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Membranous Expression of pan CD66, CD66a, CD66b, and CD66c and their Clinical Impact in Acute Leukemia: Cross Sectional Longitudinal Cohort Study in Saudi Arabia

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Abstract

CD66 and its isoforms modulate several physiologic processes and have a role in aggressiveness of malignancies. We aimed at investigating pan CD66, CD66 a, b, and c expression and their clinical implication in acute leukemia. This study included 85 cases, 50 AML, 33 ALL and 2 mixed lineage leukemia from King Abdullah Medical City, Saudi Arabia. Pan CD66, CD66a, CD66b and CD66c were detected by flow cytometry at diagnosis and pan CD66 was reanalyzed at day28. Pan CD66 and CD66c expression rate was 51.8% in B-ALL and significantly correlated with BCR/ABL gene, P-value 0.037. CD66a was detected in 11.1% and significantly associated with shorter overall survival (OS), P-value 0.045. In AML, the expression rates were 40%, 28% and 32% for pan CD66, CD66b and CD66c respectively. CD66b was significantly correlated with favorable cytogenetic and prolonged OS, P-value 0.001 and 0.025 respectively. CD66c was correlated with CD25 positivity, P-value 0.003. The expression of pan CD66 at diagnosis and day 28, were significantly correlated, P-value <0.0001. Accordingly, pan CD66 could be added to the panel for MRD. Our data were encouraging to our center to follow other centers that already included CD66c in their panel for MRD detection. CD66c may be tried as a target for monoclonal antibody therapy in CD66c positive acute leukemia. Large-scale studies are needed to verify the association of CD66b expression with cytogenetics and survival in AML.

Keywords: AML; ALL; Adhesion molecules; Pan CD66; CD66a; CD66b; CD66c; MRD

Introduction

CD66 refers to a family of heavily glycosylated glycoproteins (CD66a to CD66f) that can be [1]. Members of this family are critical modulators of several key physiologic processes, including cell adhesion, motility, and regulation of immune processes [1,2]. Additionally, they play a significant role in other cellular processes, including the inhibition of differentiation [3], inhibition of apoptosis in colon cells [4], and disruption of cell polarization and tissue architecture [3]. Carcinoembryonic antigen (CEA) regulates these activities by activating integrin signaling pathways [5].

In normal haematopoiesis, CD66a, CD66b, CD66c and CD66d are strongly expressed in the myeloid lineage on the surface of myelocytes, metamyelocytes and neutrophilic and eosinophilic granulocytes [6]. A low expression of CD66 is found on normal promyelocytes and no expression has been described for myeloblasts. For the lymphoid lineage, strong expression has been described for CD66a and CD66c on precursor B-cells and for CD66a on T-cell precursors [7].

CD66 antigens are frequently upregulated in diverse cancers, and their overexpression is often associated with poor clinical outcome and reduced survival [6]. In haematologic malignancies, CD66 is expressed on myeloid cells at different stages of maturation and in acute [8] and chronic leukaemia [9].

CD66a is the most widely distributed protein within the CEA family, is expressed on macrophages, B- cells, IL-2 activated T- cells, NK-cell, and platelets [10], is dimly expressed on promyelocytes and mature neutrophils [11] and is overexpressed in lung and gastric cancers [12]. In tumour tissue, the expression of CD66a is reduced in the early phases of many cancers including colon, prostate, liver, and breast cancers, and it is thought to be a tumour suppressor protein [13]. However, CECAM1-L, CD66a isoforms is overexpressed in other types of aggressive cancers, such as melanoma, gastric, thyroid, bladder cancers [14] and metastatic colon cancer [15], thus demonstrating its role in metastasis [14].

CD66b is highly expressed on the surface of peripheral blood eosinophils [16]. It is expressed by promyelocytes and early myelocytes, reaches maximal expression at the late myelocyte and metamyelocyte stages and then decreases at the band and segmented neutrophil stages [11].

CD66c is expressed on granulocytes and their precursors [16]. In contrast to CD66b, CD66c expression is highest at the promyelocyte stage and progressively declines during maturation [11]. It inhibits anoikis, a form of programmed cell death [17], and modulating its expression alters the malignant phenotype of cancer cells. Its deregulation was first noticed in chronic myeloid leukaemia [9] and childhood B- acute lymphoblastic leukaemia [18]. CD66c might be the most specific marker for some aggressive cancers [13].

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Acute leukaemia is an aggressive disease that involves lymphoid and myeloid progenitor cells. Leukaemic cells usually have genetic lesions that lead to altered protein expression patterns that may include changes in normal adhesion molecule expression. CD66c expression represents an example of this association where a strong correlation was found between CD66c expression in childhood and adult ALL and nonrandom genetic changes, including BCR/ABL positivity, hyperdiploidy and absence of TEL/AML1 [19]. This altered expression of adhesion molecules may affect the patient's response to treatment, overall survival or disease recurrence because they alter the adhesive qualities and survival of leukaemia stem cells in the supporting bone marrow microenvironment with a subsequent change in the sensitivity of the blasts to chemotherapy [20].

Objectives

To detect the Expression of pan CD66, CD66a, CD66b, and CD66c on the surface of malignant cell population in acute leukemia and study the relation of their expression with the initial laboratory investigation, clinical data and disease outcome, and to identify the validity of their usage in investigating minimal residual disease (MRD) or as targets for immunotherapy.

Subjects and Methods

Subjects

This study included 85 newly diagnosed cases of acute leukaemia of both sexes selected from King Abdullah Medical City, Makkah, Saudi Arabia from May 2014 to May 2016. The diagnosis was based on WHO criteria. The cases included 50 acute myeloid leukaemia (AML), 33 acute lymphoblastic leukaemia (ALL) and two mixed lineage leukaemia (MLL) cases. Secondary leukaemia and relapsed cases were excluded from the study. This study was approved by the ethics committee of Umm Al Qura University, Saudi Arabia. An informed written consent was obtained from each participant. The study conformed to the Code of Ethics of the World Medical Association (Declaration of Helsinki).

The treatment protocol of all patients followed the previously mentioned National Comprehensive Cancer Network (NCCN) guidelines [21- 23].

Samples

All cases had representative bone marrow (BM) aspiration together with trephine core biopsy specimens for evaluation of BM cellularity. EDTA peripheral blood (PB) and BM aspirate specimens were prepared for fowcytometry analysis of surface, cytoplasmic markers, and the studied adhesion molecules. Heparinised BM samples were obtained for cytogenetic and molecular studies. A four ml blood sample was collected; 2ml was placed in an EDTA tube for complete blood count, and 2 ml was placed in a plain tube for biochemical studies after serum separation

Methods

All patients were subjected to a full medical history, clinical examination, routine laboratory investigations included complete blood count and determination of liver enzymes and routine leukemia work up that included:

- Examination of stained PB and BM films together with biopsy specimen.

- Immunophenotyping was performed using the BD-FACS-Canto II System (BD- Bio Science- San Jose, CA, USA).) and reagent system (BD- FACS Setup) as previously described [24] using an extended panel of monoclonal antibodies (MoAbs) that included B cell lineage markers (CD10, CD19, CD20, CD22, cyt CD79a, Kappa and Lambda light chains, surface and cyt IgM); T cell lineage markers (CD2, surface and cyt CD3, CD5, CD7, CD4, CD8, CD1a, CD25), myelomonocytic markers (CD13, CD14, CD15, CD33, CD64, CD68, CD117, CD11c, cyto MPO) and miscellaneous markers (CD56, CD38, HLA-DR, TdT). CD45 was included in each tube to allow the identification of abnormal cell populations from the normal hematopoietic population. CD34 is used to identify early progenitors or blasts, together with weak positivity for CD45, confirm the hematopoietic origin of the tumor cells [25]. Cell populations were designated as positive for a particular surface antigen if expressed in ≥ 20% and for the intracellular antigen if ≥ 10% of blast events stained beyond an appropriate isotype cut-off. - Cytogenetic analysis: Conventional karyotype analysis was performed on metaphase cells using standard culturing and banding techniques. The results were reported according to the International System for Human Cytogenetics Nomenclature [26].

- Fluorescence in situ hybridization (FISH) was used as a complement to conventional karyotyping to detect or confirm gene rearrangements.

- Detection of *BCR/ABL* fusion gene by qualitative RT-PCR methods

Expression of pan CD66, CD66a, CD66b and CD66c by flow cytometry: Monoclonal antibodies directly labeled with fluorescent dye: Pan CD66 labelled with FITC (BD cat #551479), CD66a labelled with PE (R&D cat # FAB2244p), CD66b labelled with FITC (BD cat # 555724), and CD66c labelled with PE (BD cat # 551478) were used. A series of dilutions were tried to find the highest dilution that gave the strongest stain for the detection system. Similar to other studies, the antigen was considered positive when ≥ 20% cells in the blast region expressed the antigen [7].

Flow cytometry analysis of pan CD66 at day 28: The stability of pan CD66 expression at diagnosis was evaluated in morphologic CR specimens obtained for MRD monitoring at the end of induction therapy. Based on immunophenotypes at diagnosis, phenotypic abnormalities (co-expression patterns and aberrant expressions) were defined and used in the investigations of follow-up samples using tailored MoAb combination. Live-gate analysis was used where a total of 100-500×10^3 cells were analyzed cells. In most cases CD34/SSC or CD117/SSC gates were applied [27].

Statistical analysis

The data were analysed using SPSS program version 20. Quantitative data were presented as the mean ± SD or median (min - max) and range as appropriate. Categorical data were presented as frequency and percentage. Comparison between two groups was conducted using Student's t-test, and nonparametric Mann–Whitney U and Kruskal–Wallis tests were used for the data that were not normally distributed. Comparison between more than 2 groups was made by using the ANOVA test. The Wilcoxon signed rank test was used for comparison between pan CD66 expression at diagnosis and after induction for the same patients. A chi-square or Fisher's exact test was used for comparisons between qualitative data as appropriate. The Kaplan-Meier method was used for survival studies. For all comparisons, a two-sided alpha value was set at 0.05. Probability (P-value) < 0.05 and <0.001 were considered significant and highly significant, respectively.

Results

This study included 85 patients with acute leukaemia. They included 50 AML cases, 27 B-ALL cases, 6 T-ALL and 2 MLL cases. According to WHO classifications, 11/50 AML cases has recurrent genetic abnormalities including (4= t(8;21), 6= t(15;17) and 1= (9;11)), and 39/50 not otherwise specified . The morphological classification was M1 = 13, M2 = 17, M3=6, M4 = 4 and M5= 10. 11/27 B-ALL cases have recurrent genetic abnormalities including (4= hyperdiploidy and 7 = t (9;22)) and 16/27 not otherwise specified , and their immunophenotypic distribution was pro-B-ALL = 9, common ALL = 14 and pre-B ALL = 4. Demographic and initial laboratory investigations of the studied patients are presented in (Table1).

Expression of pan CD66 and its isoforms in the studied group

The marker expression levels in AML, B-ALL and T- ALL are presented as the mean ± SD and median (min - max) in (Table 2 and Figure 1).

In B-ALL cases, 14/27(58.1%) were positive (≥20%) for both pan CD66 and CD66c expression.

Parameters studied	AML (n=50)	ALL (n=33)
Age (years)*	44.5 (15.0-81.0)	20.0 (12.0 - 64.0)
Sex: Male (n & %)	29.0(58%)	19.0 (57.6%)
Female (n & %)	21.0(42%)	14.0 (42.4%)
Clinical presentation		
Organomegaly	3(6%)	6(18.8%)
Anaemia	20(40%)	15(46.9%)
Bleeding tendency	17(34%)	7(21.9%)
Fever	15 (30%)	16(50%)
TLC (x10⁹/L)*	20.6 (0.8-381.7)	9.8(0.8-250.0)
Hemoglobin (g/dl)*	8.0(3.8-12.7)	8.6(4.6-13.6)
Platelets (x10⁹/L)*	46.0(6.0-374.0)	53.0(6.0-306.0)
Peripheral blood blast %*	39.5(0.0-92.0)	45.0(1.0-96.0)
Bone marrow blast %*	63.0(21.0-94.0)	89.5(21.0-96.0)
LDH(U/l)*	481.0(131.0-1597.0)	416.0(153.0-5658.0)
s GOT (U/l)*	24.5(5.6- 1075.0)	27.0(11.0-132.0)
s GPT (U/l)*	30(8.0-220.0)	36.5(15.0- 153.0)

*Data are presented as median (min-max).TLC: Total leukocytic count. sGOT: Serum glutamic oxaloacetic transaminase. sGPT: Serum glutamic pyruvic transaminase. LDH: Lactate dehydrogenase.

Table 1: Demographic and initial haematological and chemical investigation of the studied patients.

Parameters studied	AML (n=50)	B-ALL (n=27)	T-ALL (n=6)	P-value^
Pan CD66 Mean ± SD Median (min-max)	23.7 ± 27.9 9.5(0.3-99.0) a &b	36.7 ± 35.5 24 (0.0-97.0) a	0.9 ± 1.5 0.8(0.0-4.0) b	0.023*
CD66a Mean ± SD Median (min-max)	6.0 ± 7.2 2.5(0.1-25.0)	8.7 ± 17.2 1.9 (0.0-62.0)	3.9 ± 8.4 3.8(0.0-14.0)	0.593
CD66b Mean ± SD Median (min-max)	20.5 ± 29.5 4.5(0.0-98.0) a	2.2 ± 2.7 1.0(0.0-10.0) b	0.2 ± 0.2 0.2(0.0-1.0) a &b	0.004*
CD66c Mean ± SD Median (min-max)	19.2 ± 23.3 7.0(0.0-98.0) a &b	31.1 ± 32.8 23 (0.0-93.0) a	2.5 ± 5.1 0.2(0.0-13.0) b	0.037

Groups sharing the same letter indicate that no statistically significant difference exists between them.
^ P-values were used to compare the mean values
* Significant

Table 2: Expression levels of pan CD66 and its isoforms among different studied groups.

The number of positive cases in relation to different phenotypes was as follows: pan CD66 and CD66c were positive in 4/9 of pre-B, 7/14 of common and 3/4 of pro-B. CD66a was positive in 2 cases of common and one case of pro-B phenotype with no significant difference between them, P-value >0.05.

All studied T-ALL cases failed to express pan CD66 or any of its studied isoforms beyond the cut-off, and only one case showed a partial expression (17%) of pan CD66 and CD66a.

According to morphological examination of AML cases, there was a significant higher expression level of pan CD66 and CD66b in M3 morphology compared to myeloid and monocytic morphology , P-value <0.001 and 0.003 respectively. The level of the studied marker expression is represented in (Figure 2).

There was no significant difference between the rate of positive expression per the cut-off (≥20%) of pan CD66, CD66a or CD66c in AML versus B- ALL. CD66b showed a significantly higher expression rate in AML as it failed to be expressed in any of the studied B-ALL cases, P- value 0.002. There was no significance difference in positive expression rate between the FAB subgroups of AML. The data are shown in (Table 3).

One of the two MLL cases expressed pan CD66 and CD66c.This patient had myeloid and B-cell lineage differentiations, whereas the other patient had myeloid/T cell markers and failed to express any of them.

Clinical presentation and initial laboratory investigations

The only difference observed was a significantly higher peripheral blood blast count in the CD66a+ AML group compared to the negative group (85.0±7 versus 37. 95±31.2), P-value 0.049. Otherwise, no significant difference was detected in terms of the clinical presentation, laboratory investigation or demographic data in B-ALL or AML.

Expression of pan CD66 and its isoforms in different cytogenetic classes

Cytogenetic analysis in AML cases revealed that 10/48(20.8%) cases had favourable profiles [t(8;21), t(15;17),and inv 16], 30/49 (62.5 %) had intermediate profiles [normal karyotype, +8,+4, +11q23, t(9;11),and t(1;9;22)], and 8/48(16.7%) were unfavourable [complex abnormality, inv 17, t (7;11), monosomy 7, hypodiploidy and t(9;22)]. Two AML cases failed karyotyping due to failed metaphase and no recurrent translocations detected by FISH. Cytogenetic analysis of B-ALL cases revealed that 3/27 (11.1%) had a favourable profile [hyperdiploidy], 13/27(48.1%) had an unfavourable profile [near tridiploidy, t (9; 22), t (17; 19), 17p- and 14q-], and 11/27 (40.7%) with an unknown prognostic category [normal karyotype, 9p21- and -12p13] [28].

In the AML group, there were significantly higher levels of the mean value of pan CD66 and CD66c expression in the favourable group compared to the intermediate group. Additionally, the mean values of CD66b expression were significantly higher in the favourable group compared to both the intermediate and unfavourable groups. The data are shown in Table 4. There was no significant difference in the number of cases that expressed the markers ≥ 20% and the cytogenetic classes (P-value > 0.05). There was no significant difference in the different cytogenetic classes in B-ALL in either the percentage of expression or the number of positive cases (P-value >0.05).

Pan CD66 and its isoforms and *BCR/ABL* gene rearrangement in B-ALL

In the B-ALL group, the *BCR/ABL* fusion gene was detected

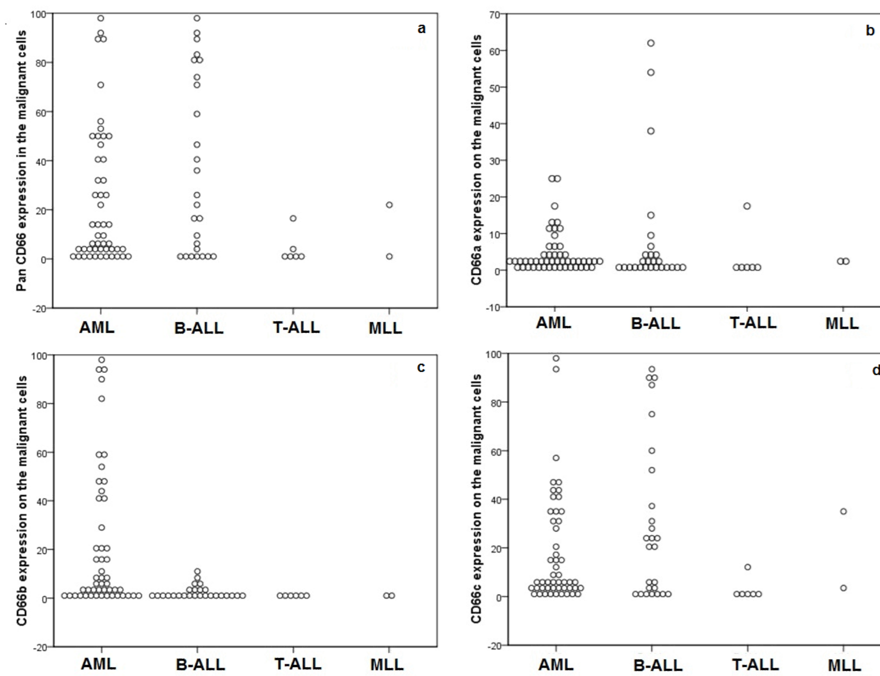

Figure 1: The expression levels of pan CD66 and its studied isoforms in the malignant cell population in acute myeloid leukaemia (AML), B- acute lymphoblastic leukaemia (B-ALL), T- acute lymphoblastic leukaemia (T-ALL), and mixed lineage leukaemia (MLL). **(a)** Pan CD66 expression is at very low levels in T-ALL compared to B-ALL. **(b)** CD66a expression shows no significant difference between the different categories. **(c)** CD66b expression revealed higher levels of expression in AML cases **(d)** CD66c expression revealed low levels of expression in T-ALL compared to B-ALL.

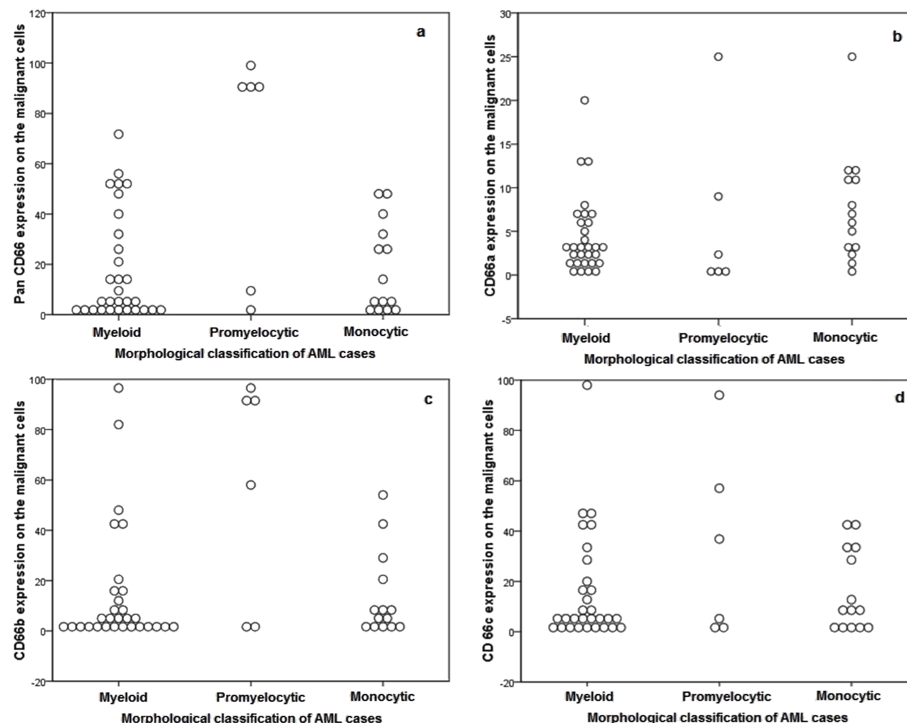

Figure 2: The expression levels of pan CD66 and its studied isoforms on the malignant cell population in acute myeloid leukemia morphological subtypes. (a) Pan CD66 expression shows significantly higher levels in promyelocytic leukemia (P-value<0.001). (b) CD66a expression shows no significant difference between the different subtypes. (c) CD66b expression was higher in promyelocytic leukemia (P-value 0.003) (d) CD66c expression shows no significant difference between the different subtypes.

Parameters studied	AML morphological subtypes #				B-ALL (n=27)	P- value^
	Myeloid (n=30)	Promyelocytic (n=6)	Monocytic (n=14)	Total (n=50)		
Pan CD66	10 (33.3%)	4(66.7%)	6(42.9%)	20 (40%)	14 (51.8%)	0.196
CD66a	1(6.7%)	1(16.7%)	1(12.5%)	3 (6%)	3(11.1%)	0.697
CD66b	6(22.2%)	4 (66.7%)	4(30.8%)	14 (28%)	0 (0%)	0.002*
CD66c	8(29.6%)	3(50.0%)	5(61.5%)	16 (32%)	14 (51.8%)	0.053

#AML-morphological subtypes: Myeloid cases included M1 and M2, promyelocytic referred to M3 morphology and Monocytic cases included M4 and M5.
^P-value between the whole AML and B-ALL groups.
* Significant

Table 3: The rate of positive expression of pan CD66 and its isoforms in the studied AML and B-ALL according to the standard cutoff for surface marker expression.

Studied parameter	Favourable N=10	Intermediate N=30	Unfavourable N=8	P- value^
Pan CD66 Mean ± SD Median(min-max)	51.4 ± 39.8 54.5 (2-99) a	14.9 ± 18.5 4.5(0-72) b	27.4 ± 21.7 26.5 (2-51) a &b	0.001*
CD66a Mean ± SD Median(min-max)	7.0 ± 10.1 3(0-25)	6.5 ± 7.4 3(0-25)	3.4 ± 2.6 4(0-7)	0.629
CD66b Mean ± SD Median(min-max)	55.5 ± 44.9 32(2-98) a	13.1 ± 20.0 3.5(0-82) b	15.6 ± 18.3 8.7(0-48) b	0.001*
CD66c Mean ± SD Median(min-max)	42.8 ± 38.7 21(1-98) a	12.1 ± 13.4 4(0-44) b	24.4 ± 19.4 23.2(3-48) a &b	0.002*

* Significance
^ P-values were used to compare the mean values
Groups sharing the same letter indicate that no statistically significant difference exists.

Table 4: Expression levels of pan CD66 and its isoforms in AML group in relation to the cytogenetic profile.

in 7/27 (25.9%) cases. Of the 14 cases that expressed pan CD66 and CD66c, 6 cases had a *BCR/ABL* gene rearrangement, with a significant correlation, P-value 0.037.

Pan CD66 and its isoforms and CD25 expression

In B-ALL, only 2 cases expressed CD25 (7.4%): one of them expressed pan CD66 and CD66c, but the other one did not, and both were *BCR/ABL* fusion gene positive.

In AML, CD25 was expressed in 5/50 (10%) cases and showed a significant positive correlation with pan CD66 and CD66c expression, P-value 0.008 and 0.003, respectively.

Pan CD66 expression at day 28

Evaluation of pan CD66 in 34(20 AML and 14 B-ALL) paired diagnosis/morphological CR specimens revealed no significant difference between the mean value of pan CD66 expression at diagnosis and day 28 in the both AML and B-ALL studied groups denoting a constant stable expression with no antigen loss (P-value >0.05).

Regarding the positive expression (≥20%) of pan CD66 in B-ALL at day 28, 13/14 cases that expressed the marker at diagnosis were still expressing it (≥20%) and the expression in the remaining case was 6% at day 28 with highly significant correlation, P-value <0.0001.

In AML cases, 14/20 cases that expressed the marker at diagnosis were still expressing it at day 28, and 5/20 showed partial expression of pan CD66 (from 10-19%), with a highly significant correlation, P-value <0.0001. The data are shown in (Figure 3).

Complete remission (CR)

The remission rate in B-ALL cases was 76.9%: 20/26 cases achieved CR, one case was missed, 3 cases died before completion of the induction therapy, and 3 cases were refractory to induction therapy. The remission rate in AML cases was 60%: 30/50 cases achieved CR, 6 cases were refractory to induction therapy, and the remaining cases died early during induction therapy. No significant difference could be detected between the patients who achieved CR and the others with respect to the expression of the studied markers in B-ALL and AML, P-value >0.05.

Overall Survival (OS)

The median (min - max) follow-up period for B-ALL cases was 31.8 (0.0-190) weeks. At the end of this period, 5/26 (19.2%) patients were dead. The median OS for the whole group was 48.6 weeks. CD66a positive cases had a shorter OS, means ± SE 26.4 ±14.1 weeks and one year OS 0.0% versus 57.6 ± 10.8 and 60.0 % in the CD66a negative with a significant difference, P- value 0.045. Otherwise, no significant differences were detected. Figure 4 represents OS curves.

The median (min - max) follow-up period of AML cases was 20.8(0-88) weeks. At the end of this period, 12 (24 %) patients were dead. The median OS for the whole group was 64.6 weeks. There was no impact of pan CD66, CD66a or CD66c positive expression on survival, P-value >0.05. The CD66b positive group had a significantly longer OS, mean ± SE 76.0 ± 7.4 weeks and one year OS of 85.7% versus 44.8 ± 4.7 and 68.4% in the CD66b negative group, P- value 0.025. Figure 5 represents OS curves.

Discussion

A proper diagnosis of acute leukaemia is essential to start efficient chemotherapy but the subsequent monitoring of MRD to detect early recurrence is equally important and is currently recommended by many guidelines [29]. In acute leukaemia, the occurrence of aberrant

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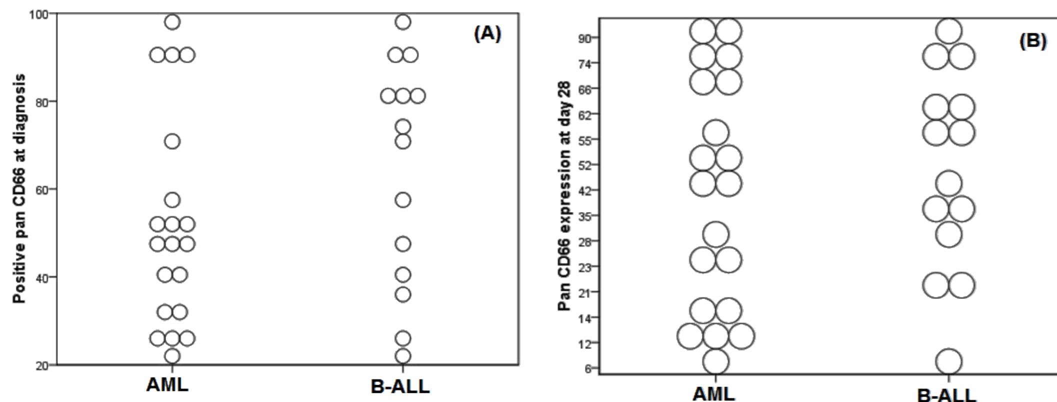

Figure 3: The expression levels of the positive pan CD66 cases in AML and B-ALL. (a) Represents the positive cases that expressed pan CD66 ≥ 20% at presentation. (b) Represents follow up of the previously positive cases at day 28. There was a highly significant correlation between the expression at presentation and at day 28 (P-value<0.0001).

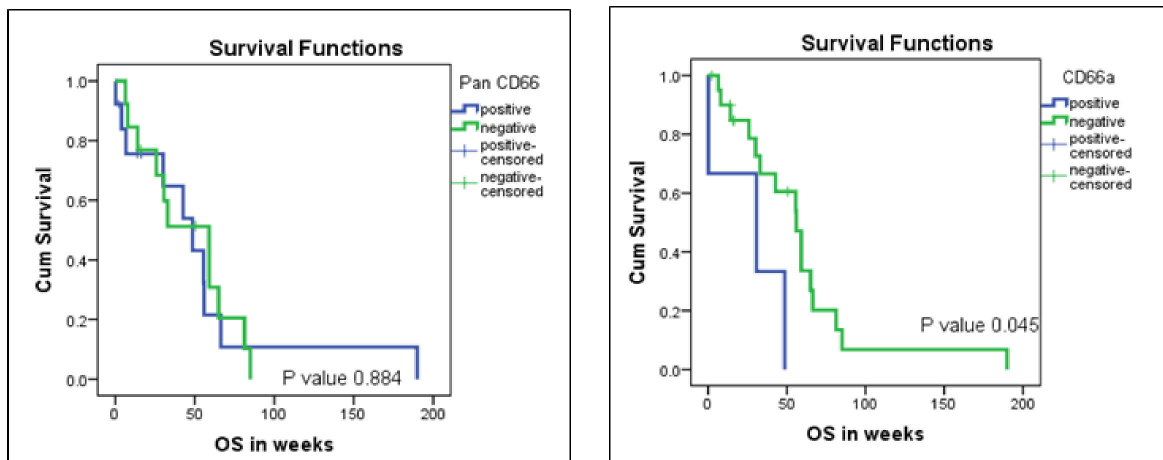

Figure 4: Impact of pan CD66 and CD66a on overall survival (OS) in B-ALL cases studied by the Kaplan-Meier method. (a) Impact of pan CD66 on OS and the same for CD66c. (b) Impact of CD66a on OS. NB. CD66b is not expressed in B-ALL.

antigen expression has been reported with varying frequency [30]. One aspect of the clinical importance of aberrant antigen expression is the ability to use them as a marker of MRD. Comparably, abnormal overexpression or downregulation of some antigens have equal clinical importance. CD66 family members are candidates for both aberrancy and overexpression in ALL and AML.

The currently studied B-ALL cases showed that the rate of pan CD66 and CD66c expression was 51.8%. Both are aberrant myeloid antigens, and they have the most frequent aberrant antigen expression compared to other myeloid antigens, such as CD13, CD33, CD117, CD14, CD15, and CD64 (data are not shown), in agreement with previous reports [9,29-32].

CD66a showed a low expression rate (11.1%), in agreement with Skubitz et al. [33].This low rate is explained by the downregulation of CD66a in early phases of many cancers, as it is suggested to be a tumour suppressor protein [13]. Comparable to the findings of previous authors, the expression of pan CD66, CD 66a, and CD66c was not restricted to certain B-ALL phenotypes and the studied markers never reached the cut-off in T-ALL cases [7].

In AML, the rate of pan CD66 expression was 40%, with the heterogeneous expression of the studied isoforms. Our results are comparable to those of Ratei et al. [7].

We reported a more frequent expression of pan CD66 in B-ALL than AML that did not reach the level of significance that was in contrast to Carrasco et al. [34] who detected a significant higher frequency in B-ALL. In agreement with a previous study, we could not detect any significant difference between the rate of positive expression of CD66a or CD66c in AML compared with B-ALL [7].

In this study, all cases that expressed pan CD66 at diagnosis were reanalysed after morphological CR. This analysis revealed a highly significant correlation between pan CD66 expression at these two different time points in both AML and B-ALL cases, indicating the stability of the marker after induction chemotherapy. We studied pan CD66 in 2 relapsed AML cases that revealed preserved stability of the marker (data are not shown). Our initial data makes pan CD66 suitable for use in the follow-up and detection of MRD in combination with the already used monoclonals.

CD66c is expressed in B-ALL cases at the same percentage of pan

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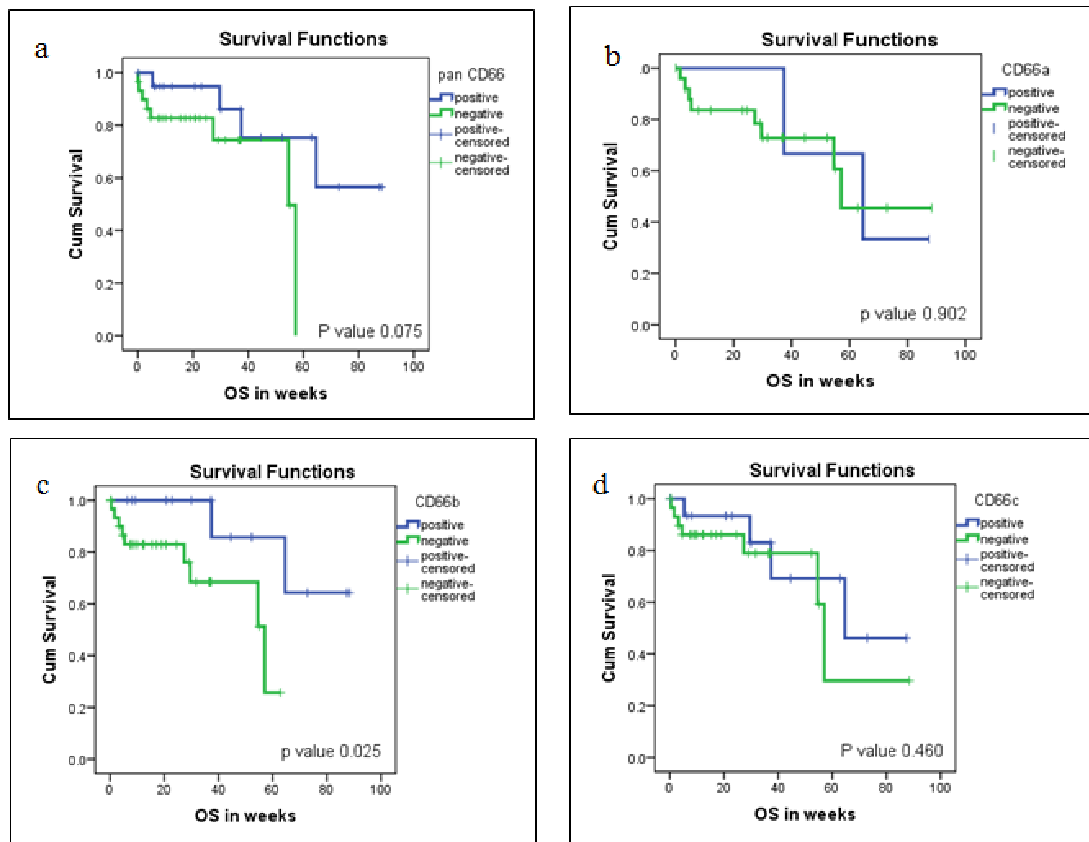

Figure 5: Impact of pan CD66 and its studied isoforms on overall survival (OS) in AML cases studied by the Kaplan-Meier method. (a) Impact of pan CD66 on OS. (b) Impact of CD66a on OS. (c) Impact of CD66b on OS (d) Impact of CD66c on OS.

CD66 but we did not examine its stability after induction therapy. However, Tang et al confirmed the stable expression pattern of CD66c and recommended its use in the recognition of abnormal leukemia cells at primary diagnosis and in monitoring of MRD during the treatment [29]. Our data were encouraging to our center to follow the EuroFlow panel for detection of MRD that included CD66c as a discrimination marker between malignant B-ALL and the normal B-cell precursors [35].

A good target for monoclonal antibody therapy should be expressed at a higher percentage and not shed during therapy to be available for treatment [36]. On the basis of our results, we can consider pan CD66 good candidate for immunotherapy in B-ALL and AML due to its high frequency of expression and molecular stability after induction therapy. However, anti-CD66 radioimmunotherapy may not be the optimal radioimmunoconjugate as its mechanism is indirect with no direct fixation on the leukemic blasts as it was tried earlier [37].

For more specific therapy, CD66c is suggested rather than pan CD66 in B-ALL. Dysregulated over-expression of CD66c plays a role in several of the hallmarks of cancer, including uncontrolled proliferation, anoikis resistance, neoangiogenesis, immune evasion, invasion and metastasis [38]. Silencing CD66c promotes anoikis with down regulation of cell survival pathway [39]. The efficacy of antibody-drug conjugate targeting CD66c was investigated in animal models where marked diminish in tumor size was reported. The only noticed adverse effect was dose- dependent reversible neutropenia because CD66c shows low level of expression in the earliest myeloid lineage (CD34+/ CD38+/ CD33+) [40]. Accordingly, targeting of CD66c may be a promising immunotherapy in B-ALL.

In the studied B-ALL, a significant correlation between CD66c expression and BCR-ABL gene rearrangement was detected, in agreement with other authors [31,32,41].This significant association enables the use of CD66c as a predictor for the *BCR/ABL* rearrangement as reported earlier [29].It is noteworthy that in our study, the expression of CD66c was not restricted to cases harbouring the *BCR/ABL* fusion gene, in agreement with Tang et al. [29]. Therefore, a lack of CD66c expression might indicate the absence of the BCR/ABL rearrangement but the vice is not verse [33]. CD66c is considered a marker for some aggressive cancers [13] that may add to the aggressiveness of Ph+ B-ALL [41].

The correlations between CD66c and BCR/ABL fusion genes prove its expression on the malignant clone. This result, together with the high frequency of CD66c positivity in B-ALL cases, strongly supports the inclusion of CD66c for MRD detection.

In AML cases, there were significantly higher levels of pan CD66 and CD66c in the favourable cytogenetic group compared to the intermediate group. CD66b showed significantly higher levels in the favourable group compared to both the intermediate and unfavourable groups, thus making CD66b a superior isoform in predicting favourable cytogenetic profiles. However, a large-scale study is needed to verify this finding.

CD25, high affinity interlukin2 receptor α chain (IL-2RA), was

expressed in 10% (5/50) of AML cases. These cases had a unique phenotype as they expressed HLA-DR, pan CD66, CD66c, CD11c (in all cases) and CD64 (in 4/5 cases), which give the AML blasts a dendritic cell-like phenotype [43,44] that enables them to interact with T-helper cells and deviate their differentiation towards T-regulatory cells. The biological role of IL-2RA was studied in lymphoma, wherein the interaction between IL-2 and IL-2RA can promote differentiation of T-cells into regulatory T-cells, leading to immune escape status [44].Therefore, a similar situation may be generated in the BM microenvironment of CD25+ AML cells. Because this issue may be fundamentally important, further investigations are needed.

CD66a expression in B-ALL had a negative impact on patient prognosis with significantly shorter overall survival. To our knowledge, no comparable results were detected in acute leukaemia. However, in the same context, pan CD66 and CD66a expression were linked to short OS in cervical and pancreatic cancer [45,46]. Short OS could be explained by the anti-apoptotic effect of CD66a [47], which make the tumour more aggressive. In AML cases, no such impact of CD66a expression was detected on OS, but it was linked to another poor prognostic factor: high peripheral blood blast, which may reflects its role in regulating cell migration [48].

CD66b expression was significantly associated with prolonged survival in AML. Interestingly, CD66b was not correlated with any of the poor prognostic factors, CD25 or CD34 expression, high total leukocyte count or blast count, but it was highly expressed with favourable cytogenetics. These findings are taken together with the results of previous authors who measured CD66b in the immature compartment of myeloblast in MDS and stated that CD66b is an independent prognostic factor associated with longer OS irrespective of CD34 or CD117 expression. However, further investigations are needed to clarify similar results in AML [49].

Conclusions

Pan CD66 and CD66c were expressed in 51.8% of B-ALL cases, and their expression was linked to one of the poor prognostic factors, the *BCR/ABL* fusion gene. In AML, pan CD66 was expressed in 40% of AML cases with a predominance of both CD66b and CD66c. The clinical significance of CD66 isoform expression in AML was heterogeneous. Whereas CD66a expression is correlated with high PB blast count, CD66b is associated with favourable cytogenetics and prolonged OS, and CD66c is correlated with CD25 expression.

Recommendations

Our initial results regarding CD66c expression in B-ALL are encouraging for our center to follow the Euro Flow panel for MRD detection. Also, CD66c could be a monoclonal antibody therapy research area in B-ALL; especially it is correlated with the poor prognosis factors: *BCR/ABL* fusion gene. Further investigations are needed to study the stability of CD66c expression after morphological CR and during relapse and to evaluate its biological value as a target molecule for immunotherapy.

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Author Contributions

MMI: Designed the study, shared in clinical selection of cases, performed the laboratory investigations, shared in statistical analysis, preparing tables, and figures and critically reviewed the manuscript. **AZ**: Shared in the study design, and performed statistical analysis, writing the first draft of the main manuscript text, tables and figures. **NABA:** Was involved in the clinical selection of cases, the performance of laboratory investigations and collection of patient data. **HKM**: Shared in study design and study plans, performance of laboratory investigations and preparation of the master tables including patient data and collected the scientific materials. All authors researched literature, shared in manuscript writing, revised, edited, and approved the final version of the manuscript.

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