Monocyte Analysis: The Secret to CMML?

Jennifer Mills

Objectives

Session Objectives

01

Monocytes 101

Overview of monocytes in health and disease

02

CMML

Overview of CMML and current and historical diagnostic criteria.

03

Monocyte Subsets

Monocyte subsets and their role in disease states.

04

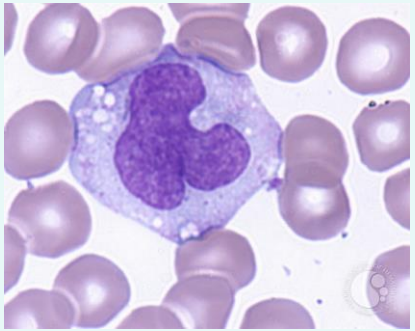
Monocyte Subset Analysis.

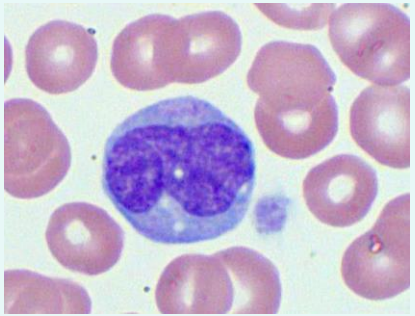
The application of monocyte subsets in CMML diagnosis.

Monocytes 101

Monocytes: Basics

- Peripheral blood leucocyte accounting for ~5% of total nucleated blood cells.
  - **Reference range: 0.2 to 1.0 x10*9/L**
  - Monocytes circulate for up to 3 days before migrating to tissues through interactions with vessel walls.
  - They release cytokines that trigger immunological actions in areas of damage/infection.
  - In tissues they differentiate to macrophages, so act as antigen presenting cells.
  - Key roles in regulate inflammation and activate adaptive responses..
- Post-resolution, monocytes release growth factors and mediators to initiate tissue remodelling.





Monocytes: In disease: Non-Malignant

- Acute and persistent (>3 months) monocytosis can have several possible causes.
- Non-malignant causes include infective and reactive conditions.

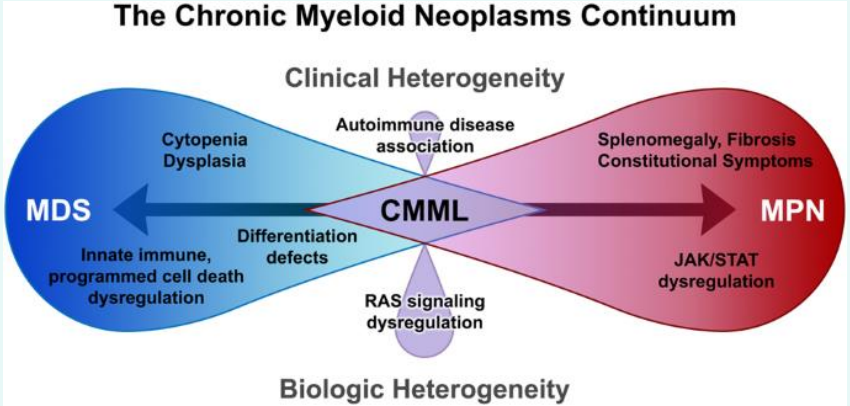
Cause	Subtypes
Infective.	Tuberculosis.
	Viral infection.
	Malaria.
	Subacute bacterial endocarditis.
	Congenital syphilis.
Immune Disorders.	Collagen vasculitis.
	Inflammatory bowel disease.
	Sarcoidosis.
	Immune Thrombocytopenia.
	Chronic neutropenia.

Cause	Subtypes
Myocardial Infarction	N/A
Iatrogenic Causes.	Cytokine therapy.
	Steroids.
	Radiation therapy.
Bone marrow recovery (early sign)	Chemotherapy.
	Bone marrow transplant.
Post splenectomy.	N/A

Chronic Myelomonocytic Leukemia

Chronic Myelomonocytic Leukaemia

- CMML is a clonal haematopoietic disorder, the principal features of which are dysplasia and monocytosis.
  - Classified by the World Health Organisation (WHO) as a Myelodysplastic/Myeloproliferative crossover neoplasm (MDS/MPN).
- **Epidemiology**:
  - Median age of onset is 71-74 years
  - Slight male preponderance
  - Incidence rate of just 4-12.8 cases per 100,000
- CMML is clinically heterogeneous, with dysplastic and proliferative forms, and has a varied clinical course.
  - 15-20% risk of AML transformation



WHO (2016) vs WHO (2022)

CMML

2016 World Health Organization (WHO) recommended diagnostic criteria for chronic myelomonocytic leukemia (CMML)

1	Presence of persistent (>3 months) peripheral blood monocytosis > 1 x10^9/L, with monocytes cor blood cell count differential.
2	Not meeting WHO criteria for *BCR-ABL1* driven chronic myeloid leukemia, essential thrombocy primary myelofibrosis *.
3	No evidence for *PDGFRA* or *PDGFRB* rearrangements, and the absence of *FGFR1* rearrangemer the context of concomitant eosinophilia **.
4	< 20% blasts/blasts equivalent (promonocytes, monoblasts and myeloblasts) in the peripheral blo
5	Dysplasia in one or more myeloid cell lineages. If myelodysplasia is absent or minimal, the diagn made if the other requirements are met and (see point 6)

Requirements for diagnosis
- Pre-requisite criteria must be present in all cases.
- If monocytosis is ≥ 1 × 10^9/L: one or more supporting criteria must be met.
- If monocytosis is ≥0.5 and <1 × 10^9/L: supporting criteria 1 and 2 must be met.

Prerequisite criteria
1. Persistent absolute (≥0.5 × 10^9/L) and relative (≥10%) peripheral blood monocytosis.
2. Blasts constitute <20% of the cells in the peripheral blood and bone marrow.[a]
3. Not meeting diagnostic criteria of chronic myeloid leukaemia or other myeloproliferative neoplasn
4. Not meeting diagnostic criteria of myeloid/lymphoid neoplasms with tyrosine kinase fusions.[c]

Supporting criteria
1. Dysplasia involving ≥1 myeloid lineages.[d]
2. Acquired clonal cytogenetic or molecular abnormality.
3. Abnormal partitioning of peripheral blood monocyte subsets.[e]

CMML Classification 2016 vs. 2022

2016		
Classification	Subtype	Features.
CMML 0-2 (WHO 2008 Morphological Classification)	CMML-0	• <2% Peripheral circulating blasts. • <5% Bone marrow blasts.
	CMML-1	• 2%-4% Peripheral circulating blasts. • 5%-9% Bone marrow blasts.
	CMML-2	• >5% Peripheral circulating blasts. • 10%-19% Bone marrow blasts. • And/or the presence of Auer rods.
Proliferative vs. Dysplastic. (FAB Classification, included in the WHO 2016 classification)	Proliferative	• White Blood Cell Count: ≥13×10^9/L • Leucocytosis/monocytosis. • Hepatosplenomegaly. • Constitutional symptoms. • Higher incidence of RAS pathway mutations.
	Dysplastic	• Peripheral blood cytopenia. • White Blood Cell Count: <13×10^9/L • Symptoms of cytopenia: easy bruising, infections, and transfusion dependence.
Treatment vs. De Novo	Treatment	• Increased cytogenetic abnormalities.
	De novo CMML	• Variable presentation. • More common than T-CMML.

CMML Classification 2016 vs. 2022

2022
Subtyping criteria
- Myelodysplastic CMML (MD-CMML): WBC < 13×10^9/L
- Myeloproliferative CMML (MP-CMML): WBC ≥ 13×10^9/L
Subgrouping criteria (based on percentage of blasts and promonocytes)
CMML-1: <5% in peripheral blood and <10% in bone marrow
CMML-2: 5–19% in peripheral blood and 10-19% in bone marrow

CMML

Current Diagnostic Limitations.

Limitations

Lack of pathognomonic criteria

High dependence on morphology

Genetic Abnormalities lack specificity.

Monocytosis is common finding in malignant (haem and non) and reactive conditions.

Highly subjective, especially relating to blast count (including promonos) and the risk of missing dysplastic change.

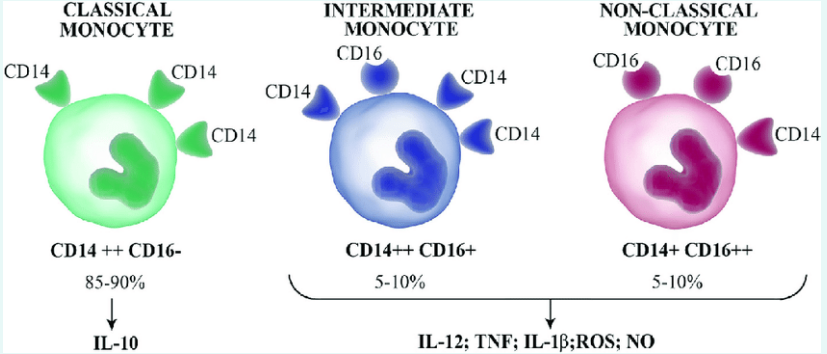
ASXL1 and TET2 etc. are seen across myeloid disorders.

Molecular abnormalities have been seen in elderly patients without any neoplastic disease

Monocyte Subsets

Monocyte Subsets: General

- Since 2010, The Nomenclature Committee of the International Union of Immunological Societies has recognised 3 monocyte subsets: classical (cMo), intermediate (iMo) and nonclassical (ncMo)
  - Monocyte subsets are defined by their expression of CD14 and CD16.
  - These subsets have distinct functional profiles.
- Studies show that intermediate and non-classical monocyte subsets (i.e., CD16+ populations) expand in inflammatory disorders.
  - This illustrates a relationship between CD16+ monocytosis and reactive change.
  - This expansion occurs at the expense of classical monocytes.



Monocyte Subsets: Intermediate

Subtype.	Monocyte %	Phenotype.	Functions
Intermediate	• 5%	• CD14+ • CD16+	• Cytokines: IL-6, IL-8. • Key Function: T-cell stimulation and reactive oxygen species production. • Secondary Function: Stimulation/regulation of angiogenesis.

Subtype	Condition.	Supporting Study.
iMo	**Sepsis.**	One study showed an increase in iMo by 11.2% compared to controls (Poehlmann et al., 2009).
	Crohn's Disease.	iMo was increased 3.7-fold in patients with active Crohn's Disease compared to controls (Grip et al., 2007).
	Rheumatoid Arthritis.	iMos have been shown to increase by 5% in Rheumatoid arthritis compared to healthy controls (Rossol et al., 2011).
	Asthma.	iMos increase with disease severity, though statistical significance was only seen between healthy controls and severe asthmatics (iMo were 12 times higher than controls) (Moniuszko et al., 2009).
	Stroke.	iMos peaked at 48 hours post-stroke and were associated with mortality. (Urra et al., 2009).

Monocyte Subsets: Non-Classical

Subtype.	Monocyte %	Phenotype.	Functions
Non-Classical	• 10%	• $CD14^{dim}$ • CD16+	• Cytokines: TNF-α, IL-1β, IL-6, IL-8. • Key Function: T-cell proliferation and stimulation. • Exhibit “patrolling” behaviour in peripheral blood.

Subtype.	Condition.	Supporting Study.
ncMo	Periodontitis	ncMo were increased in chronic and aggressive cases and chronic periodontitis patients showed a statistically significant increase (Controls: 8.5±1.0%, chronic periodontitis: 13±1.3%) (Nagasawa et al., 2003).
	Coronary Artery Disease (CAD)	ncMos were significantly increased in stable CAD (Controls: 9.5% (2.8–17.2%), CAD Patients: 13.4% (5.2–21.1%)) (Tallone et al., 2011).
iMo and ncMo	Tuberculosis	CD16+ subtypes were significantly increased in tuberculosis patients compared to immunised and non-immunized controls (Castaño et al., 2011).
	Hepatitis B	CD16+ monocyte expansion was increased in cases of active infection and correlated with the level of liver injury when compared to ALT ($r=0.617$, $P<0.001$) (Zhang et al., 2011).
	HIV.	CD16+ subtypes were increased in untreated HIV; however, only iMos were positively correlated with viral load ($P = 0.001$, $r = 0.346$) (Han et al., 2009).

Monocyte Subsets: Classical

- Classical monocytes make up the largest proportion of monocytes in peripheral blood.
- They have no known association between classical monocytes and expansion in reactive disorders.
  - Classical monocytes typically decrease in favour of CD16+ve monocyte expansion in reactive conditions.
- Classical monocytes have been shown to expand in clonal haematopoietic disorders, such as CMML.

Subtype.	Monocyte %	Phenotype.	Key Functions
Classical	• 85%	• CD14+ • CD16−	• Cytokines released: G-CSF, IL-10, CCL2, IL-6, IL-8. • Key function: Phagocytosis.

Subset Analysis and CMML.

Immunophenotyping and CMML.

- CMML is driven by aberrancies within the developmental environment.
  - This results in phenotypic abnormalities that can be exploited.
  - An ideal diagnostic solution would be immunophenotyping to identify a clonal monocyte population

- Possible CMML markers:
  - **CD56**
    - Seen in up to 80% of cases (Hudson, Burack & Bennett, 2018).
    - CD56 can discriminate between CMML and MDS.
    - Overexpression of CD56 is common in many haematological malignancies, including AML.
    - CD56 is seen in 30% of reactive monocytosis cases
  - **CD2.**
    - An insensitive marker with expression only seen in 10-40% of cases (Lacronique-Gazaille et al., 2007; Santos, Franzon & Koga, 2012; Bain & Béné, 2019; Hudson, Burack & Bennett, 2018;).
  - Diminished expression of: **HLA-DR** (reduced in 50% of CMML cases), **CD13, CD15, CD36 and CD64** (Santos, Franzon & Koga, 2012; Hudson, Burack & Bennett, 2018).
    - Similar patterns are seen in MDS and MPNs (Valent et al., 2019).

Monocyte Subsets and CMML

Subset Analysis

- Selimoglu-Buet, et al., published their work on CMML identification using monocyte subset analysis in 2015.
    - **This study showed that classical monocytes expand to >96.6% in CMML compared to 84.0% in age-matched controls.**
- This expansion occurs at the expense of intermediate and nonclassical monocytes, providing a discriminatory between CMML and monocytosis.
- The assay discriminated between MDS and CMML with a sensitivity of 71.76% and a specificity of 86.21%.
- The assay can also differentiate CMML and MPN with monocytosis. (Patnaik et al., 2017b).
    - One study illustrated that 100% of MPN patients have a classical monocyte fraction of <92% (mean 77%) and 93% of CMML patients have a classical fraction of ⩾94% (mean 95.6%).
- Three years after the assay's development, Tarfi et al., (2018) organised a French multicentre validation of the assay.
    - Thirty centres were included, with 329 files analysed.
    - **This study confirmed previous findings, with a sensitivity of 93.6% for CMML in patients with classical monocytes of >94%.**

Monocyte Subset Assay

Selection of "live cells" and exclusion of doublets.
SCC vs FSS
FSC-A vs FSC peak

Rough Selection of Monocytes.
CD45+/SSC^{intermediate}

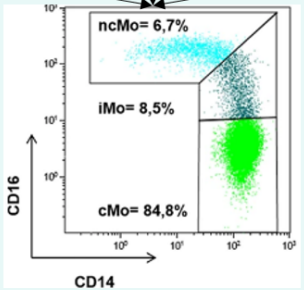
Exclusion of Other Cell Types

- **T-Cells** CD2+/SSC^{low}
- **NK Cells** CD56+/SSC$^{low/int}$
- **B-cells** CD24+/SSC^{low}
- **Basophils/NK Cells** CD14-/CD16-
- **Granulocytes** CD24+/SSC$^{int/high}$ CD16/SSC^{high}

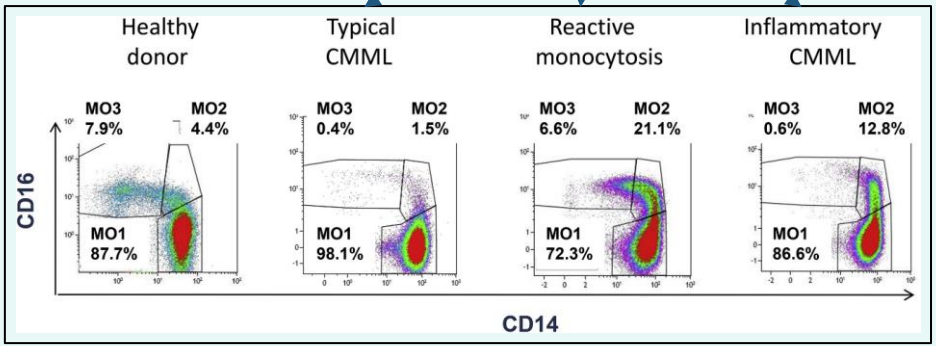
Reassess Monocyte Selection.
CD45+/SSC^{intermediate}

Monocyte Separation.
CD14 vs CD16

- **Classical.** CD14++/CD16-
- **Intermediate.** CD14++/CD16+
- **Nonclassical.** CD14^{dim}-/CD16+



Inflammatory CMML has a characteristic "bulbous" shape. However, expansion of other subsets can result in cMO fractions below the established cut off.



My Project

Subset Analysis

3rd Year Project for Clinical Science (Msc.)

- **Aim : Produce a flow cytometric assay for use in PHUT to differentiate between reactive and malignant monocytosis.**
  a) Identify differences in monocyte subset populations between non-reactive, reactive and CMML patients using the assay developed by Selimoglu-Buet et al. (2015)
  b) Establish sensitivity and specificity of Classical Monocyte percentages to differentiate between reactive and malignant monocyte populations and compare these values to those provided in the literature.

NHS
Portsmouth Hospitals University
NHS Trust

National School of Healthcare Science

Manchester Metropolitan University

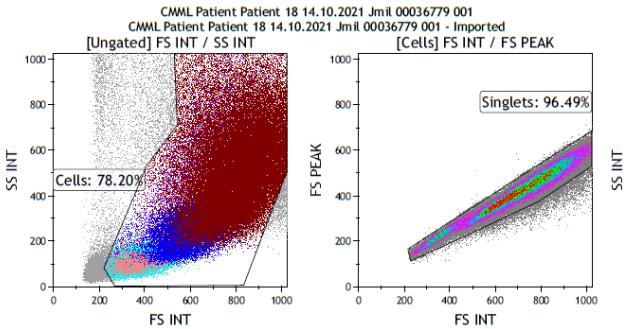
My Project: Set Up

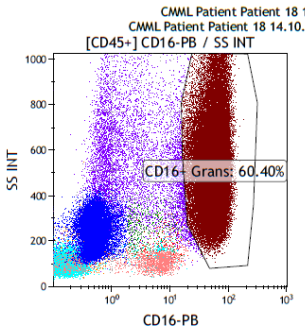
Subset Analysis

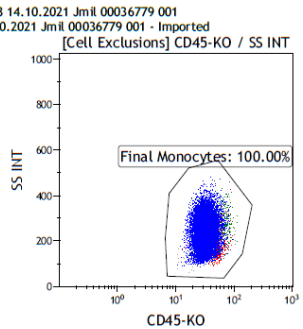
- Assay set up followed original protocol, with adjustments made to match local protcols.
- N=20 Fully anonymised EDTA peripheral blood samples were selected for reactive, normal/nonreactive and CMML patients.
    - Samples later found to not meet the inclusion criteria were removed, as were samples with <10,000 events in the final monocyte gate, as recommended by the multicentre trial (Tarfi *et al.*, 2018).

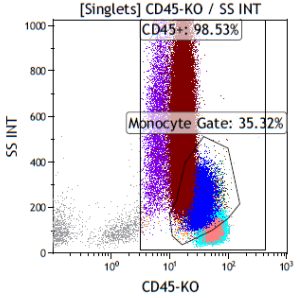
Criteria used to select samples for the three test groups.			
Criteria	Patient Group.		
	Normal/Nonreactive	Reactive	Known CMML
Age (years)	>18		
Gender	A mix of male and female where possible.		
CRP (mg/L)	0-7	>100	N/A
Monocyte Count ($x10^9$/L)	>0.5-≤1.0	>1.0 (for <3 months)	N/A
Clinical History	No significant reactive or haematological clinical history.	Established reactive cause preferable, e.g., sepsis or inflammation	CMML confirmed by haematology e.g., clinic letters/MDT report.

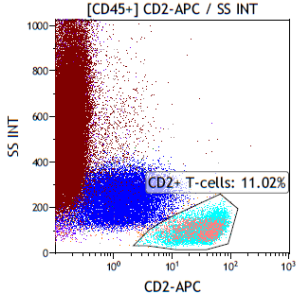
Note that high CRP was not included in previous studies, instead a clinical diagnosis of a reactive condition was used as inclusion criteria.

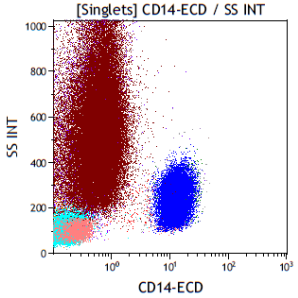


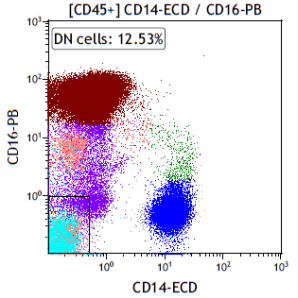


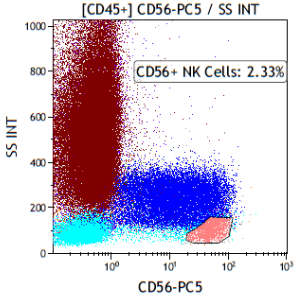


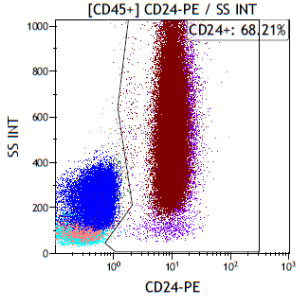


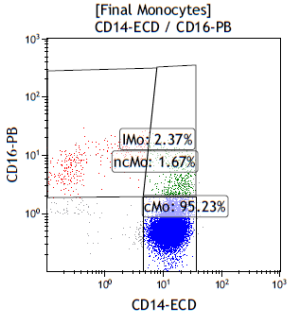


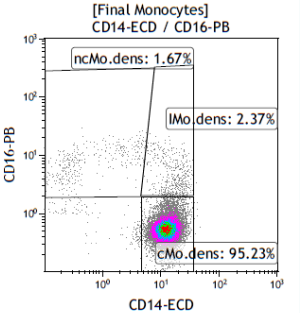


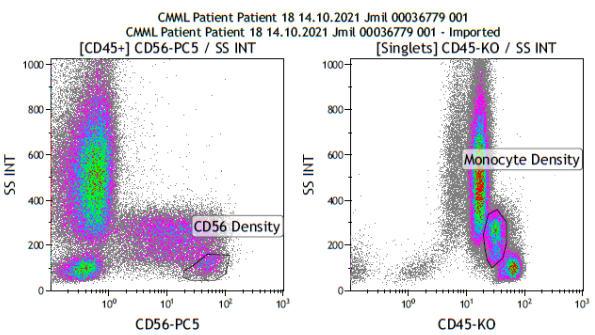










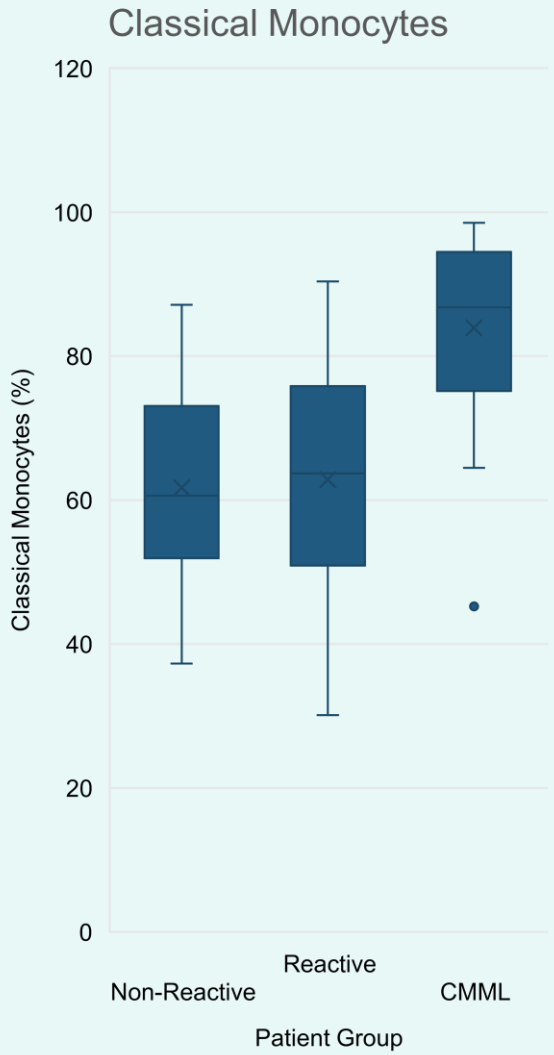


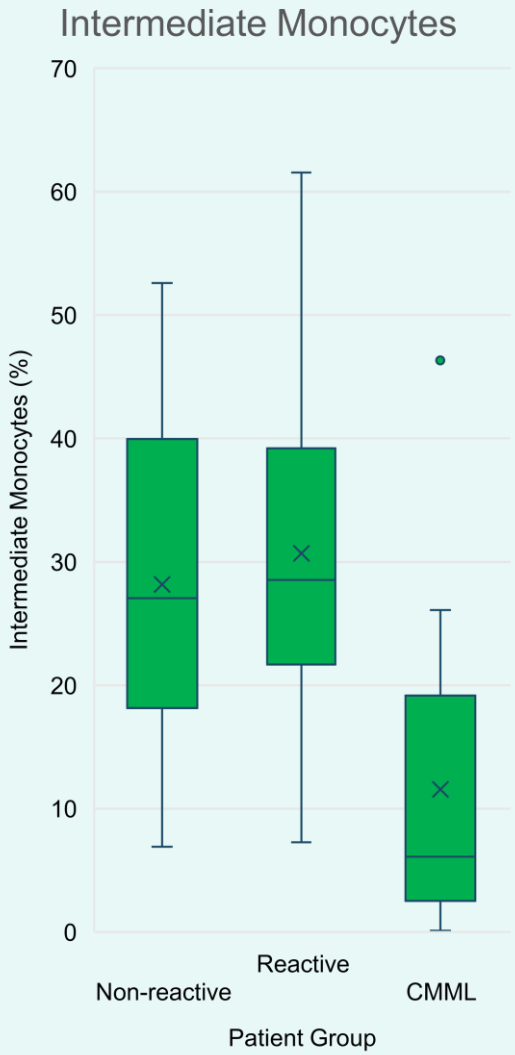
[Final Monocytes] Number	20,003
[cMo] % Gated	95.23
[cMo] CD14-ECD Median	12.98
[IMo] % Gated	2.37
[IMo] CD14-ECD Median	17.41
[ncMo] % Gated	1.67
[ncMo] CD14-ECD Median	0.43

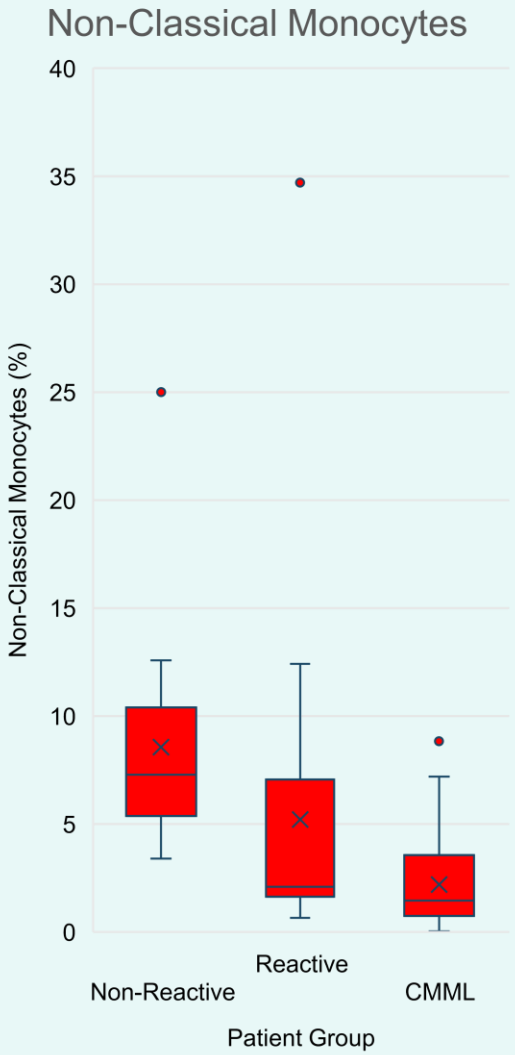
Results

Subset Analysis

Summary statistics for the three tested groups.				
Criteria		Patient Type		
		Non-Reactive (n=20)	Reactive (n=20)	CMML (n=20)
Gender	Male	9	14	13
	Female	11	6	7
	M: F	0.82	2.33	1.86
Age Group (Years)	<30	3	0	0
	30-50	6	4	0
	50-80	8	14	12
	>80	3	2	8
Monocyte Count (x10*9/L)	Average	0.755	1.59	5.38
	Range	0.5-1	1.1-2.6	0.2-18.3
Total White Cell Count (x10*9/L)	Average	8.04	17.32	16.78
	Range	4.8-10.5	8.3-42.3	2.7-65
CRP mg/L	Average	3.25	186.1	7.278
	Range	0-7	102-285	1-25
CD56+ Monocytes	Cases.	1	5	15


Classical Monocytes
120
100
80
60
40
20
0
Classical Monocytes (%)
Reactive
Non-Reactive
CMML
Patient Group


Intermediate Monocytes
70
60
50
40
30
20
10
0
Intermediate Monocytes (%)
Reactive
Non-reactive
CMML
Patient Group


Non-Classical Monocytes
40
35
30
25
20
15
10
5
0
Non-Classical Monocytes (%)
Reactive
Non-Reactive
CMML
Patient Group

My Findings

Subset Analysis

- **Classical Monocytes**
  - CMML patients had a significantly higher proportion of classical monocytes than non-reactive and reactive patients ($p < 0.001$; Kruskal-Wallis)
  - There was no significant difference in classical monocyte % between non-reactive and reactive groups ($p=0.97$; Mann-Whitney).
- **Non-Classical Monocytes**
  - CMML non classical monocytes were lower than reactive patients, but this was not significant ($p=0.062$)
  - CMML ncMos were significantly lower than non-reactive patients ($p<0.01$; Mann-Whitney).
  - ncMos of <1.13% has been suggested as a diagnostic marker of CMML, however, in this study, ncMo was higher than this value at 2.21%
    - The original study identified no statistical benefit in using cMo and ncMo as a ratio compared to cMo alone.
- **Intermediate Monocytes**
  - iMo were significantly lower in CMML compared to other groups ($p<0.01$ for both, Mann-Whitney)

Concordance with Literature Values.

Subset Analysis

- The >94% literature cut-off for classical monocytes in CMML gave a sensitivity of 25% and specificity of 100%.
  - This exceeds the specificity's given in other studies of 88.2-95.1% (Hwang et al., 2020; Tarfi et al., 2018; Selimoglu-Buet et al., 2015).
  - Sensitivity was much lower compared to 90.6-93.6%,
    - This could result in a high false-negative rate.
  - **Positive predictive value** (PPV): 100%
  - **Negative predictive value** (NPV): 72.74%
  - Assay accuracy was established at 75%.
- Studies completed in Korea and Australia, met with differing success.
  - In some studies, the >94% cMo cut-off was validated, with a sensitivity of 93.8% and specificity of 88.2%
  - Other studies presented a sensitivity of 75% and a specificity of 95.4% which was an unsuitable diagnostic cut-off (Hwang et al., 2020; Murali et al., 2020; Pophali et al., 2019; Arenillas et al., 2018).

Subset Analysis

Locally Derived Values

- **A locally established cut of >71.96% was derived.**

- **Sensitivity and specificity**: 90% and 72.5%
- **PPV and NPV:** 60.71% and 90.62%
- **Accuracy:** 76.67%.

- As a screening assay >94% cMos is still valuable as an inclusionary marker, as it could be used to justify further testing, but could not exclude CMML if negative.

- In contrast, the >71.96% cut-off for cMo's gave PPV and NPV of 60.71% and 90.62%.
  - If this value was used, clinicians could be confident in the use of the assay to identify true negative patients, who could in theory then be discharged from haematological clinics.
  - It could not confirm CMML and patients would require further invasive testing.

Limitations

Subset Analysis

- The local assay set up produced higher %CV's (~15-26%), compared to the original studies.
  - Possible reasons for this, including:
    - Low numbers within the study
    - Lack of age matched healthy control.
- Monocyte subset gates in the CD14 vs CD16 plot was manually placed according to the position of the cMo population on the density plot, resulting in a high level of subjectivity.
- The original assay utilises CD56 to exclude CD56+ Natural Killer cells (NK.)
  - CD56+ve monocytes were seen in 75% of the CMML group and 25% of the Reactive group.
- **>94% classical monocytes in CMML is likely not a universal value.**
  - Variability in these values has been seen across multiple replicating centres.
  - Classical monocytes can be reduced to below the >94% cut-off in cases of concomitant autoimmune disorders (seen in 20% of cases).
- There is evidence of normalisation of classical monocytes in CMML when treated with Azacytidine and hypomethylating agents (Hwang et al., 2020; Selimoglu-Buet et al., 2015).
  - The numbers of patients in my study were too low to analyse the effects these different subgroups.
  - Further work is needed to establish possible variations within cMos in CMML subclassifications.

Conclusions

- It has been consistently shown that expanded classical monocyte populations are correlated with CMML diagnoses.
- The cut off >94% provided by the original study is likely not universal though it does have benefits as a screening tool.
- There is still work to be done around monitoring using this assay, as well as the impact of treatments and transformation to AML.
- The assay's integration into the 2022 WHO diagnostic criteria means that the assays introduction into routine use would be invaluable.

Thanks!

Do you have any questions?