rich areas) and analysing the chromosomes under high power microscopy. The unique staining patterns of the chromosomes allows the cytogeneticist to evaluate the entire karyotype for large scale mutations. Some significant drawbacks of conventional karyotyping include the requirement for actively dividing cells (in order for culturing to occur), resolution constraints which may result in failed identification of smaller cryptic re-arrangements, the substantial resource requirement (including time, skilled staff and specialized equipment) and the limited number of nuclei that can be analysed.

The introduction of FISH in the 1980’s was lauded as further evolution in cytogenetic analysis. The technique utilizes fluorescently labelled probes to detect specific nucleic acid sequences within morphologically preserved chromosomes. This more targeted approach allows for more nuclei to be analysed (usually 200) in the same amount of time as karyotyping, thus improving the sensitivity from 5% (karyotyping) to 0.5% (FISH). The specific molecular design of the probes and higher resolution end point analysis techniques used by FISH also result in greater specificity which may accordingly allow for the identification of cryptic re-arrangements (i.e those not identified by karyotyping). FISH is also less labour intensive and does not require actively dividing cells, thus allowing for the identification of mutations in a wider range of cell types (terminally differentiated cells). However, FISH only provides information on the targeted abnormality as opposed to the whole karyotype and has limited utility in identifying small genetic (gene-specific) abnormalities, which is why it is not usually used as a standalone technique in AML genetic diagnostics.

In the studies of Grimwade et al., cohorts of AML patients were retrospectively analysed to link cytogenetic findings to clinical outcomes, including complete remission (CR) rates, relapse risk and overall survival (OS). Patients were consequently divided into 3 cytogenetic risk groups, namely; favourable, intermediate and adverse. Further study of these groups led to the development of a cytogenetic risk stratification system known as the original Medical Research Council classification (MRC-C) system, which was subsequently revised in 2010 (revised MRC-C) (see Table 1 for both). The original MRC-C provided evidence supporting the use of cytogenetic findings as the cornerstone for AML risk stratification. Consequently, it offered a much needed standardized, objective and evidence-based guide for clinicians to make critical consolidation therapy decisions regarding the appropriateness, timing and nature of stem cell transplants in a patient’s first complete remission (CR1).
Although the global use of both MRC cytogenetic risk stratification systems has had a notably positive impact on therapy decisions and subsequent clinical outcomes in AML, unfortunately a significant number of AML cases cannot be stratified as they lack any cytogenetic aberrations (i.e. have a normal karyotype without evidence of abnormal FISH analysis). These cases are known as cytogenetically normal AML (CN-AML). According to the Association for Clinical Cytogenetics (ACC), a “normal karyotype” in AML requires ≥10 fully analysed metaphases via conventional karyotyping. If <10 metaphases are analysed, it should be regarded as a failed analysis.

Data from Europe and the United States of America show CN-AML frequencies of up to 47% in adults with higher rates in developing countries such as Brazil and India (see Table 2). However, surprisingly, data from a study by Marshall et al suggests a significantly lower CN-AML frequency in South Africa. To date, this study is the only one to describe CN-AML frequencies in a South African population. This overall remarkable lack of South African data coupled with the fact that this study was a single centre study performed on a relatively small (n = 160, with notable further fallout due to failed karyotyping) and ethnically unique population (predominantly Black African patients, 71.25%; n = 114/160) may explain the comparatively lower CN-AML frequencies they found. Further studies are therefore required to investigate these findings.

### 1.3 Cyto malignant normal AML

<table>
<thead>
<tr>
<th>Risk Category</th>
<th>Genetic abnormality</th>
<th>Revised MRC-C (2010)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Favourable</td>
<td>t(15;17)(q22;q21)</td>
<td>t(15;17)(q22;q21)</td>
</tr>
<tr>
<td></td>
<td>t(8;21)(q22;q22)</td>
<td>t(8;21)(q22;q22)</td>
</tr>
<tr>
<td></td>
<td>inv(16)(p13q22)</td>
<td>inv(16)(p13q22)/t(16;16)(p13;q22)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Normal</td>
<td>Entities not classified as favourable or adverse</td>
</tr>
<tr>
<td></td>
<td>+8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>del(7q)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>del(9q)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abnormal 11q23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>All other structural/numerical abnormalities</td>
<td></td>
</tr>
<tr>
<td>Adverse</td>
<td>-5</td>
<td>abn(3q) [excluding t(3;5)(q21/25;q31/35)]</td>
</tr>
<tr>
<td></td>
<td>-7</td>
<td>inv(3)(q21q26)</td>
</tr>
<tr>
<td></td>
<td>Del(5q)</td>
<td>t(3;3)(q21;q26)</td>
</tr>
<tr>
<td></td>
<td>Abnormal 3q</td>
<td>add(5q), del(5q), -5</td>
</tr>
<tr>
<td></td>
<td>Complex karyotype</td>
<td>-7, add(7q)/del(7q) [Excluding cases with favourable karyotype]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t(6;11)(q27;q23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t(10;11)(p11/13;q23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t(11q23) [Excluding t(9;11)(p21/22;q23) and t(11;19)(q23;p13)]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t(9;22)(q34;q11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-17/abn(17p)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complex (&gt;3 unrelated abnormalities)</td>
</tr>
</tbody>
</table>
CN-AML is a particularly challenging disease to prognosticate for the purpose of clinical decision making, not only because it per definition lacks any clinically informative cytogenetic abnormalities, but also due to its highly variable clinical features, molecular landscape and treatment outcomes. However, Schlenk et al first addressed this challenge by demonstrating variable survival rates associated with different gene-specific mutations (nucleophosmin \((NPM1)\), fms-like tyrosine kinase \((FLT3)\) and \(CCAAT/enhancer binding protein alpha \((CEBP\alpha)\)) within a cohort of 872 adults with CN-AML. These correlations thus verified the utility of certain molecular assays for the purpose of risk stratification and subsequent therapy decisions in the context of CN-AML.

The three most studied gene-specific mutations in CN-AML are those of the \(NPM1\), \(FLT3\) and \(CEBP\alpha\) genes. The prognostic impact of these mutations was shown by Taskesen et al using survival statistics from a large cohort of 1182 adults with CN-AML (see Figure 1). Additional studies have mirrored these findings and the prognostic significance of these 3 mutations is now universally acknowledged resulting in their inclusion in the ELN 2010 and subsequent 2017 genetic risk stratification guidelines (see Table 3).

Beyond these mutations, three additional gene-specific mutations have been shown to potentially be of prognostic significance in CN-AML, namely those of the runt-related transcription factor 1 \((RUNX1)\), additional sex combs like transcriptional regulator 1 \((ASXL1)\) and tumour protein p53 \((TP53)\) genes.
The consequent incorporation of these six prognostically informative gene-specific mutations into both the WHO 2016 AML classification system\(^2\) (see Figure 2) and ELN 2017 genetic stratification system\(^3\) (see Table 3), emphasizes the rapid progression in the area and application of genetic techniques for diagnostic and treatment purposes in CN-AML over the past 10 years. The biological and clinical significance of these six mutations in CN-AML will be discussed in detail in the section below.

### Table 3. ELN 2010 and 2017 genetic risk stratification systems

<table>
<thead>
<tr>
<th>Risk Category</th>
<th>ELN 2010 genetic risk stratification system(^2)</th>
<th>ELN 2017 genetic risk stratification system(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Favourable</strong></td>
<td>t(8;21)(q22;q22); RUNX1-RUNX1T1</td>
<td>t(8;21)(q22;q22.1); RUNX1-RUNX1T1</td>
</tr>
<tr>
<td></td>
<td>inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11</td>
<td>inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11</td>
</tr>
<tr>
<td></td>
<td>Mutated NPM1 without FLT3-ITD (normal karyotype)</td>
<td>Mutated NPM1 without FLT3-ITD or with FLT3-ITD(^{low})</td>
</tr>
<tr>
<td></td>
<td>Mutated CEBPα (normal karyotype)</td>
<td>Biallelic mutated CEBPα</td>
</tr>
<tr>
<td><strong>Intermediate I:</strong></td>
<td>Mutated NPM1 and FLT3-ITD (normal karyotype)</td>
<td>Mutated NPM1 and FLT3-ITD(^{high})</td>
</tr>
<tr>
<td></td>
<td>Wild-type NPM1 and FLT3-ITD (normal karyotype)</td>
<td>Wild-type NPM1 without FLT3-ITD or with FLT3-ITD(^{low}) (without adverse-risk genetic lesions)</td>
</tr>
<tr>
<td></td>
<td>Wild-type NPM1 without FLT3-ITD (normal karyotype)</td>
<td>t(9;11)(p21.3;q23.3); MLLT3-KMT2A(^{A})</td>
</tr>
<tr>
<td><strong>Intermediate II:</strong></td>
<td>t(9;11)(p22;q23); MLLT3-MLL</td>
<td>Cytogenetic abnormalities not classified as favourable or adverse</td>
</tr>
<tr>
<td></td>
<td>Cytogenetic abnormalities not classified as favourable or adverse</td>
<td></td>
</tr>
<tr>
<td><strong>Adverse</strong></td>
<td>inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1</td>
<td>t(6;9)(p23;q34.1); DEK-NUP214</td>
</tr>
<tr>
<td></td>
<td>t(6;9)(p23;q34); DEK-NUP214</td>
<td>t(6;9)(p23;q34.1); KMT2A rearranged</td>
</tr>
<tr>
<td></td>
<td>t(11)(v;v;q23); MLL rearranged</td>
<td>t(9;22)(q34.1;11.2); BCR-ABL1</td>
</tr>
<tr>
<td></td>
<td>-5 or del(5q); -7; abnl(17p); complex karyotype</td>
<td>inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−5 or del(5q); −7; −17/abn(17p)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complex karyotype(^{b}), monosomal karyotype(^{c})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wild-type NPM1 and FLT3-ITD(^{high})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutated RUNX1(^{d})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutated ASXL1(^{e})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutated TP53(^{g})</td>
</tr>
</tbody>
</table>

- \(^{a}\)Low, low allelic ratio (<0.5); \(^b\)high, high allelic ratio (≥0.5)
- \(^{c}\)The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations.
- \(^{d}\)Three or more unrelated chromosome abnormalities in the absence of 1 of the WHO-designated recurring translocations or inversions
- \(^{e}\)Defined by the presence of 1 single monosomy (excluding loss of X or Y) in association with at least 1 additional monosomy or structural chromosome abnormality (excluding core-binding factor AML)
- \(^{f}\)These markers should not be used as an adverse prognostic marker if they co-occur with favourable-risk AML subtypes.
- \(^{g}\)TP53 mutations are significantly associated with AML with complex and monosomal karyotype
1.4 **PROGNOSTICALLY SIGNIFICANT MOLECULAR MUTATIONS IN CN-AML**

1.4.1 *FLT3* mutations

The *FLT3* gene is found on the long arm of chromosome 13 (13q12.2) and codes for a receptor tyrosine kinase which is normally expressed by haematopoietic stem cells (HSC). Binding of its cognate ligand, cytokine FLT3 ligand, results in auto-phosphorylation of amino acids (aa) in its intracellular domain and consequential signal transduction via protein phosphorylation, promoting the survival, proliferation and differentiation of HSC.

*FLT3* mutations are typically classified into 2 categories (see Figure 3 for schematic illustration): (1) **Internal tandem duplication (ITD)** mutations of 3-300bp (always in-frame), which occur within or near the juxta-membrane (JM) domain of the receptor and are seen in up to 23% of adult AML cases. **FLT3-ITD** mutations cause constitutive activation of the tyrosine kinase in the absence of its cognate ligand, resulting in inappropriate and sustained pathway activation;
Point mutations, which result in single aa substitutions in the activation loop of the tyrosine kinase domain (TKD) are seen in only 7% of adult AML.\textsuperscript{36,37} \textit{FLT3-TKD} mutations have no prognostic/risk stratification significance in CN-AML as yet and will not be discussed further.

\textit{FLT3-ITD} mutations can be seen in up to one-third of CN-AML cases (see Table 4),\textsuperscript{39} with several large retrospective European studies indicating that the presence of a \textit{FLT3-ITD} mutation portends a more adverse prognosis specifically in CN-AML.\textsuperscript{16,39–41} The degree of prognostic adversity depends on both the \textit{FLT3-ITD} allelic ratio (AR) as well as the concurrent \textit{NPM1} mutation status (explained in section 1.4.2).\textsuperscript{3,42,43} \textit{FLT3-ITD} mutations are usually detected using standard PCR followed by capillary electrophoresis, with the AR calculated as the ratio of the area under the peaks of the mutant and wild-type amplicons (\textit{FLT3-ITD}/\textit{FLT3-wt}). The ELN 2017 guidelines\textsuperscript{3} suggest that a low \textit{FLT3-ITD} AR (<0.5) is associated with a better prognosis (favourable/intermediate risk) as opposed to a high AR (\geq 0.51) (intermediate/adverse risk). This prognostic significance has resulted in both the \textit{FLT3-ITD} mutation status and AR being incorporated into the ELN’s 2017 genetic risk stratification system (see Table 3).\textsuperscript{3}

While a strong positive correlation has been demonstrated between \textit{FLT3-ITD} and \textit{NPM1} mutations in CN-AML,\textsuperscript{25,39,44,45} there appears to be a strong negative correlation between \textit{FLT3-ITD} and two other prognostically significant mutations in CN-AML, namely; \textit{ASXL1}\textsuperscript{46} and \textit{TP53} mutations.\textsuperscript{47} Moreover, the favourable prognosis associated with \textit{CEBPα} double mutations (\textit{CEBPaα}) (discussed in section 1.4.3) is negated by the presence of a \textit{FLT3-ITD} mutation.\textsuperscript{40} Practically, this means that testing for \textit{CEBPα}, \textit{ASXL1} and \textit{TP53} mutations in \textit{FLT3-ITD} mutated CN-AML is of little to no prognostic benefit. Therefore, \textit{FLT3-ITD} mutation analysis also has the ability to guide further molecular testing in CN-AML, which is of particular benefit in a resource and financially limited setting such as South African state hospitals.

In addition to the frequency of CN-AML in South Africa, Marshall et al\textsuperscript{24} have also published the only local data on \textit{FLT3-ITD} mutations in adult CN-AML. Their data suggest a lower frequency of \textit{FLT3-ITD} mutations compared to European data, but on par with some other countries (see Table 4). This frequency needs to be studied in larger local populations before it can be confirmed and potential reasons for it explored.
Table 4. Frequencies of FLT3 mutations in CN-AML in different geographic populations

<table>
<thead>
<tr>
<th>Data origin</th>
<th>FLT3 mutations in CN-AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td>32.3%39</td>
</tr>
<tr>
<td>USA</td>
<td>18.3%48</td>
</tr>
<tr>
<td>India</td>
<td>10.7%23</td>
</tr>
<tr>
<td>Brazil</td>
<td>16.1%49</td>
</tr>
<tr>
<td>South Africa</td>
<td>17.6%24</td>
</tr>
</tbody>
</table>

1.4.2 NPM1 mutations

The NPM1 gene is found on the long arm of chromosome 5 (5q35.1) and codes for a nucleolar protein which shuttles between the nucleolus, nucleus and cytoplasm as it has structural domains allowing for both nuclear and cytoplasmic localization.\(^{50}\) The primary NPM1 functions, that have been described thus far, include ribosome biogenesis, maintenance of genomic stability, regulation of the TP53-dependent stress response and modulation of growth suppressive pathways.\(^{51}\)

The vast majority (98%) of NPM1 mutations in AML are insertions/deletions in exon 12 causing a net gain of 4bp, resulting in aa frame shifts and the generation of a novel C-terminal domain.\(^{41,44}\) Accordingly, six major variants of NPM1 mutations (Types A-F) have been described (see Table 5).\(^{50}\) Type A mutations are by far the most common (roughly 80% of cases) and describe a duplication mutation of TCTG (956 – 959bp) resulting in a frame shift causing the last 7 aa to be replaced by 11 alternate ones.\(^{44,52}\) While the base changes may vary between subtypes, the end result is the loss of 1 or 2 key tryptophan (W) residues and the generation of a novel C-terminal domain (aa VSLRK).

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>Sequence</th>
<th>Predicted protein</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>GATCTCTG........GCAGT....GGAGGAAGTCTCTTTAAGAAAATAG</td>
<td>DLWQWRKSL</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>GATCTCTGTCTGCGAGT....GGAGGAAGTCTCTTTAAGAAAATAG</td>
<td>DLCLAVEE VSLRK</td>
<td>72-80.3%</td>
</tr>
<tr>
<td>B</td>
<td>GATCTCTGCTTCGAGT....GGAGGAAGTCTCTTTAAGAAAATAG</td>
<td>DLCAVVE VSLRK</td>
<td>8-12%</td>
</tr>
<tr>
<td>C</td>
<td>GATCTCTGGCGTGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG</td>
<td>DLCAVVE VSLRK</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>D</td>
<td>GATCTCTGCTCGCGAGT....GGAGGAAGTCTCTTTAAGAAAATAG</td>
<td>DLCAVVE VSLRK</td>
<td>3.1-7%</td>
</tr>
<tr>
<td>E</td>
<td>GATCTCTG....GCAGTCTCTTCAGCCCGAACGTCTCTTTAAGAAAATAG</td>
<td>DLWQSLAQ VSLRK</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>F</td>
<td>GATCTCTG....GCAGTCTCTGGAGAACGTCTCTTTAAGAAAATAG</td>
<td>DLWQSLK VSLRK</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

Nuclear localization of wild-type NPM1 is determined by the dominance of the nuclear localization signal (NLS) over the nuclear export signal (NES).\(^{53}\) However, mutations of NPM1 result in both the production of an additional leucine-rich NES motif (novel C-terminus), as well as loss of key tryptophan residues in the NLS.\(^{54}\) The resultant reversed balance promotes nuclear export of NPM1, limiting its activity within the nucleus and thus critically altering its function.\(^{54}\) The molecular mechanisms by which this functional change in NPM1 results in leukemogenesis include haplo-insufficiency induced genomic instability,\(^{41,55}\) impaired Arf\(^{56,57}\) and MYC\(^{58}\) dependent cell cycle regulation and retarded caspase dependent apoptotic functioning.\(^{59}\) Despite these numerous leukemogenic mechanisms, studies suggest that an additional cooperative event is required for leukemogenesis.\(^{41,55,60,61}\)
Importantly, NPM1 mutations are considered to be predominantly associated with a normal karyotype, and are consequently observed with a significant frequency of up to 45% in CN-AML (see Table 6). Limited local data shows an unexpectedly low frequency (23.5%) compared to other international studies, which again needs to be confirmed and investigated.

<table>
<thead>
<tr>
<th>Data origin</th>
<th>NPM1 mutations in CN-AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td>45.7%&lt;sup&gt;63&lt;/sup&gt;</td>
</tr>
<tr>
<td>USA</td>
<td>33.5%&lt;sup&gt;64&lt;/sup&gt;</td>
</tr>
<tr>
<td>India</td>
<td>31.51%&lt;sup&gt;65&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brazil</td>
<td>32.7%&lt;sup&gt;49&lt;/sup&gt;</td>
</tr>
<tr>
<td>South Africa</td>
<td>23.5%&lt;sup&gt;24&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NPM1 mutations are known to confer a more favourable prognosis in CN-AML.<sup>43</sup> This favourable prognosis and largely unique biological profile associated with NPM1-mutated AML have earned it its own AML classification category within the WHO 2016 (see Figure 2).<sup>2</sup> However, it has been shown in many studies that the degree of prognostic favourability depends also on the presence and allele burden of a co-occurring FLT3-ITD mutation.<sup>3,42,43</sup> The ELN 2017 guidelines<sup>3</sup> (Table 3) show that the favourable risk stratification associated with an NPM1 mutation is consequently demoted to intermediate risk in the presence of a FLT3-ITD mutation, with a high AR, highlighting the importance of testing for both when risk stratifying a case of CN-AML.

NPM1 and FLT3-ITD mutation assays are fairly easily performed. Both initially entail the extraction of genomic DNA (or total RNA) from a bone marrow or peripheral blood sample, followed by amplification of the genetic area of interest (exon 12 and exons 14-15, respectively) using PCR, and end point assessment by means of size variant analysis using capillary electrophoresis. Sensitivity to 5% mutant alleles is achieved using this technology, which is adequate for AML diagnostics. NPM1 mutation-specific probes can also be used in a real-time PCR approach to allow for greater sensitivity (<0.01%) and minimal residual disease monitoring.<sup>66–68</sup> Testing for both NPM1 and FLT3-ITD mutations according to the ELN 2017 guidelines<sup>3</sup> allows clinicians to prognosticate many cases of CN-AML into favourable, intermediate and adverse risk categories. This enables them to consequently make important decisions regarding consolidation treatment, particularly the appropriate application of allo-SCT. The relative simplicity and affordability of the assays (R500-800 each), coupled with their high mutational frequencies, WHO 2016<sup>2</sup> classification importance, prognostic significance and clinical utility in CN-AML make them both essential tests to perform in all cases of CN-AML.

Of additional practical importance is the fact that NPM1 mutations in CN-AML are considered mutually exclusive of other prognostically significant gene specific mutations, such as those of the CEBPα,<sup>21,62,69</sup> RUNX1,<sup>70</sup> ASXL1,<sup>71–73</sup> and TP53<sup>74</sup> genes. Testing for these specific mutations in NPM1-mutated CN-AML would therefore be of little to no prognostic, financial or resourceful benefit. NPM1 mutation testing can thus also be used to guide further molecular testing for prognostically informative mutations in CN-AML. This is particularly useful in a resource and financially limited setting such as South Africa.

1.4.3 CEBPα mutations

CEBPα is a leucin zipper transcription factor encoded by the intronless single-exon CEBPα gene found on the long arm of chromosome 19 (19q13.1).<sup>75</sup> Its functionality within the haematological system is
restricted to myelomonocytic cells and it is specifically up-regulated during granulocytic differentiation.\textsuperscript{75,76}

\textit{CEBP}\textsubscript{a} mutations are diverse in nature encompassing insertions, deletions and single nucleotide polymorphisms\textsuperscript{77,78}. Despite the majority of mutations clustering in two main hot spots (N-terminus and C-terminal aa), they can occur across the entire coding region (see Figure 4).\textsuperscript{77,78} N-terminal mutations (5' region of the gene) usually introduce a premature stop codon (via a frame shift mechanism), preventing translation of the full length p42 \textit{CEBP}\textsubscript{a} protein and leading to the production of a shorter p30 isoform. This short isoform inhibits the function of the full-length protein through a dominant negative mechanism.\textsuperscript{79} C-terminus mutations (3' region of the gene) are usually in-frame insertions or deletions, which disrupt DNA binding or dimerization functions.\textsuperscript{80} \textit{CEBP}\textsubscript{a} mutations ultimately lead to unbalanced proliferation and differentiation of the affected mutant cell.\textsuperscript{80}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure4.png}
\caption{Schematic illustration displaying \textit{CEBP}\textsubscript{a} mutations locations in AML. (Taken from Su et al.\textsuperscript{77}) (TAD: Transactivating domain)}
\end{figure}

\textit{CEBP}\textsubscript{a} mutations are classified as either single allele mutations (\textit{CEBP}\textsubscript{asm}) or double allele mutations (\textit{CEBP}\textsubscript{adm}). \textit{CEBP}\textsubscript{adm} can occur as a consequence of a homozygous mutation in either the N-terminal or C-terminal encoding region of the gene or as a combination of 2 mutations at both sites within the same cell. Either way, the affected cell does not produce any functional protein, which results in a unique gene expression profile distinguishing these cases from other AML subtypes, including \textit{CEBP}\textsubscript{asm} leukemias.\textsuperscript{81} \textit{CEBP}\textsubscript{adm} are also associated with a more favourable prognosis in CN-AML (see Table 3 and Figure 1.).\textsuperscript{75,81} This biological uniqueness and favourable prognosis has earned \textit{CEBP}\textsubscript{adm} AML recognition as a distinct entity in the 2016 WHO classification (Figure 2)\textsuperscript{2} and \textit{CEBP}\textsubscript{a} mutation testing is strongly recommended in the 2017 ELN diagnostic guidelines (Table 3).\textsuperscript{3}

In a European cohort of 1182 adults with CN-AML, Taskesen et al\textsuperscript{25} demonstrated a \textit{CEBP}\textsubscript{a} mutational frequency of 12.8\% (\textit{CEBP}\textsubscript{asm}: 5\%; \textit{CEBP}\textsubscript{adm}: 9\%). Kappala et al\textsuperscript{82} reported a \textit{CEBP}\textsubscript{adm} mutational frequency of 7.14\% in a South African CN-AML population, however, the cohort was particularly small (n = 14) and they utilized a micro-array only approach to determine cytogenetic and molecular findings.\textsuperscript{82} Additional studies in South Africa are thus required to strengthen these findings.
Much like FLT3-ITD and NPM1 mutation testing, CEBPα mutation analysis is also a PCR based test, however, there are important methodological differences to note. Firstly, the GC rich nature of the CEBPα gene as well as the significant length over which the mutations can occur (see Figure 4) requires it to be divided and amplified as at least two separate fragments. Secondly, the significant heterogeneity of CEBPα mutation types (including single base changes) requires the use of sequencing-based analysis as opposed to the far simpler fragment length analysis methods used for NPM1 and FLT3-ITD mutation testing. For these reasons, CEBPα testing is more expensive and labour intensive.

The difficulties associated with CEBPα testing may be mitigated by the fact that not all patients with CN-AML require CEBPα testing and the potentially limited effects on clinical decision making. As explained above (sections 1.4.1 and 1.4.2) CEBPα testing is of little to no benefit in CN-AML with mutated NPM1 and/or FLT3-ITD. Practically, this implies that a significant proportion of CN-AML cases will not benefit from CEBPα testing, thus saving the costs, resources and labour needed for testing. Additionally, the patients in which CEBPα testing is truly indicated may benefit greatly as it has clear utility in identifying favourable risk patients (CEBPadm) who may not require a high risk and costly allo-SCT in CR1.

Furthermore, by virtue of the fact that single and double allele CEBPα mutations are also considered mutually exclusive of RUNX170 mutations, CEBPα testing also has the potential to guide further testing for RUNX1 mutations.

1.4.4 The adverse risk mutations

1.4.4.1 RUNX1 mutations

RUNX1, also known as Acute Myeloid Leukemia 1 (AML1) or Core-Binding Factor Subunit Alpha-2(CBFA2) is a transcription factor encoded by the RUNX1 gene located on the long arm of chromosome 21 (21q22).³⁰,³⁵ RUNX1 performs its integral orchestrating role in haematopoiesis by interacting with other transcription factors, as well as the promoter regions of multiple genes including those which code for growth factors, surface receptors, signalling molecules, and transcription activators.³⁴

The RUNX1 gene is a frequent target of somatic mutations in myeloid neoplasms. RUNX1 mutations can be broadly classified into two categories, namely; (1) monoallelic chromosomal translocations and (2) mono- or biallelic somatic mutations.³⁵

The most common monoallelic chromosomal translocation in de novo AML involving RUNX1 is t(8;21)(q22;q22). This balanced translocation results in a fusion protein, namely RUNX1-RUNX1T1 and is classified as a separate entity in the WHO 2016.²,³⁵,³⁶

Mono- or biallelic somatic mutations of the RUNX1 gene in CN-AML include insertions, deletions and single nucleotide polymorphisms. The runt homology domain (RHD) encoded over exons 3, 4 and 5 appears to be a mutational hotspot, however, RUNX1 mutations have been shown to occur across all 8 exons in CN-AML (see Figure 5).³⁸ This makes the analysis of these mutations particularly complex, with the most efficient methodology being high through-put sequencing such as next generation sequencing (NGS), which is unfortunately currently an expensive and scarce technology in South Africa.
The frequency of RUNX1 mutations in CN-AML ranges from 6.3 – 13.9%,\textsuperscript{88–90} although the frequency in South Africa is currently unknown. The presence of a RUNX1 mutation in CN-AML is considered to be an unfavourable prognostic indicator as it has been shown to be associated with poorer event free survival (EFS), relapse free survival (RFS) and OS compared to cases of CN-AML with wildtype-RUNX1.\textsuperscript{70,88,90} Gene expression analysis has provided insight into the possibility that RUNX1 mutations may share a distinct biological profile which overlaps with other high risk AML categories, although this has not been comprehensively studied.\textsuperscript{89} This prognostic significance and potential biological uniqueness has led to its inclusion in both the ELN 2017\textsuperscript{3} genetic risk stratification system as an adverse prognostic indicator as well as the WHO 2016\textsuperscript{2} AML classification system as a provisional entity, namely ‘AML with mutated RUNX1.’

1.4.4.2 ASXL1 mutations

ASXL1 is a chromatin binding protein belonging to the polycomb group and trithorax complex family, which is coded by the ASXL1 gene found on the long arm of chromosome 20 (20q11.21). Through chromatin remodeling, the proteins in this family are responsible for both activation and repression of gene transcription in localized areas of the genome.\textsuperscript{72,91,92} The precise molecular role of wild type
ASXL1 in haematopoiesis and how mutated ASXL1 leads to leukemogenesis is still being investigated, however, developing findings suggest that ASXL1 acts as a tumour suppressor protein.93

ASXL1 mutations in myeloid malignancies are either frame-shift or nonsense mutations (creating stop codons) primarily found in exon 12 and are virtually always heterozygous in nature.71 More than 50% of mutations are frameshift mutations due to a duplication of a guanine nucleotide.31,94 Mutations in other exons have been found, however they are rare.95 See Figure 6 below for an illustration of the location and type of mutations in the ASXL1 protein.92

![Figure 6. Distribution of ASXL1 mutations along the protein. (Taken from Gelsi-Boyer et al)](image)

The reported frequency of ASXL1 mutations in CN-AML is 8.9%,46 although the frequency in South Africa is currently unknown. In adult AML, ASXL1 mutations have been associated with poor OS and EFS as well as reduced CR.71,72 Testing is possible by PCR and sequence analysis, but due to the large size of exon 12 several PCR reactions would be required to cover the mutational hot-spot adequately. This makes it a rather expensive and resource intensive assay and analysis is better suited to NGS methods.

1.4.4.3 TP53 mutations

TP53 is a tumour-suppressor protein coded by the TP53 gene found on the short arm of chromosome 17 (17p13.1). TP53 plays an integral role in maintaining genomic stability in response to DNA damage, by either inducing apoptosis or arresting the cell cycle and initiating DNA repair mechanisms.96 It is the loss of genomic stability associated with TP53 mutations which results in tumorigenesis and
explains why it is the most frequent mutation found in human malignancies, as well as its association with chemotherapy resistance.\textsuperscript{97,98} 

\textit{TP53} mutations are known to occur across all 11 exons of the gene, however, there is a noticeable predilection for the DNA binding domain encoded by exons 5, 6 and 7.\textsuperscript{99} The vast majority (70-80\%) of mutations are missense mutations, however, frame-shift, nonsense and deletion mutations are also well described.\textsuperscript{32,47,98} See Figure 7 below for an illustration of the location and type of mutations in the \textit{TP53} protein.\textsuperscript{99}

![Figure 7. Distribution of TP53 mutations along the protein. (taken from Haferlach et al\textsuperscript{99})](image)

\textit{TP53} mutations occur in AML with a frequency of 6.4 - 11\%.\textsuperscript{32,47,97} Numerous studies have shown that \textit{TP53} mutations are highly associated with complex karyotypes.\textsuperscript{32,47,97,98} In logical contrast, rates of \textit{TP53} mutations in CN-AML are lower and appear to be between 1.8 – 5\%.\textsuperscript{74,97} The frequency of these mutations in South African CN-AML are currently unknown. \textit{TP53} mutated AML is considered to have an adverse prognosis, as multiple studies have shown that the mutation is associated with significantly lower OS- and RFS rates.\textsuperscript{32,47,74,97,98}

Due to the large expanse of DNA over which mutations can occur, NGS is realistically the only suitable methodology to adequately detect these mutations.

\subsection*{1.4.4.4 Practical aspects of RUNX1, ASXL1 and TP53 mutation analysis in CN-AML}

Much like \textit{CEBP\textalpha} mutation analysis (section 1.4.3), the heterogeneity of RUNX1, ASXL1 and TP53 mutations also commands the use of sequencing based assays for analysis (ideally NGS), meaning that they are costly, resource dependent and labour intensive mutations to test for.

Furthermore, RUNX1, ASXL1 and TP53 mutation analysis is currently only indicated in cases of CN-AML which initially stratify as intermediate risk according to their NPM1, FLT3-ITD and \textit{CEBP\textalpha} mutation status. One exception to this are those with mutated NPM1 and FLT3-ITD(high AR) which remain in the intermediate risk category. RUNX1, ASXL1 and TP53 mutation assays are used to identify adverse
risk cases of CN-AML within this group. Identifying such cases is clinically relevant as their associated adverse prognosis would favour an allo-SCT (matched or haplo-identical) in CR1 over less intensive consolidation strategies (such as chemotherapy or auto-SCT) which may be considered in intermediate risk CN-AML patients.3

To justify the introduction of RUNX1, ASXL1 and TP53 mutation testing in CN-AML in a resource limited setting such as South African state hospitals, the cost and resource dependence of the assay would have to be strongly outweighed by both the likelihood of a positive result and the ability to perform an allo-SCT as consolidation therapy in CR1 should either of the mutations be found.

Currently, the previously mentioned mutation prevalence rates of 6.3 – 13.9%, 8.9% and 1.8 – 5% for RUNX1, ASXL1 and TP53 respectively, in CN-AML, indicate that the pre-test probability of a positive result for any of these mutations is low. Novitzky et al.100 also demonstrated in a cohort of 90 transplant eligible South African patients with AML, that following extensive HLA typing and donor searching, only 37 (41%) had a sibling matched donor available and only 32 (35%) eventually underwent a matched allo-SCT.

These important realities clearly call into question the practical relevance of using expensive, labour intensive and resource dependent molecular assays to specifically identify these 3 adverse risk mutations within a resource constrained setting such as South Africa. Further local studies would be essential to justify their introduction.

1.5 RISK STRATIFICATION AND TREATMENT OF CN-AML

1.5.1 Overview of CN-AML risk stratification and treatment

The management of a newly diagnosed CN-AML patient depends on several variables including eligibility for intensive induction therapy, age, genetic risk group and haemopoietic stem cell transplant (HSCT) donor options.3

Intensive chemotherapy has a significant risk of treatment related mortality (TRM) and accordingly, patients with advanced age, poor performance status or significant co-morbidities may not be eligible. Those deemed unfit may instead receive alternative treatment to slow disease progression and improve quality of life.101

Intensive treatment, for eligible patients, is divided into two phases: the induction phase and the subsequent consolidation phase.102 Induction therapy is standard for all AML cases (except acute promyelocytic leukaemia), whereas the choice of post remission therapy (consolidation therapy) is determined by the patient’s risk of relapse, which is currently informed by measurable residual disease monitoring and genetic risk stratification.3

1.5.2 Risk stratification at Groote Schuur Hospital

Measurable residual disease monitoring (by flow cytometric or PCR based methodology) is now a recognized determinant of adverse risk AML and has accordingly been incorporated into the ELN 2017 AML response criteria.3 Nonetheless, its application in terms of treatment time points, assay methodology and data interpretation remain plagued by poor standardization between institutions.103 No PCR based MRD assays for AML are currently available at GSH, except for quantitative reverse
transcription BCR-ABL testing. On the other hand, pre-consolidation flow cytometric MRD monitoring has grown in utility at GSH since its introduction approximately five years ago. However, its unstandardized, uncomprehensive (limited panels used) and unvalidated application means that genetic risk stratification is still largely favoured when consolidation treatment decisions are made.

Current GSH genetic prognostication guidelines (see Table 7) are almost identical to the ELN 2010 guidelines (see Table 3) with two exceptions: firstly, GSH guidelines do not subcategorize cases into 2 separate intermediate subgroups and secondly, the CEBPa mutation status is omitted as this assay has not been available at GSH as a diagnostic test. Table 7 describes both the GSH and ELN 2017 genetic risk stratification systems and Table 8 highlights their divergent clinical application. The risk adapted application of the aforementioned consolidation modalities, according to both the current GSH and ELN 2017 treatment guidelines, will now be further contrasted.
Table 7. Genetic risk stratifications used in current GSH and ELN 2017 guidelines

<table>
<thead>
<tr>
<th>Risk Category</th>
<th>GSH genetic risk stratification system (Based on ELN 2010 genetic risk stratification system&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>ELN 2017 genetic risk stratification system&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Favourable</td>
<td>t(8;21)(q22;q22); RUNX1-RUNX1T1, inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBF8-MYH11</td>
<td>t(8;21)(q22;q22.1); RUNX1-RUNX1T1, inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBF8-MYH11</td>
</tr>
<tr>
<td></td>
<td>Mutated NPM1 without FLT3-ITD (normal karyotype)</td>
<td>Mutated NPM1 without FLT3-ITD or with FLT3-ITD&lt;sup&gt;low†&lt;/sup&gt;, Biallelic mutated CEBPα</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Mutated NPM1 and FLT3-ITD (normal karyotype), Wild-type NPM1 and FLT3-ITD (normal karyotype), Wild-type NPM1 without FLT3-ITD (normal karyotype), t(9;11)(p22;q23); MLLT3-MLL, Cytogenetic abnormalities not classified as favourable or adverse</td>
<td>Mutated NPM1 and FLT3-ITD&lt;sup&gt;high†&lt;/sup&gt;, Wild-type NPM1 without FLT3-ITD or with FLT3-ITD&lt;sup&gt;low†&lt;/sup&gt; (without adverse-risk genetic lesions), t(9;11)(p21.3;q23.3); MLLT3-KMT2A&lt;sup&gt;†&lt;/sup&gt;, Cytogenetic abnormalities not classified as favourable or adverse</td>
</tr>
<tr>
<td>Adverse</td>
<td>inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1, t(6;9)(p23;q34); DEK-NUP214, t(v;11)(v;q23); MLL rearranged, -5 or del(5q); -7; abnl(17p); complex karyotype</td>
<td>t(6;9)(p23;q34.1); DEK-NUP214, t(v;11q23.3); KMT2A rearranged, t(9;22)(q34.1;q11.2); BCR-ABL1, inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1), -5 or del(5q); -7; -17/abnl(17p), Complex karyotype&lt;sup&gt;§&lt;/sup&gt;, monosomal karyotype&lt;sup&gt;†↑&lt;/sup&gt;, Wild-type NPM1 and FLT3-ITD&lt;sup&gt;high†&lt;/sup&gt;, Mutated RUNX1&lt;sup&gt;§&lt;/sup&gt;, Mutated ASXL1&lt;sup&gt;§&lt;/sup&gt;, Mutated TP53&lt;sup&gt;§&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

- <sup>†</sup>Low, low allelic ratio (<0.5); <sup>††</sup>high, high allelic ratio (≥0.5)
- <sup>†</sup>The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations.
- <sup>§</sup>Three or more unrelated chromosome abnormalities in the absence of 1 of the WHO-designated recurring translocations or inversions
- <sup>↑</sup>Defined by the presence of 1 single monosomy (excluding loss of X or Y) in association with at least 1 additional monosomy or structural chromosome abnormality (excluding core-binding factor AML)
- <sup>†</sup>These markers should not be used as an adverse prognostic marker if they co-occur with favourable-risk AML subtypes.
- <sup>§</sup>TP53 mutations are significantly associated with AML with complex and monosomal karyotype.

"..."
1.5.3 **Induction therapy**

Induction therapy is the backbone of all AML management regimens and its intent is to achieve a morphologic leukaemia-free state (MLFS) followed by a CR. Table 8 demonstrates that the specific induction treatment protocol recommended by both the ELN 2017 and current GSH guidelines is standard across all genetic risk groups and consists of a combination of chemotherapeutic agents, including cytarabine (Ara-C), an anthracycline (daunorubicin, idarubicin or mitoxantrone) and etoposide (GSH only).

1.5.4 **Consolidation therapy**

Consolidation therapy follows induction treatment and aims to maintain a CR (i.e. prevent relapse). Therefore, only patients who achieved and retained a CR following induction treatment are eligible for consolidation therapy. Three general consolidation treatment options in CN-AML CR1 are: chemotherapy, auto-SCT and allo-SCT.

Chemotherapeutic consolidation involves the administration of high or intermediate dose cytarabine typically given over several days typically in an in-patient setting. This process is usually repeated for a total of 3-4 cycles over a period of 4-6 months. Patients generally require supportive measures such as blood products, nutritional support and antimicrobials in order to mitigate the myelosuppressive and cytotoxic adverse effects of the chemotherapy. Notable disadvantages of this consolidation method include its strict inapplicability in adverse risk CN-AML as well as its lengthy overall duration. Conversely however, it is generally cheaper than allo-SCT with a superior risk-benefit ratio (i.e NRM/M versus reduction of relapse risk) in favourable risk patients.

Allo-SCT from a matched donor entails the mobilization and infusion of haematopoietic stem cells from a healthy HLA matched donor into the myeloablated patient. The procedure has four general phases:

1. A suitably fully matched donor must be found by HLA typing. Simply, the suitability of a donor is directly proportional to genetic compatibility between numerous HLA genetic loci. The nature of inheritance patterns for HLA haplotypes means that donor searches typically begin with first-degree relatives (matched related donors) and if unsuccessful move to local and international stem cell registries (matched unrelated donors). Once a suitably matched donor has been found, stem cell mobilization and harvesting must occur. Before infusion of the harvested stem cells, myeloablative conditioning of the patient must take place. Myeloablation is a highly toxic procedure which requires hospital admission and extensive supportive care. Following stem cell infusion haematological recovery must occur before discharge and outpatient follow up.

Haplo-identical allo-SCT follows the same procedure described above, however, the acceptable number of matched HLA loci between a related donor and patient is reduced in order to improve the chances of finding a donor. This overall reduced compatibility unfortunately drastically increases the risk of graft-vs-host-disease (GVHD), which in turn increases the risk of NRM/M. It is therefore generally reserved for patients without a fully matched (related or unrelated) donor option.

Despite the well described superior anti-leukemic effects associated with allo-SCT, its high NRM/M coupled with the cost and difficulty of finding appropriate donors means that it can only be applied in certain situations and thus highlights the need for alternative consolidation options.
virtue of these potent anti-leukemic effects, allo-SCT is the first line consolidation therapy option in intermediate risk CN-AML and the only viable consolidation therapy option in adverse risk CN-AML.\textsuperscript{3}

Auto-SCT involves the mobilization, harvesting and post-myeloablative re-infusion of a patient’s own haematopoietic stem cells. Compared to chemotherapeutic consolidation, auto-SCT offers a more condensed treatment with subsequently shorter hospitalization, thus making it the preferred first line consolidation modality in favourable risk CN-AML at GSH (see Table 8). Nevertheless, the financial benefits of reduced hospitalization may be negated by the cost associated with the mobilization, harvesting and infusion of stem cells. Compared to allo-SCT, auto-SCT does not require a donor search (nor the additional associated cost of HLA typing) and its inherently less potent anti-leukemic effects result in much lower rates of NRM/M. Auto-SCT’s reduced toxicity generally shortens hospitalization and reduces long term complications. Conversely, this comparatively weaker anti-leukemic effect means that auto-SCT is strictly inapplicable as a consolidation modality in adverse risk CN-AML. Its clinical utility in CN-AML is therefore restricted to patients with favourable or intermediate risk genetics.\textsuperscript{3,109}

The choice of consolidation treatment in CN-AML CR1 therefore depends primarily upon the specific genetic risk stratification category, as well as HSCT-factors such as the availability of both a donor and the required specialized facilities. Practically, this decision involves weighing up each modality’s probability of maintaining a CR state and its potential for causing NRM/M in a particular patient, in addition to the financial and resource implications.
1.5.5 Treatment of CN-AML: ELN 2017 versus current GSH guidelines

It is evident from Table 7 that there are clear differences between the ELN 2017 and current GSH guidelines regarding the genetic stratification of CN-AML into the respective favourable, intermediate and adverse risk groups.

As discussed above in section 1.5.4, in order to stratify as favourable risk CN-AML, GSH risk stratification guidelines require only *NPM1* and *FLT3-ITD* (excluding AR) mutation analysis, whereas the ELN 2017 guidelines encourage additional molecular testing, including both *FLT3-ITD* AR and *CEBPα* mutation analysis (Table 7). Unfortunately, this simplified GSH approach means that two specific molecular sub-groups of CN-AML patients, namely ‘mutated *NPM1* and *FLT3-ITD* (low AR)’ and...
those with \textit{CEBPa}d\textsubscript{m} mutations are being assigned to the intermediate risk group, rather than the favourable risk group. The potential practical implications of this are twofold. Firstly, it may mean that these specific patients are being considered for consolidation by matched related allo-SCT as opposed to cheaper, less toxic and similarly effective alternatives. Secondly, the use of this simplified strategy may be underestimating the true incidence of favourable risk CN-AML. If this proves to be so, it may encourage the revision of institutional ‘favourable risk CN-AML’ treatment guidelines by considering the potential benefits that chemotherapeutic consolidation may have over the current first line consolidation treatment (auto-SCT).

Of further concern is the fact that patients with ‘wild-type NPM1 and FLT3-ITD (low AR)’, who would be stratified as intermediate risk according to the ELN 2017\textsuperscript{3} are being classified as adverse risk according to current GSH guidelines (Table 7). As mentioned earlier, allo-SCT is strictly recommended in adverse risk patients. Therefore, this disparity has important potential clinical implications, as in the absence of a matched sibling donor such patients would be considered for haplo-identical SCT, rather than for cheaper and less toxic alternatives which would be appropriate in intermediate risk CN-AML (chemotherapy or auto-SCT). Consequently, in these patients, the current GSH guidelines may lead to inappropriate TRM as well as the unnecessary use of resources.

Unlike the ELN 2017 guidelines,\textsuperscript{3} the choice of consolidation treatment in CN-AML at GSH is currently not informed by mutation analysis for \textit{RUNX1}, \textit{ASXL1} and \textit{TP53} mutations. The acceptability of molecular mutation analysis for these three genes in a resource limited setting is contentious, as has been discussed above. However, it is worth noting that by introducing \textit{NPM1}, \textit{FLT3-ITD} and \textit{CEBPa} mutation analysis according to the ELN 2017 GSH guidelines\textsuperscript{3}, the true number of patients who would benefit from further testing for these three mutations could be identified. Should this group be significant in number, it may encourage further investigation into the incidence of these mutations in the local population. This could guide the acceptability of the introduction of their testing into GSH guidelines. Therefore, the more modern and refined ELN 2017 guidelines\textsuperscript{3} highlight both financial and clinical shortcomings in the practical application of the current GSH prognostication system. The addition of \textit{FLT3-ITD} allelic ratio and \textit{CEBPa} analysis (as per these ELN 2017 guidelines\textsuperscript{3}) has the potential to address these issues.

\subsection*{1.6 Conclusion}

Following international studies of large patient cohorts, the utility of genetic techniques (cytogenetic and gene-specific mutations) in the risk stratification of adult AML and particularly CN-AML is well established and rapidly developing.

Genetic techniques are routinely used for the diagnosis of AML in South Africa. However, data on the cytogenetic and molecular profile of AML, and the CN-AML subgroup specifically, in South Africa is limited. To date, the largest South African study on genetic findings in AML was performed by Marshall et al.\textsuperscript{24} on a small Gauteng based cohort (160 patients), which found a significantly lower frequency of CN-AML as well as associated specific genetic mutations (\textit{NPM1} and \textit{FLT-ITD}) compared to larger European studies.

These potential discrepancies are important to investigate. A true lower incidence of these genetic mutations may indicate a unique AML genetic landscape amongst South African patients. An artefactual finding, caused by small patient cohorts due to karyotyping difficulties is also essential to investigate for quality improvement purposes.
From a clinical point of view, the accurate identification of gene specific mutations in CN-AML allows for the risk-adapted application of consolidation therapy. The appropriate choice of consolidation therapy is therefore not only essential for optimal patient outcomes but may also reduce the costs, resource expenditure and NRM/M associated with less appropriate consolidation treatment modalities when patients are not stratified adequately.

Therefore, in order to both define our local CN-AML and reap the much needed benefits of accurate genetic risk stratification, current genetic testing practices need to be reviewed and the applicability of molecular testing must be studied.

1.7 MMED STUDY AIM AND OBJECTIVES

The primary aim is to describe CN-AML in a South African state cohort population according to demographic, pathological and genetic findings as well as basic clinical outcomes. To achieve this aim, the study comprises several objectives.

(1) By describing this largely understudied group (in SA) according to the above variables, we wish to investigate the hypothesis that there are both lower rates of CN-AML, as well as associated FLT3-ITD and NPM1 mutations, in South Africa compared to European populations.

(2) We want to use these molecular findings to audit current clinical and laboratory practises according to institutional guidelines.

(3) Our final objective is to determine the utility of a relatively new, yet globally accepted genetic based prognostication system (ELN 2017) in a South African tertiary institution that currently uses treatment guidelines based on an outdated genetic prognostication system (ELN 2010). In so doing we wish to refine institutional molecular diagnostic and treatment strategies.

This study has the potential to not only provide a much needed clinicopathological description of CN-AML in our demographically unique setting, but it may also enable us to optimize diagnostic, prognostic and treatment strategies in CN-AML within our resource-limited setting.

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Part B: Manuscript

This manuscript was compiled in accordance with the author guidelines stipulated by the American Journal of Hematology (Appendix D).

**Cytogenetically Normal Acute Myeloid Leukaemia at a single centre in South Africa**

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**Running title:** Cytogenetically Normal Acute Myeloid Leukaemia at a single centre in South Africa

**Key words:** Cytogenetically normal acute myeloid leukaemia, CN-AML, European LeukaemiaNet, Risk stratification

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Introduction

Acute myeloid leukaemia (AML) is the most common adult acute leukaemia\(^1\) and is defined as a clonal haematological disease characterized by impaired differentiation and enhanced proliferation of myeloid progenitors, with subsequent accumulation of malignant cells in the peripheral blood (PB) and bone marrow (BM).\(^2\)

AML is a heterogeneous disease in terms of its clinicopathological, genetic and prognostic features. The utility of cytogenetic techniques (conventional karyotyping and fluorescence in situ hybridization (FISH)) for both classifying and prognosticating AML is well established and culminated in the development of both the WHO 2008 AML classification scheme,\(^3\) as well as the 2010 revised MRC prognostication system.\(^4\) The 2010 MRC prognostication system\(^4\) stratified patients into 3 broad groups according to cytogenetic findings, namely; favourable, intermediate and adverse. Risk stratification ultimately allowed for a standardized approach to the choice of consolidation treatment based on the anticipated risk of relapse.

However, cases without an identifiable cytogenetic abnormality, known as cytogenetically normal AML (CN-AML) remained a challenge, as they represented not only a large AML cytogenetic category,\(^5,6\) but are also a biologically and prognostically heterogeneous group.\(^7\) Fortunately, the application of advanced molecular techniques allowed for the identification of gene-specific mutations, which in turn assisted with further sub-classification and risk stratification of CN-AML cases. The significance of these findings is highlighted by the formal incorporation of certain gene-specific mutations into the WHO 2016 AML classification scheme\(^8\) and European Leukaemia Net (ELN) 2010\(^9\) and 2017\(^10\) AML risk stratification systems. The prognostically significant gene-specific mutations currently recognized in the ELN 2017 guidelines\(^10\) include nucleophosmin (NPM1), fms-like tyrosine kinase internal tandem duplication (FLT3-ITD), CCAAT/enhancer binding protein alpha (CEBPα), runt-related transcription factor 1 (RUNX1), additional sex combs like 1 (ASXL1) and tumour protein P53 (TP53) mutations.

South African (SA) data on AML and particularly CN-AML is limited, however, the findings from the largest SA adult AML study to date by Marshall et al.\(^11\) suggest significantly lower frequencies of CN-AML, as well as the associated NPM1 and FLT3-ITD mutations compared to European\(^7,12,13\) and North American cohorts.\(^14–16\) In this retrospective study we aimed to investigate this potential discrepancy by describing an adult CN-AML population in the Western Cape state health care system. We wanted not only to characterize this population according to standard AML clinico-pathological features, but also establish the local relevance of genetic testing in this AML subgroup by reporting mutational frequencies (NPM1, FLT3-ITD and CEBPa mutations), as well as potential difficulties associated with the cytogenetic diagnosis and molecular prognostication of CN-AML in a SA setting.

Furthermore, Groote Schuur Hospital’s (GSH) current AML post-remission treatment is still loosely guided by the now outdated ELN 2010 genetic risk stratification system\(^9\) and differs from that recommended by the ELN 2017 guidelines\(^10\) primarily due to restricted state-approved therapeutics, limited measurable residual disease monitoring options and transplant related hindrances. Consequently, we additionally aimed to investigate the basic clinical outcomes of the CN-AML cohort, to establish the success of the current strategies and determine the exigency and potential utility of a more up-to-date risk stratification system (in line with the ELN 2017 system),\(^10\) within a resource limited hospital setting, such as those experienced in SA and many other developing countries.
Methods

Selection of patients and samples used for DNA analysis

Between January 1st 2005 and December 31st 2018, all adults with newly diagnosed de novo AML (according to WHO criteria), who presented to GSH from Western Cape health care centres were included in this study. Patients with secondary AML (those whose AML diagnosis was preceded by a confirmed diagnosis of a myeloproliferative neoplasm, myelodysplastic syndrome, aplastic anaemia or exposure to chemotherapy and/or radiotherapy) were excluded. As discussed further below, based on cyogenetic (karyotype and/or FISH) findings these patients were subsequently divided into 3 subgroups, namely: ‘clonal cytogenetic abnormalities’, ‘CN-AML’ and ‘cytogenetically unknown’. A retrospective review of laboratory reports and clinical folders was then performed in order to gather all clinico-pathological findings on the CN-AML patients throughout the course of their follow-up. Although the CN-AML patients were our population of interest, a similar review of the ‘cytogenetically unknown’ group was also performed for the sole purpose of determining the reason for their lack of cytogenetic data.

Diagnostic BM DNA from the CN-AML patient cohort was either extracted from stored unstained aspirate slides using the Qiagen mini-blood extraction kit (Qiagen, USA) or retrieved from archived DNA samples, which had been extracted from diagnostic EDTA BM aspirate samples using the Maxwell 16 system (Promega, USA) (National Health Laboratory Service (NHLS), GSH Molecular Haematology Laboratory). Only DNA samples that yielded >25 ng/μl with 260:280 nm ratios of >1.7 were used in the PCR analysis described below. Ethics approval for retrospective access to patient information and additional genetic testing was obtained from the University of Cape Town (UCT) Health Faculty Human Research Ethics committee (HREC. 110/2019).

Genetic analysis

A: Cytogenetically informative tests

Diagnostic G-banding karyotype analysis and FISH testing were conducted at the NHLS/GSH human cytogenetics diagnostic laboratory, using South African National Accreditation System (SANAS) accredited standard procedures. Karyotyping was performed on cultured BM samples in accordance with standards stipulated by the Association for Clinical Cytogenetics (ACC). Karyotypes were reported in agreement with the International System for Human Cytogenetic Nomenclature criteria. At the time of diagnosis, the specific choice of FISH analysis was determined in a case by case fashion, guided by morphology and/or immunophenotyping and included testing for the following rearrangements where appropriate: RUNX1/RUNX1T1, CBFβ/MYH11, BCR/ABL1, PML/RARA (all dual-fusion probes) and KMT2A (break-apart probe). CN-AML was defined as the absence of any karyotypic aberration in ≥10 fully analysed metaphases with no accompanying evidence of an abnormal FISH result.

B: NPM1, FLT3-ITD and CEBPα mutation PCR analysis

For the identified CN-AML patients, NPM1, FLT3-ITD and CEBPα mutation analysis was performed on DNA extracted from diagnostic samples where possible. All cases with available DNA were tested for NPM1 exon 12 mutations and FLT3-ITD mutations in exons 14 and 15. These assays were performed using PCR/capillary electrophoresis methodology capable of detecting all mutation types associated with an overall 4bp insertion in NPM1 (mutation types A-D) and FLT3-ITDs of between 3-300bp. The FLT3-ITD allelic ratio (AR) was calculated as the ratio of the area under the curve of mutant to wild type alleles (FLT3-ITD/FLT3wt). Testing was performed by the NHLS/GSH Molecular Haematology
laboratory, using in-house methodology (see Appendix A) with both assays having a limit of detection of 5% mutated alleles and both being SANAS accredited for diagnostic use.

**CEBPα** mutation analysis was subsequently performed only on cases found to be negative for both **NPM1** and **FLT3-ITD** mutations. Testing was performed according to the PCR/Sanger sequencing methodology described by Behdad et al,\textsuperscript{19} using the recommended reagents and PCR conditions specifically for the GC-rich amplicons. The entire **CEBPα** coding region was analysed and compared to the NCBI reference genomic sequence (NG_012022.1) to identify any mutations. The assay was found to reliably detect **CEBPα** mutations to a sensitivity of 20% allelic load, with peak heights being used to determine single (sm) and double mutant (dm) where appropriate.

**Genetic risk stratification**

Following molecular mutation analysis, genetic risk stratification of patients in accordance with ELN 2017 guidelines\textsuperscript{10} was performed. As mutation analysis of **RUNX1**, **ASXL1** and **TP53** genes was not performed, we categorized our CN-AML cohort into 2 risk groups; favourable and non-favourable, where the non-favourable group represented a combination of both the intermediate and adverse risk categories from the comprehensive ELN 2017 genetic stratification system.\textsuperscript{10}

**Patient therapy**

**A: Induction**

The choice of intensive or non-intensive therapy depended primarily upon each patient’s age (<60 years vs. ≥60 years), performance status and personal choice. All patients who were either unfit for intensive treatment or failed induction therapy were offered hydroxurea, blood products and additional standard care measures (i.e: analgesia, anti-microbials, IV fluid etc.) where appropriate. Furthermore, some of these patients were given supplementary non-intensive chemotherapy such as decitabine, mitoxantrone and combination therapy with both 6-mercaptopurine and methotrexate.

All intensively managed patients were induced with a “3+7” regimen for initial induction therapy (daunorubicin 75 mg/m\textsuperscript{2}/day, cytarabine 100 mg/m\textsuperscript{2}/day and etoposide 100 mg/m\textsuperscript{2}/day). Induction treatment response was assessed by BM aspiration and trephine biopsy performed on days 14 and 28. Response to treatment was defined using the ELN 2017 ‘Response Criteria in AML’.\textsuperscript{10} Patients with a partial remission who were fit for further intensive treatment were offered re-induction salvage therapy with FLAM (fludarabine 30 mg/m\textsuperscript{2}, mitoxantrone 10 mg/m\textsuperscript{2} and cytarabine 2 g/m\textsuperscript{2}). Conversely, patients deemed unfit for further intensive treatment, as well as those with evidence of resistant disease had their intensive therapy discontinued and were offered the aforementioned non-intensive alternatives.

**B: Consolidation**

First-line consolidation therapy for all eligible patients consisted of an additional cycle of intensive chemotherapy followed by stem cell transplantation (SCT). This intensive chemotherapy (for all patients except those re-induced with a salvage regimen) was another single cycle of induction treatment except using a lower dose of Daunorubicin (60 mg/m\textsuperscript{2}/day). Those who achieved a successful induction response with salvage therapy were given an additional cycle as part of their consolidation before proceeding to stem cell transplantation. Two AML genetic prognostication systems were used over the study period to determine the first line choice of SCT for eligible patients, namely; the original MRC (1998)\textsuperscript{5} and ELN 2010 systems.\textsuperscript{9} Favourable risk patients were offered an autologous (auto) SCT in first complete remission (CR1). Intermediate and adverse risk patients were offered an allogeneic (allo) SCT as first-line treatment if a matched sibling donor was available.
Intermediate risk patients without a matched sibling donor had the second line option of an auto-SCT. Finally, consolidation by chemotherapy was offered as a last line therapy to favourable and intermediate risk patients who failed auto-SCT due to unsuccessful stem cell mobilization.

**Determination of clinical outcomes**

CN-AML patients in our cohort were treated according to the therapy strategy detailed above. The clinical end points of interest for this study included induction response and overall survival (OS). ELN 2017 response criteria were used to define induction outcomes. 1) A morphological leukaemia free-state (MLFS) at the day 28 post-induction assessment was considered a successful induction response; 2) Unsuccessful induction responses were classified as either induction death (death related to treatment and/or hypoplasia within 30 days of induction) or resistant disease (failure to eliminate disease, including partial remissions). OS was calculated from the date of the diagnostic BM to the date of death. Patients without definitive dates of death, who were not being treated with curative intent, had their last day of medical follow-up used as their date of death. Patients in remission but lost to follow up (LTFU) were censored on the day of their last medical follow up. A comparative review of patient folders, laboratory results and electronic hospital clerking data was used to determine the most accurate date of last medical follow up for all relevant patients. Surviving patients were censored on 31/12/2019. OS curves were calculated using the Kaplan-Meier survival function and compared by Long Rank test. Fisher’s exact test was used to compare categorical data. A P-value of less than 0.05 was considered statistically significant.
**Results**

**Demographic, clinical and cytogenetic characteristics**

A total of 218 patients with newly diagnosed *de novo* AML were identified during the 13 year assessment period (2005-2018). Of the 218 patients, we found that 154 (71%) had a successful diagnostic karyotype available and 64 (29%) did not, due to either failed karyotyping (23%, n=50) or unrequested karyotyping (6%, n=14). As shown in Figure 1, following further review of all additional FISH results we found that 114 patients (52%) had “clonal cytogenetic abnormalities” with FISH analysis identifying cytogenetic abnormalities in 16 patients who lacked a diagnostic karyotype. Fifty-six patients (26%) were classified as “CN-AML”, with 14 of these also being confirmed with negative FISH for either *RUNX1/RUNX1T1, CBFB/MYH11, KMT2A, PML/RARA, BCR/ABL1* and *FIP1L1/PDGFRA* rearrangements where suspected. The remaining 48 patients (22%) were “cytogenetically unknown” due to either failed karyotyping (with/without additional negative FISH results) (n=36; 17%) or no cytogenetic testing (karyotyping or FISH) requested at diagnosis (n=12; 5%).

![Cytogenetic classification of entire de novo AML cohort (n = 218) according to karyotype and FISH](image)

The relatively high proportion of “cytogenetically unknown” cases was investigated to determine the reasons for the lack of cytogenetic data. The only documented reason for “failed karyotyping” was “no or insufficient metaphases” being cultured due to either poor sampling (i.e. haemodilute specimen) and/or a hypoproliferative specimen. Unfortunately, differentiating between these 2 possibilities, as well as other possible pre-analytical factors (e.g. delivery times) was retrospectively not possible. On the other hand, reasons for “unrequested cytogenetic testing” (karyotyping and FISH) included inadequate bone marrow aspirate sample collection (dry tap) (n=3), therapeutic insignificance due to advanced patient age (n=5), early death (n=1) and unknown reasons (n=3). By excluding the “cytogenetically unknown” cases, a final CN-AML frequency of 33% (n=56/170) was identified. This is the patient cohort which was analysed further.

A median age of 50 years was found for the CN-AML cohort and 44 years for those patients <60 years of age (n=39) at diagnosis. No male or female dominance was observed (50%). Using the French-American-British (FAB) classification system, we identified patients exhibiting characteristics from subtypes M0-M7. Notably, the rates of M0, M1 and M5 FAB subtypes for the entire cohort were...
12.5%, 37.5% and 3.5% respectively. Table 1 demonstrates pre-treatment demographic and clinicopathological features for the CN-AML cohort.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients (n=56)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>50</td>
</tr>
<tr>
<td>Range</td>
<td>17 - 84</td>
</tr>
<tr>
<td><strong>Sex - No. (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>28 (50)</td>
</tr>
<tr>
<td>Females</td>
<td>28 (50)</td>
</tr>
<tr>
<td><strong>White cell count</strong></td>
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</tr>
<tr>
<td>Median — ×10⁹/l</td>
<td>22</td>
</tr>
<tr>
<td>Range — ×10⁹/l</td>
<td>0.66 - 484.1</td>
</tr>
<tr>
<td><strong>Neutrophil count</strong></td>
<td></td>
</tr>
<tr>
<td>Median — ×10⁹/l</td>
<td>1.12</td>
</tr>
<tr>
<td>Range — ×10⁹/l</td>
<td>0.01 - 53.83</td>
</tr>
<tr>
<td><strong>Hemoglobin</strong></td>
<td></td>
</tr>
<tr>
<td>Median — g/dl</td>
<td>7.15</td>
</tr>
<tr>
<td>Range — g/dl</td>
<td>3 - 12.2</td>
</tr>
<tr>
<td><strong>Platelets</strong></td>
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</tr>
<tr>
<td>Median — ×10⁹/l</td>
<td>54.5</td>
</tr>
<tr>
<td>Range — ×10⁹/l</td>
<td>2 - 324</td>
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<tr>
<td><strong>Peripheral-blood blasts</strong></td>
<td></td>
</tr>
<tr>
<td>Median — %</td>
<td>48</td>
</tr>
<tr>
<td>Range — %</td>
<td>0 - 98</td>
</tr>
<tr>
<td><strong>Bone marrow blasts</strong></td>
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<tr>
<td>Median — %</td>
<td>67.6</td>
</tr>
<tr>
<td>Range — %</td>
<td>20 - 96</td>
</tr>
<tr>
<td><strong>French-American-British (FAB) subtype No. (%)</strong></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>7 (12.5)</td>
</tr>
<tr>
<td>M1</td>
<td>21 (37.5)</td>
</tr>
<tr>
<td>M2</td>
<td>13 (23)</td>
</tr>
<tr>
<td>M4</td>
<td>10 (18)</td>
</tr>
<tr>
<td>M5</td>
<td>2 (3.5)</td>
</tr>
<tr>
<td>M6</td>
<td>1 (2)</td>
</tr>
<tr>
<td>M7</td>
<td>2 (3.5)</td>
</tr>
</tbody>
</table>
Risk Stratification

A: Molecular analysis

Due to the introduction of regular molecular testing for CN-AML at GSH only in 2009, some patients in the cohort did not have suitable samples to allow for further molecular stratification. As a result, of the entire CN-AML cohort, 44 patients (78.6%) were assessed for the presence of NPM1 exon 12, FLT3-ITD and CEBPα mutations according to the schematic shown in Figure 2A, with the distribution of the mutations shown in Figure 2B and the frequencies of the clinically relevant mutations presented in Table 2.

NPM1 and FLT3-ITD mutations were found in 39% and 34% of cases respectively. The majority of NPM1 positive patients (71%) had co-occurring FLT3-ITD mutations, with a third (29%) having the favourable prognostic effect of the NPM1 mutations modified by a FLT3-ITD (high AR) mutation. Patients that were negative for both NPM1 and FLT3-ITD mutations (n = 24) were subsequently tested for CEBPα mutations, as the CEBPα mutation status of this specific CN-AML sub-group has been shown to be of prognostic relevance.10,20 As seen in Figure 2B, most patients successfully tested were wild-type for the CEBPα sequence (73%) with a clinically relevant double (or bi-allelic) mutation found in only 2/22 patients.
Figure 2. Molecular results (A) Systematic \textit{NPM1}, \textit{FLT3-ITD} and \textit{CEBP\alpha} testing of CN-AML cases (B) Frequencies and distribution of \textit{NPM1}, \textit{FLT3-ITD} and \textit{CEBP\alpha} mutations
B: ELN risk stratification

Triple mutation testing (NPM1, FLT3-ITD and CEBPα mutation analysis) was successful in 42/44 of patients with available DNA and allowed us to unequivocally risk stratify 50% of these CN-AML patients according to the ELN 2017 guidelines. Details of this stratification are highlighted in Figure 3. Favourable risk genetics were found in 33.3% of patients, with only 2 patients (4.7%) falling definitively into the adverse prognostic group. According to the ELN 2017 guidelines, CN-AML patients that are “NPM1wt/FLT3-ITD negative/CEBPαwt(or sm)” or “NPM1wt/FLT-ITD(low AR)” would be placed in the intermediate risk category only if they additionally test negative for adverse risk genetic mutations, which for CN-AML would include ASXL1, TP53 and RUNX1 (non-translocating mutations). These adverse risk mutations were not investigated in this study due to the lack of sufficient residual DNA for the complex PCR analysis required to comprehensively investigate these genes. We were therefore unable to be certain that this group (21/42) did not contain a few additional adverse risk mutations.

To be able to further investigate the impact of genetic risk stratification on our CN-AML cohort, we chose to risk stratify using a simplified approach (Figure 3) effectively combining the intermediate and adverse risk groups into a single “non-favourable risk” group. This also allowed for larger subgroups for further analysis.

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>Positive cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPM1 Exon 12 (44 cases successfully investigated)</td>
<td>17 (39)</td>
</tr>
<tr>
<td>FLT3-ITD (44 cases successfully investigated)</td>
<td>15 (34)</td>
</tr>
<tr>
<td>Allelic ratio &lt;0.5</td>
<td>8 (18)</td>
</tr>
<tr>
<td>Allelic ratio ≥ 0.5</td>
<td>7 (16)</td>
</tr>
<tr>
<td>CEBPα (22 cases successfully investigated)</td>
<td>6 (27)</td>
</tr>
<tr>
<td>CEBPαsm</td>
<td>4 (18)</td>
</tr>
<tr>
<td>CEBPαdm</td>
<td>2 (9)</td>
</tr>
</tbody>
</table>

Table 2. Frequencies of molecular mutations in CN-AML
Figure 3. Mutational subgroups according to the two risk stratification approaches used.
**Treatment and Survival analyses**

In addition to clinicopathological features and NPM1/FLT3-ITD/CEBPα mutational frequencies, we also sought to investigate basic treatment responses and OS in our CN-AML cohort. As mentioned previously, during the study period (2005-2018), post-remission treatment of CN-AML patients was guided by genetic based risk stratification systems (MRC 5 and ELN 2010), patient performance status, matched stem cell donor availability and stem cell mobilization success. Figure 4 summarizes the treatment pathway followed by patients in our CN-AML cohort specifically.

![Figure 4. Summary of induction and post-remission treatment in the whole CN-AML cohort](image)

**A: Treatment response**

Supplementary Table 1 details the induction and consolidation treatments for the entire CN-AML cohort as well as the three risk categories, namely: ‘favourable’, ‘non-favourable’ and ‘unknown’. Thirty-nine percent (22/56) of all CN-AML patients received non-intensive treatment, with the main reason being advanced age at diagnosis (≥60 years; n=17/22). Of the 58.9% (33/56) who were intensively treated, only a third achieved a successful induction treatment response and could proceed to post-remission therapy (Table S1). The relatively high proportion (60.6%) of patients who failed induction treatment was investigated further to determine the reasons for this (Table S1). It was found that half of these patients died during the induction phase and an equal proportion had resistant disease (Table S1). Table 3 highlights the initial treatment responses between the two stratifiable genetic risk categories (favourable and non-favourable) (n=42). While no statistically significant difference in successful induction responses was seen between these two retrospectively stratified categories (p=0.32) a highly significant difference in both induction death and resistant disease rates was noted (p=0.007) (Table 3).
Regarding the value of additional gene-specific mutation testing, of the 11 stratifiable patients that received CR1 consolidation therapy, 5 patient’s post-remission therapy would have remained unchanged, and at least 3 patients may have been offered alternative post-remission therapies should a triple mutation testing approach (NPM1/FLT3-ITD with AR/CEBPα), in line with ELN 2017 guidelines, have been used to inform treatment at the time. This included therapy choices for 1 patient re-stratified to favourable risk and 2 patients re-stratified to adverse risk. The remaining 3 patient’s consolidation treatment cannot be commented on in this regard as they would require RUNX1/ASXL1/TP53 mutation analysis in order to be conclusively stratified. Relapse and re-treatment statistics were not investigated further due to the small number of patients available for analysis (n=9).

### Supplementary Table 1. Therapy responses in all patients and genetic risk groups (n = 56)

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>All patients (n=56)</th>
<th>Favourable Risk (n=14)</th>
<th>Non-favourable Risk (n=28)</th>
<th>Unknown Risk (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction treatment, no. (%)</td>
<td>33 (58.9)</td>
<td>11 (78.6)</td>
<td>18 (64.3)</td>
<td>4 (28.6)</td>
</tr>
<tr>
<td>Non-intensive treatment, no. (%)</td>
<td>22 (39.3)</td>
<td>3 (21.4)</td>
<td>9 (32.1)</td>
<td>10 (71.4)</td>
</tr>
<tr>
<td>Missing data, no. (%)</td>
<td>1 (1.8)</td>
<td>0</td>
<td>1 (3.6)</td>
<td>0</td>
</tr>
</tbody>
</table>

**Induction Treatment Response (Day 28)**

| Successful, no. (%) | 12 (36.4) | 6 (54.5) | 5 (27.8) | 1 (25) |
| Unsuccessful, no. (%) | 20 (60.6) | 5 (45.5) | 12 (66.7) | 3 (75) |

i) Induction death, no. (%) | 10 (50) | 5 (100) | 3 (25) | 2 (66.6) |

ii) Resistant disease, no (%) | 1 (50) | 0 | 9 (75) | 1 (33.3) |
| Missing data, no. (%) | 1 (3) | 0 | 1 (5.5) | 0 |

**Consolidation Treatment**

| Chemotherapy-only | 3 (25) | 3 (50) | 0 | 0 |
| Re-induction and Auto-SCT in CR1, no. (%) | 7 (58.3) | 2 (33.3) | 4 (80) | 1 (100) |
| Re-induction and Allo-SCT in CR1, no. (%) | 1 (8.3) | 1 (16.7) | 0 | 0 |
| Missing data, no. (%) | 1 (8.3) | 0 | 1 (20) | 0 |

### Table 3. Therapy responses in stratifiable patients (n = 42)

<table>
<thead>
<tr>
<th>Initial Treatment</th>
<th>Favourable Risk (n=14)</th>
<th>Non-favourable Risk (n=28)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction treatment, no. (%)</td>
<td>11 (78.6)</td>
<td>18 (64.3)</td>
<td>0.32</td>
</tr>
<tr>
<td>Non-intensive treatment, no. (%)</td>
<td>3 (21.4)</td>
<td>9 (32.1)</td>
<td>0.007</td>
</tr>
<tr>
<td>Unknown, no. (%)</td>
<td>0</td>
<td>1 (3.6)</td>
<td>0</td>
</tr>
</tbody>
</table>

**Induction Treatment Response (Day 28)**

| Successful, no. (%) | 6 (54.5) | 5 (27.8) |
| Unknown, no (%) | 0 | 1 (5.5) |

Unsuccessful, no (%) | 5 (45.5) | 12 (66.7) | 0.32 |

i) Induction death, no. (%) | 5 (100) | 3 (25) |
| ii) Resistant disease, no (%) | 0 | 9 (75) | 0.007 |
B. Survival analysis

The median follow-up time to determine survival statistics was 77 days (range 1 to 3718 days), with Figures 5A and 5B showing the Kaplan-Meier survival estimates for the entire cohort as well as the 2 separate risk groups (intensively treated patients) respectively. Of the entire cohort, 47/56 patients (83.9%) died during this period; the median overall survival was 77 days (95% CI, 44 to 139) and the 1 and 2-year rates of overall survival were only 24% (95% CI 14-36) and 16% (95% CI, 8 to 27%) respectively. No significant difference in overall survival was found between the intensively treated favourable risk and non-favourable risk groups (p=0.16) (Figure 5B).

Figure 5. Kaplan-Meier survival estimates. (A) All CN-AML patients (n=56). (B) All stratifiable intensively treated CN-AML patients (n=29)
Discussion

CN-AML remains a particular therapeutic challenge, as it not only lacks a common molecular signature but also represents a large AML subgroup with notably heterogeneous clinical outcomes. However, the prognostic importance of specific molecular mutations in CN-AML has come to light and begun to address this issue by informing the application of allo-SCTs in a risk-adapted manner. Unfortunately, the applicability of a molecular based stratification system for CN-AML in SA remains elusive, primarily due to exceedingly limited and seemingly divergent local CN-AML data reported by Marshall et al compared to larger European cohorts and secondly, but perhaps more importantly, the limited availability of comprehensive molecular testing that is required for this stratification. With the intention of better understanding our local CN-AML population and also refining institutional guidelines (diagnostic and therapeutic) we wanted to describe a local CN-AML population according to demographic, clinico-pathological, molecular and basic treatment outcomes and consequently determine the local applicability of a triple mutation testing (NPM1, FLT3-ITD and CEBPa mutations) approach, in line with the ELN 2017 genetic prognostication system. This study represents the largest clinico-pathological, molecular and outcomes based characterization of SA CN-AML patients to date.

The cytogenetic classification of an AML cohort is dependent upon the availability of diagnostic cytogenetic test results, particularly a karyotype. We found a much higher local rate of unknown diagnostic AML karyotypes (29%) compared to those reported in large European cohorts (roughly 16%). Additionally, the rate of failed diagnostic AML karyotyping we found (23%) was more than double that of approximately 10% reported by Fischer et al, Grimwade et al and Löwenberg et al. These findings are important to consider as they demonstrate that karyotyping difficulties (failed and unrequested analyses) contributed to a large fallout of our cytogenetically analysable cases (notably reducing our local cytogenetic sub-group sizes) and highlight a sizeable proportion of our cohort who were ineligible for risk-adapted therapy (informed by karyotype) should intensive treatment have been considered. FISH analysis, although useful, showed limited capacity in addressing this by identifying specific cytogenetic abnormalities in those without a diagnostic karyotype.

We showed that failed karyotyping (23%) contributed more to the lack of karyotype data as opposed to unrequested karyotyping (6%). Failed karyotyping typically occurs due to biological factors (e.g. poorly proliferative capacity of the leukaemic clone) or systematic errors in the pre-analytical/analytical phases of testing. Systematic errors are largely controllable through standard operating procedures, however, pre-analytical issues still occur due to the fact that variables affecting the procurement, quality, storage and transport of specimens are notoriously challenging to standardize. Unrequested karyotyping had a smaller effect on case fallout in our study and was justified in our institution at the time by the therapeutic insignificance of karyotyping in AML patients who had either demised, were unfit for intensive treatment or had a PML/RARA re-arrangement already identified by FISH or RT-PCR. However, given both the academic responsibility of our institution as well as the broadening of effective treatment approaches for patients previously deemed “unfit for intensive therapy”, we have mandated diagnostic karyotyping in all new AML cases. Similarly, the notable number of CN-AML cases we found that had no diagnostic DNA available for further gene-specific molecular testing (21.4%) is troubling, given both the prognostic importance of these mutations, as well as the emergence of targeted treatments (e.g. FLT3 inhibitors) which may be available to SA state patients in the future. Revision of our institutional guidelines and laboratory pre-analytical standard operating procedures is clearly required to focus on timeous delivery of adequate volumes of ‘first pull’ BM aspirate samples (or PB if BM aspirate is not an option) for karyotyping and DNA extraction, in all newly diagnosed AML patients. This may notably reduce the future number of
AML cases without a cyrogenetic and/or molecular profile at GSH and consequently allow for more effective patient management.

No significant differences in median age, sex and cell counts (PB and BM) were observed between our CN-AML cohort and other international cohorts.7,20,25,26 However, noteworthy variances in FAB findings were demonstrated. We found consistently higher rates of M0 and M1 cases and lower rates of M5 cases compared to international CN-AML cohorts.7,25–27 These dissimilarities may be attributed to differences in laboratory diagnostic methodology, cytomorphological judgement28,29 and population ethnicities.30–33

The frequency of CN-AML in our local cohort (33% of AML cases) was similar to European findings, especially those of Grimwade et al (41%)4 and Schlenk et al (45%).7 The frequencies of NPM1 and FLT3-ITD mutations at 39% and 34% respectively, were also akin to findings from numerous large European CN-AML cohorts (approximately 50% and 31% respectively).7,12,13,20 The marginally lower rate of CN-AML we observed may be explained by a difference in local AML driver mutation frequency, a finding which was reported by Blanshard et al,34 who found slightly higher rates of t(8;21)(q22;q22) and t(15;17)(q24;q21) re-arrangements compared to others,4,35 following analysis of the ‘cytogenetically abnormal’ patients in our local AML cohort. Additionally, as was proposed by Marshall et al,11 the comparatively higher proportion of non-Caucasian patients in the SA health care system36,37 may also have contributed to this. Such a hypothesis is supported by Sekeres et al18 who found lower frequencies of CN-AML in African American patients compared to Caucasian Americans. Similarly, ethnic variation in NPM1 mutational frequencies between Caucasians and Asians is described by Rau et al,39 however a lack of such data on African populations does not allow for extrapolation of this observation. Although some data on race was available to us, thorough ethnic characterization of our population was retrospectively not possible and its effect on the rates of CN-AML and NPM1 mutations we found remains undetermined. In contrast to other local findings, our rates of CN-AML and associated NPM1 and FLT3-ITD mutation rates were almost double those reported by Marshall et al (18%, 23.5% and 17.6% respectively).11 We primarily attribute this to the almost 3 fold difference between our cohort sizes, with our larger cohort probably giving a more accurate indication of the rates in this disease group.

Comparing the rate of CEBPa (sm and dm) mutations we found to European CN-AML cohorts20,40–42 would be misleading, primarily because the targeted approach by which we performed CEBPa testing (i.e only in CN-AML patients negative for both NPM1 and FLT3-ITD mutations) differed notably from these European studies who employed CEBPa testing in all CN-AML patients. However, by assuming all NPM1 mutated cases in our analysable cohort (17/44) were CEBPa wild type (based on the well described mutually exclusive relationship between NPM1 and CEBPa mutations in AML14,43–45) we estimate the CEBPa mutation rate in our CN-AML cohort to be 13-25% which is similar to the 8-18% reported in these aforementioned European cohorts.20,40–42

The notable prevalence of these 3 prognostically relevant mutations (NPM1, FLT3-ITD and CEBPa) in our CN-AML cohort indicated their potential use in risk stratification of the CN-AML group. However, this alone does not justify the cost and resources required to test for them, particularly in an immensely cost-restrictive state health care system. The testing costs must be justified by their ability to conclusively risk stratify patients and consequently inform post-remission therapy.

The triple-mutation approach conclusively risk stratified half of the cases in our cohort, with patients categorised into all 3 risk groups (favourable, intermediate and adverse). FLT3-ITD and NPM1 mutation status were most useful in this regard. Although comparatively less impactful, the addition of CEBPa testing allowed for the identification of the remaining 14.7% of favourable risk patients.
These findings showed that a triple testing approach has the potential to inform consolidation treatment in a significant proportion of our local CN-AML patients, being particularly useful in identifying favourable risk patients, who may, as per ELN 2017 treatment guidelines accordingly avoid an allo-SCT in their first complete remission (CR1) provided no evidence of measurable residual disease is present.

Unfortunately, half of our cohort remained inconclusively stratified (potentially being either intermediate or adverse risk) using this approach, which poses a key therapeutic challenge for local CN-AML. ELN 2017 treatment guidelines recommend HLA-matched allo-SCTs as first line consolidation therapy in CR1 for both intermediate and adverse risk patients. However, realistically HLA matched donor options are hard to come by for SA patients due to: low numbers of HLA matched siblings, small sizes of African stem cell registries, as well as profound ethnic (HLA related), logistical and financial obstacles associated with procuring stem cells from established European or North American donor registries. Moreover, state funded SA SCT facilities and SCT resources are also incredibly limited. It is thus vital to consider alternative second line consolidation treatment options for intermediate and adverse risk patients.

By virtue of its potent anti-leukemic effects, haplo-identical (haplo) SCT is strongly advised as a second line consolidation therapy for adverse risk patients. Conversely, cheaper alternative second line consolidation modalities (chemotherapy or auto-SCT) with comparatively lower burdens of non-relapse related mortality and morbidity may be considered ahead of haplo-SCT in intermediate risk patients. Therefore, in order to both optimize clinical outcomes and mitigate the adverse effects (clinical and financial) of inappropriate haplo-SCTs, it is imperative to utilize a risk stratification approach which clearly differentiates between intermediate and adverse risk patients. In this specific regard, the triple-mutation testing approach was not impactful in our cohort as the majority (75%) of patients with non-favourable (intermediate or adverse) risk genetics required further mutation analysis for RUNX1, ASXL1 and TP53 mutations in order to be conclusively stratified at diagnosis. On one hand, international studies suggest particularly low frequencies (<14%) of these 3 mutations in CN-AML, however, such studies are yet to be performed on a local CN-AML cohort. The biological complexity of mutations in these 3 genes (particularly RUNX1 and TP53) means that modern molecular methods such as next generation sequencing (NGS) are more suited to their analysis. However, the accessibility and expense of such technology remains the primary obstacle to its comprehensive introduction in a local state health care setting. A limited introduction (i.e case by case basis) may however be more feasible and worth investigating further specifically in cases which require extensive mutation analysis for the purpose of risk stratification.

We found comparatively dismal CN-AML OS statistics compared to European cohorts studied by both Schlenk et al and Taskesen et al. We primarily attribute this to the significant proportion (30%) of non-intensively treated elderly patients (≥ 60 years) included in our study. The recent growth in both non-intensive and intensive treatment options and approaches for the elderly suggests that OS in this age group might improve considerably should they be adopted locally. The poor OS in our cohort is likely also due to the notably higher rate of unsuccessful induction (due to induction death or resistant disease) that we found compared to these aforementioned European studies, which may be due to delayed presentation, poorer performance status, more co-morbidities and lower socio-economic status amongst SA state patients. No clear reason for the statistically significant difference in induction death rates between the 2 risk groups could be elucidated. These treatment response and survival statistics are concerning and warrant further investigation to identify and address the specific cause/s behind them. In addition to such steps, we also believe that using the ELN 2017 genetic risk stratification system in combination with our already established flow cytometric measurable residual
disease monitoring would greatly assist local clinicians in making post-remission treatment decisions (particularly regarding haplo-SCTs) and would consequently contribute towards improved CN-AML outcomes.

A few limitations of our study must be acknowledged. In addition to the retrospective design and single-institution execution of the study, the cohort size of the intensively treated patients (and its prognostic sub-groups) is notably smaller than many of the European study cohorts referenced and may accordingly account (at least in part) for the highlighted differences in clinical outcomes.

The concerning number of cytogenetically unknown AML cases and CN-AML cases without available DNA that we encountered, emphasizes the need for both revision and better implementation of our institutional cytogenetic and molecular diagnostic guidelines for AML. While the triple-mutation testing was not able to conclusively risk stratify all of the CN-AML, this more cost-friendly approach was capable of definitively stratifying 50% of cases, which could in turn aid in addressing the dismal outcome of patients we observed. Our findings did however, highlight that a significant proportion of non-favourable risk (intermediate or adverse risk) CN-AML patients require additional testing for adverse risk mutations which are better suited to an NGS approach (RUNX1, ASXL1 and TP53 mutations) in order to be definitively stratified for post-remission therapy. We therefore recommend: NPM1 and FLT3-ITD mutation testing in all intensive treatment eligible CN-AML patients, CEBPa testing only in those who are “NPM1wt/FLT3-ITD negative” and NGS testing (for ASXL1, RUNX1 and TP53 mutations) in those who are “NPM1wt/FLT3-ITD negative/CEBPawt(or sm)” or “NPM1wt/FLT-ITD(low AR).” The incorporation of CEBPa and NGS testing in such a case by case manner would be a great step towards introducing evidence based practices in a resource effective manner.

Acknowledgements

This work was possible due to a grant from the National Health Laboratory Service Research Trust. The researcher would like to acknowledge contributions made by Ms Jenna Oosthuizen (statistical data analysis) and the UCT division of human genetics. Finally, the researcher would also like to acknowledge the enormous support and invaluable assistance from study supervisor Associate Professor Karen Shires and co-supervisor Dr Lee-Ann Phillips.

References


Part C: Supporting Documentation

Appendix A: University of Cape Town Human Research Ethics Committee Approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

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Telephone (021) 406 6492
Email: sumayyah.urfeldien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

11 March 2019

HREC REF: 110/2019

Dr L Phillips
Division of Pathology
Haematology Laboratory
ICH Building
Red Cross Children’s Hospital
Rondebosch

Dear Prof Phillips

PROJECT TITLE: ACUTE MYELOID LEUKEMIA IN SOUTH AFRICA: A SINGLE CENTRE RETROSPECTIVE REVIEW (SUB-STUDY: R019/2018)

Thank you for your response letter dated 06 March 2019, addressing the issues raised by the Human Research Ethics Committee (HREC).

The HREC thank you for your thoughtful justification and recognition of limitations of using race as a demographic variable for analysis in this study.

It is a pleasure to inform you that the HREC has formally approved the above-mentioned study.

Approval is granted for one year until the 30 March 2020.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure Form if the study is completed within the approval period.

(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator must obtain appropriate institutional approval, where necessary, before the research may occur.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Appendix B: University of Cape Town Human Research Ethics Committee Approval for extension
Appendix C: Western Cape Department of Health Ethics Approval

Dr. E. Verburgh
Medicine: Haematology
E-mail: Estelle.Verburgh@uct.ac.za

Dear Dr. Verburgh,

RESEARCH PROJECT: DATABASE- Haematology Patient Database

Your recent letter to the hospital refers.

You are granted permission to proceed with your research, which is valid until 30 July 2021.

Please note the following:

a) Your research may not interfere with normal patient care.
b) Hospital staff may not be asked to assist with the research.
c) No additional costs to the hospital should be incurred i.e. Lab, consumables or stationary.
d) No patient folders may be removed from the premises or be inaccessible.
e) Please provide the research assistant/field worker with a copy of this letter as verification of approval.
f) Confidentiality must be maintained at all times.
g) Should you at any time require photographs of your subjects, please obtain the necessary indemnity forms from our Public Relations Office (E45 OMB or ext. 2187/2188).
h) Should you require additional research time beyond the stipulated expiry date, please apply for an extension.
i) Please discuss the study with the HOD before commencing.
j) Please introduce yourself to the person in charge of an area before commencing.
k) On completion of your research, please forward any recommendations/findings that can be beneficial to use to take further action that may inform redevelopment of future policy / review guidelines.
l) Kindly submit a copy of the publication or report to this office on completion of the research.

I would like to wish you every success with the project.

Yours sincerely

[Signature]

DR. BERNADETTE EICK
CHIEF OPERATIONAL OFFICER
Date: 27 August 2018

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The title page should contain the complete title of the manuscript, the names, degrees, and affiliations of all authors, as well as the name, address, phone, fax and email of the person to whom all correspondence should be addressed. The title page should also include the abstract word count, text word count, the number of tables and figures, a short running title, and three to six keywords to index the content.

When submitting the manuscript online, provide the names, affiliations, and email addresses of three preferred reviewers at institutions other than those of the authors.

The total number of words in the text, as well as the number of figures and tables should be listed at the bottom of the title page.

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