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RESEARCH ARTICLE

BONE MARROW ANGIOGENESIS AND MICROVESSEL DENSITY IN PATIENTS WITH APLASTIC ANEMIA

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ABSTRACT

Introduction: The present study was conducted with the aim to diagnose and categorize aplastic anemia (AA) according to the severity as non severe (NsAA), severe (sAA) and very severe (VsAA) and to study microvessel density using immunohistochemical stain using CD 34 and morphometric analysis of microvessel density by computer assisted quantitative analyser and to compare it in the three subcategories of aplastic anemia with control group. ***Material and Methods:*** The present prospective study was conducted in the Department of Pathology at Pt. B.D. Sharma PGIMS, Rohtak. Thirty cases of AA patients and 10 cases of control were taken. AA patients were diagnosed on the basis of complete blood count, peripheral blood smear, bone marrow aspiration examination and bone marrow trephine biopsy (using Jamshidi's needle). AA patients were categorized as sAA, NsAA and VsAA using camitta et al criteria. IHC using CD 34 was performed to look for angiogenesis and to calculate microvessel density (MVD). ***Results:*** Majority of patients (56%) of AA were between 1st and 3rd decade of life. Females were more commonly affected with a male to female ratio (11/19) of 0.58:1. In Aplastic anemia patients (Group I), using Camitta et al criteria 30 cases were categorized into NsAA (10 cases), sAA (12 cases) and VsAA (8 cases). MVD was calculated using CD 34 which is a good marker of angiogenesis. MVD showed a decreasing trend with increase in severity of the disease. ***Conclusion****:* The study shows that Aplastic anemia is associated with reduced bone marrow angiogenesis and mean MVD score shows a decreasing trend from non severe to very severe Aplastic anemia.

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INTRODUCTION

Aplastic anemia (AA) is a life threatening disorder that is characterized by deficiency of pluripotent hematopoietic progenitor cells with consecutive bone marrow (BM) aplasia and peripheral pancytopenia1. Aplastic anemia is pancytopenia with hypocellular bone marrow in the absence of infiltration and fibrosis having cellularity <25% however usually cellularity is even <10% (Marsh *et al.,* 2009). The incidence of AA shows geographical variability. The incidence of AA varied from 10- 52.7% among patients with pancytopenia (Khunger *et al.,* 2002). Factors that temporarily or permanently injure the bone marrow which further affect the blood cell production are radiation and chemotherapy treatments, exposure to toxic chemicals, use of certain drugs, autoimmune disorders, viral infection, pregnancy and idiopathic (Nissen, 1989). Recently the fact that angiogenesis may have an important role in aplastic anemia has attracted considerable attention and this has lead to conducting of new studies regarding the role of angiogenesis in AA (Erdem *et al.,* 2006). The induction of angiogenesis is a complex and dynamic processes mediated by many pro-angiogenic and anti-angiogenic molecules. Disruption of the balance between pro-angiogenic and anti-angiogenic factors in favour of pro-angiogenic factors leads to formation of new vascular structure. VEGF is one of the most relevant angiogenic factors as its expression shows close correlation with the vessel density in several human tumors and inhibition of VEGF by specific antibodies suppresses tumor growth (Jackson and Blythe, 2008). Angiogenesis is quantified through the staining of blood vessels with various endothelial markers. Various endothelial cell markers used for quantification of angiogenesis include CD31, CD34, CD105 and factor VIII (Wang *et al.,* 2008). Morphometry means measurement of form, and is defined as the quantitative description of geometric features of structure with any dimension (Baak, 1991). This is more restricted definition of morphometry than that of Weibel, who includes stereology in the definition of morphometry (Weibel, 1979). Measurement has several advantages over conventional visual assessment; objectivity, reproducibility and the ability to detect changes not immediately apparent to the naked eye. This concept of measurement is called morphometry. The sequence of steps involved in the analysis of microscopic image is 'Microscopic image CCD video camera Video frame grabber Image processing Imagesegmentation Interactive measurement Data

recording Data analysis. Morphometry enables the pathologist to obtain densitometric as well as morphometric parameters like the number of vessels with a certain dimension range, the vessel luminal area, vessel luminal perimeter and the number of immune-stained areas per microscopic field (Hamilton and Allen, 1995). The present study was conducted to diagnose and categorise aplastic anemia according to the severity and to study microvessel density using immunohistochemical stain CD 34 and to compare the microvessel density in three subcategories of aplastic anemia with control group.

MATERIALS AND METHODS

The present study was conducted in Pt. B.D. Sharma, PGIMS, UHS, Rohtak from year 2017 to 2019. The study comprised of patients diagnosed as AA on the basis of complete blood count, peripheral blood smear, bone marrow aspiration examination and bone marrow trephine biopsy (using Jamshidi's needle). The bone marrow aspiration and biopsy was performed after an informed consent of the patient in the Department of Medicine and was processed routinely. Thirty cases of AA patients and 10 cases of control were taken. All the sections were stained for Haematoxylin and Eosin stain. All AA cases were categorised in NsAA, sAA, and VsAA using Camitta *et al.* criterion (Camitta *et al.,* 1982) (Table 1). Bone Marrow cellularity was calculated according to Tuzuner and Bennet reference standards (Tuzuner and Bennett, 1994).

Control: Control patients constituted the bone marrow biopsies of patients with iron deficiency anaemias and marrow for staging of Hodgkins lymphoma.

Inclusion Criteria: Only newly diagnosed cases and who were not yet started on any treatment were included in the study.

Exclusion Criteria: Patients on immunosuppressive therapy were excluded from the study.

Immunohistochemical analysis: IHC was performed on 3-5 µm in thick sections from 10% formalin fixed paraffin-embedded specimens, according to the streptoavidin-biotin immunoperoxidase technique (Dako-cytomation). Multiple slides were evaluated, and ideal section was used for IHC staining Positive and negative controls were run simultaneously. Strong brown nuclear immunoreactivity was considered as positive staining.

Control for IHC staining: Sections of normal bone marrow biopsy were used as positive controls for anti CD34 antibodies. Negative controls were obtained by substituting the primary antibody with an antibody of irrelevant specificity.

Statistical Analysis

A retrospective study was carried out for all the variables included in the study. Chi square test was used to compare the categorical values. P value less than 0.05 was accepted as clinically significant using SPSS version 20.0. Morphometric Analysis: The quantitative morphometric studies were done by image analysis. Images provided by a charged device video camera coupled with Olympus BX51 microscope at a magnification 400 X were stored on a host computer based on Pentium 4 processor with operating system Microsoft Windows Vista/ XP through a digital frame grabber and processing was done by image analysis software Image Pro Plus Version 6.3. Microvessel (MV) was defined as any highlighted endothelial cell or endothelial cell cluster, tumor cells and other connective tissue elements. Vessel lumen was not necessary for a structure to be defined as microvessel. Large vessels and vessels in the periosteum or bone were excluded because vascularisation is not representative of neo-angiogenesis in these areas. Areas of staining with no discrete breaks were counted as a single vessel. MVD was assessed by light microscopy in representative areas with highest number of capillaries and small venules (neovascular ''hot spots'') according to the method that was first described by Weidner et al.13 The sections were scanned first at low magnification (100X), and the most intense area of neovascularisation (hot spot) was identified. Microvessel (MV) counts were performed on 400 X. The MVD were determined by two independent observers in each case. Computer assisted image analysis was performed on all cells, tissues and vessels expressing antibody staining, avoiding confounding background and included all positive staining vessels.

RESULTS

Out of total 40 cases taken in the study; 10 cases were taken as control and total of 30 cases of AA were categorized using Camitta et al criteria and it was observed that 10 cases were categorised as non-severe (NsAA), 12 as severe AA (sAA) and 8 cases as very severe AA (VsAA) (Figure 1). Maximum number of cases of AA were between the age group of 10 to 30 years constituting total of 17/30 patients suggesting 56% of total cases. (Figure 2) Cases from almost all age groups were taken to represent as controls. Out of total 30 cases of AA, 63% were females, suggesting mild female preponderance. In control group, 60% were males. Fifty three percent (16/30) of AA cases had TLC count ranging between 1000- 2000. Only 2 (6.5%) patients had count less than 500/cumm and only 4 patients (13%) had more than 2000/cumm. Twenty three percent of AA cases had ANC <200µL, 33% cases had ANC between 200-500µL and 43% cases had ANC >500 µL. All patients had thrombocytopenia with approximately 77% (23/30) patients had platelet count less than 30,000/cumm. Quantum of corrected reticulocyte count suggested that 60% of cases had reticulocyte count <0.5%, 23% cases had reticulocyte count between 0.5-1% and 16% cases had reticulocyte count more than 1%. All the cases showed reduction in bone marrow cellularity in AA cases with and only 3% had cellularity more than 30%. On IHC with CD34 staining, No. of vessels varied from 2 to 8 as per high power field when an average of 10 views were taken. MVD varied from 8.33 to 33.33/mm2. Mean microvessel density of 7.41+/- 3.9 was observed in AA cases with median of 6.25 and range from 2.08 to 14.5. Mean micro- vessel density of 23.74 +/- 7.9 with median of 25 and range from 8.3 to 33.3 was observed in controls.

NsAA patients showed: Median TLC count of 2050, median ANC count of 800, reduced platelet count of 33.5K and reticulocyte count of 0.59 on PBF. Bone marrow biopsy showed cellularity with a median of 20-25% and mean average no. of vessels of 2 with mean MVD of 8.5 with median of 9.3 (Table 2).

sAA patients showed: Median 1500 TLC, <500 ANC, median 15K platelet count, reticulocyte count of 0.41 on PBF.

Table 1. Camitta *et al.* classification for Aplastic anemia

Severity	Criteria
Severe (sAA)	Bone marrow cellularity < 25% (or if <30%, 25-50% residual hematopoietic cells) and atleast two of the following: •Peripheral blood absolute neutrophil count (ANC) <500/µl •Peripheral blood platelet counts <20,000/µL •Peripheral blood corrected reticulocytes <1%
Very Severe(vsAA)	As severe, but peripheral blood absolute neutrophil count (ANC) <200/µL
Non Severe (NsAA)	Hypocellular bone marrow with peripheral blood cytopenias not fulfilling criteria for severe or very severe aplastic anemia

Table 2. Parameters for non-severe aa cases

S. No.	TLC	ANC	Platelet	Reticulocytes	Cellularity(%)	Avg No. of Vessels	MVD
1	1100	800	30K	0.41	25-30	3	12.5
2	3400	510	35K	1.3	25-30	0.5	2.08
3	3000	1050	35K	0.56	30-35	3	12.5
4	2000	1160	45K	1.2	25-30	1	4.16
5	2000	740	20K	1.4	15-20	1	4.16
6	2100	504	25K	0.22	20-25	1.5	6.25
7	2000	800	50K	1.2	20-25	2	8.3
8	2100	840	32K	0.17	10-15	2.5	10.41
9	3500	1200	25K	0.54	10-15	3	12.5
10	2000	540	55K	0.63	0-5	3	12.5
Median	2050	800	33.5K	0.59	20-25	2.25	9.35
Mean	2320	814	35.2K	0.76		2.05	8.53

Table 3. Parameters for severe aa cases

S.No.	TLC	ANC	Platelet	Reticulocytes	Cellularity(%)	Avg. No. of Vessels	MVD
1	1000	400	22K	0.17	20-25	2	8.3
2	1200	360	12K	0.14	10-15	2.5	10.4
3	2000	500	15K	0.56	20-25	3.5	14.5
4	1000	300	10K	0.5	15-20	1.5	6.25
5	1200	480	15K	1.32	20-25	1	4.16
6	1500	330	12K	0.58	15-20	1	4.16
7	1500	600	10K	0.2	25-30	1	4.16
8	4000	920	8K	0.32	20-25	3.5	14.5
9	2000	600	15K	0.58	20-25	1.5	6.25
10	1100	495	32K	0.21	20-25	1	4.16
11	2000	500	20K	0.65	20-25	0.5	2.08
12	1500	450	20K	0.21	15-20	1.5	6.25
Median	1500	487.5	15K	0.41	20-25	1.5	6.2
Mean	1666.7	494.6	15.9	0.45		1.7	7.1

Table 4. Parameters for very severe aa cases

S. No.	TLC	ANC	Platelet	Reticulocytes	Cellularity (%)	Avg. No. of Vessels	MVD
1	1000	200	20K	0.2	20-25	1	4.16
2	1000	200	10K	0.18	15-20	0.5	2.08
3	450	90	18K	0.06	05 - 10	1.5	6.25
4	800	160	15K	0.12	05 - 10	2	8.3
5	1100	220	10K	0.1	10 -15	1.5	6.25
6	800	80	18K	0.12	0 - 5	0.5	2.08
7	200	80	13K	0.21	25-30	2.5	10.4
8	2000	100	10K	0.21	05-10	3	12.5
Median	900	130	14K	0.15	7.5-12.5	1.5	6.25
Mean	918.7	141.25	14.25K	0.15		1.56	6.50

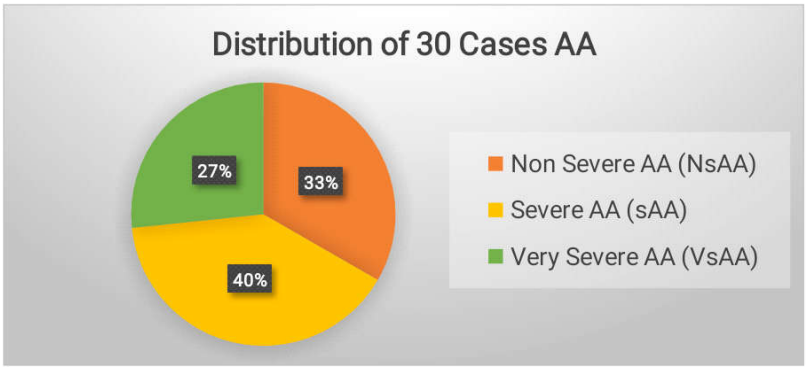

Figure 1. Distribution of 30 Cases AA

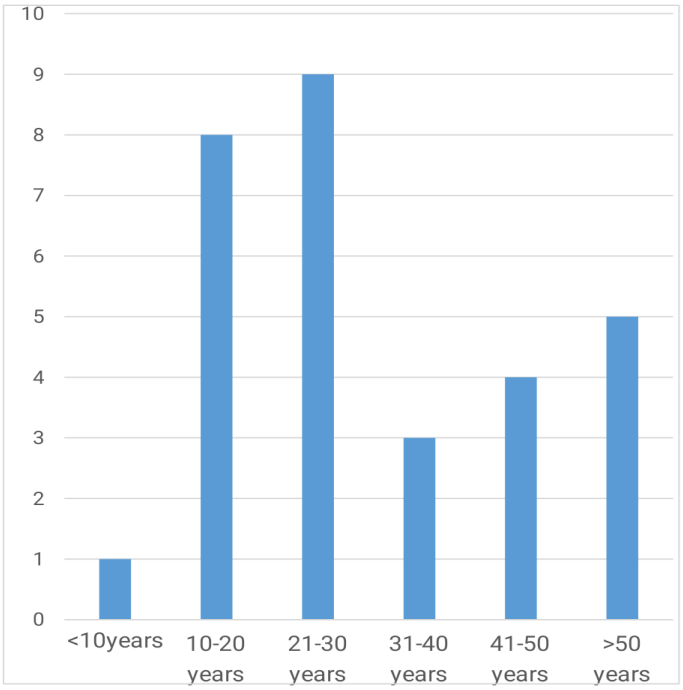

Figure 2. Demography on the basis of age

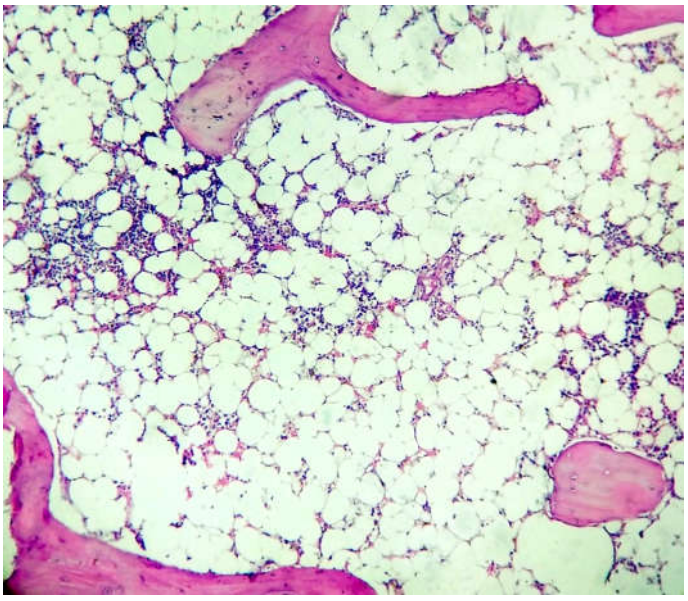

Figure 3. Bone marrow sections from AA patient with increase in fat: cell ratio (H & E;200X)

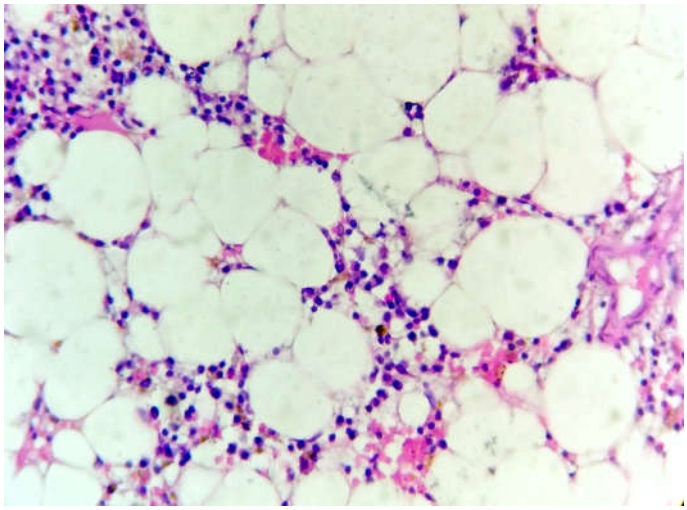

Figure 4. Increased fat: cell ratio in AA patient (H & E; 400X)

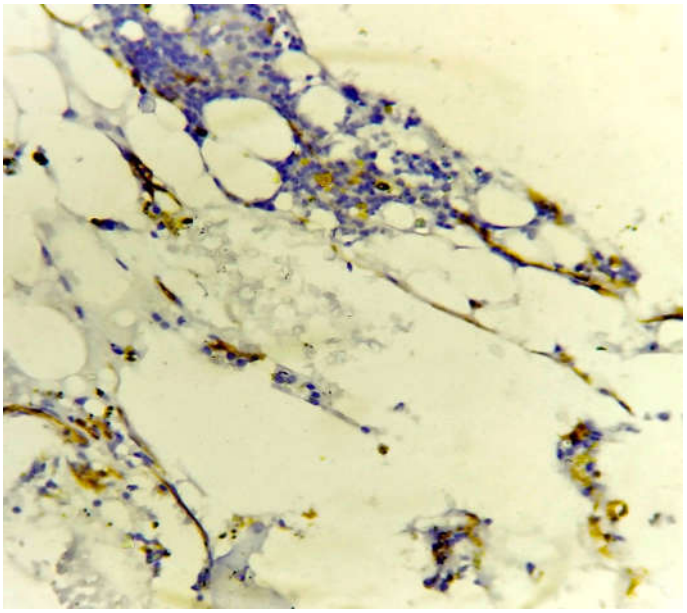

Figure 5. Reduced number of microvessels in severe Aplastic anemia (CD 34; 400X)

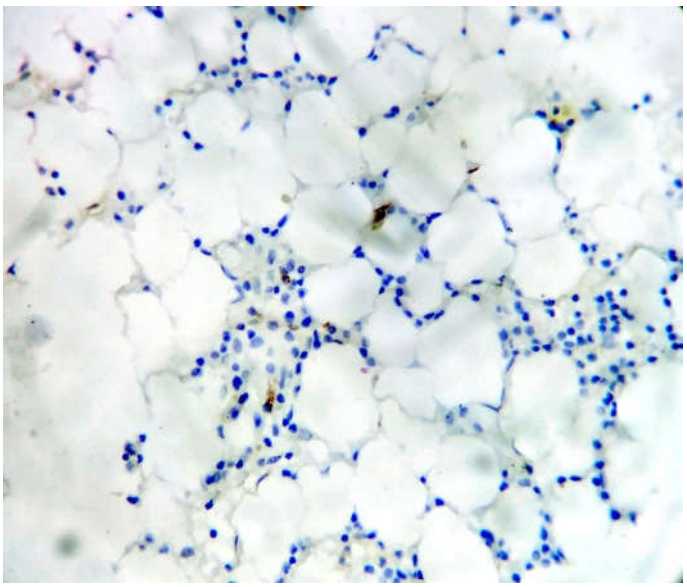

Fig. 6. Morphometric representation of reduced number of microvessels in severe Aplastic anemia (CD34; 400X)

Bone marrow biopsy showed cellularity with a median of 20-25% and mean no. of vessels of 1.7 and mean MVD of 7.1 were observed (Table 3).

VsAA patients showed: Median ANC count of 130, < 15K platelets, < 1000 TLC, markedly reduced reticulocyte count of 0.15 on PBF. Bone marrow biopsy showed reduced cellularity with a median of 7.5-12.5% and mean no. of vessels of 1.56 with mean MVD of 6.5 with median of 6.25 were observed (Table 4). The parameters suggested that TLC, ANC and corrected reticulocyte count shows reducing trend as severity of AA increases. Platelet count also show reducing trend as severity increase however no much interval difference was seen in sAA and VsAA cases. Cellularity reduces markedly in VsAA. The Bone marrow micro vessel density was significantly lower (Fig 3&4) in patients with AA as compared to controls (P value <0.001) with further mild reducing trend of MVD was observed in AA cases as severity increases with however no significant difference was observed in sAA and VsAA.

DISCUSSION

AA is classified as non-severe (NsAA), severe (sAA) and very severe (VsAA) based on the degree of the peripheral blood cytopenia and classified on the basis of camitta et al criteria taking into account the bone marrow cellularity, peripheral blood, absolute neutrophil count, platelet count and corrected reticulocyte count. Total of 30 diagnosed cases of AA, on the basis of complete blood count, peripheral blood smear and bone marrow aspiration examination were further evaluated by bone marrow trephine biopsy, performed using Jamshidi's needle and was processed routinely. Only newly diagnosed cases and who were not yet started on any treatment were included in the study. Patients who were on immunosuppressive therapy were excluded from this study. Ten cases as control were also analysed. All the sections were stained for H&E stain. The cases were categorised in NsAA, sAA, and VsAA on basis of reticulocyte count and cellularity. IHC staining for CD 34 was done on all cases included in the study and average no. of vessels and microvessel density were further analysed with Olympus BX 51 using objectives at 40x (being 0.24 for 100 eyepiece small divisions). In our study majority of (56%) patients of AA were between 1st and 3rd decade of life. The youngest patient was 9 years old and the oldest patient was 60 years old with mean age of 27.5 years. Our study showed slight female preponderance in AA cases with a male to female ratio (11/19) of 0.58:1. All 30 cases of AA in our study were subcategorised into NsAA, sAA and VsAA (10,12 and 08 respectively) on the basis of Camitta et al criteria which is mostly widely accepted standard for the diagnosis of Aplastic Anemia. Our study suggested that median values of TLC, ANC, Platelets, reticulocyte and bone marrow cellularity showed a decreasing trend in various subcategories of AA (Table 2, 3 and 4) Same criteria was used by the Fureder *et al.* (2006), Wu *et al.* (2015), Gupta *et al.* (2017), Somasundaram *et al.* (2009) and Yoon *et al.* (2012) for sub-classification of AA. Their studies also suggested reducing trend in median values of ANC, Platelets and corrected Reticulocytes, However Yoon et al18 emphasized that ANC should be an essential and not an optional criteria for diagnosis and classification of AA contrary to the study of Camitta *et al.* (1982) which also relates well with our study.

Bacigalupo *et al.* (2017) also in their study emphasized the importance of ANC for predicting the severity of AA and further assessment of prognosis during the treatment, thus we also conclude ANC being a good marker for the assessment of severity of AA. Our study also suggests reducing TLC in subcategories of AA. All the cases diagnosed as AA and the control group were subjected to IHC staining for CD 34 antibody and average number of blood vessels and mean vessel density was analysed. Controls which included cases with Iron deficiency anemia and marrow for staging with Hodgkin's Lymphoma showed a mean vessel number of 5.7+/-1.9, MVD of 23.74+/-7.91. However in cases of AA the mean vessel of 1.78+/-0.95 and MVD of 7.4+/-3.9 was observed suggesting reduced angiogenesis. Our results were in concordance with the studies of Fureder *et al.* (2006), Wu *et al.* (2015). They observed a mean bone marrow MVD in AA significantly lower than that in control group ($P<0.001$). Further average number of vessels and mean vessel density was analysed in subcategories of AA. There was a decreasing number in the average no. of vessels per field as well as decreasing trend of MVD with the increasing severity of the disease. Somasundaram *et al.* (2009), Gupta *et al.* (2017) and Ji *et al.* (2006) also observed in their study that increasing severity of the disease is associated with decrease in the microvessel density hence reduced angiogenesis. In our study a statistically significant difference in MVD of NsAA vs sAA and NsAA vs VsAA whereas there was no statistically significant decrease in MVD of sAA vs. NsAA was observed. Our study is in concordance with the study of Somasundaram *et al.* (2009) and Ji *et al.* (2006) They also observed that the MVD of sAA vs NsAA patients and the MVD of VsAA vs. NsAA being significantly different. However there was no significant difference between sAA and VsAA. However the study of Gupta *et al.* (2017) and Fureder *et al.* (2006) in their study reported significant difference in MVD of controls and AA patients however no apparent difference in the MVD when patients in various subcategories of AA (NsAA vs. sAA vs. VsAA) were compared. These findings probably hint towards decreasing angiogenesis with increasing severity but many more studies with more number of cases will be required to solve the dilemma.

Conclusion

Our findings demonstrate that Camitta *et al.* classification of AA on the basis of peripheral blood absolute neutrophil count, platelet count, corrected reticulocyte count and bone marrow cellularity is essential with ANC being the true indicator of classification and not an optional criterion, with further assessment of TLC also being a good tool for classification. The MVD is a good marker of angiogenesis in patients with AA and there is a significant decrease in angiogenesis in bone marrow as the severity of AA increases.

REFERENCES

Baak JPA. 1991. Manual of quantitative pathology in cancer diagnosis and prognosis. Berlin: Springer-Verlag, 7-18

Bacigalupo A. 2017. How I treat acquired aplastic anemia. Blood, 129(11):1428-36.

Camitta BM, Storb R, Thomas ED. 1982. Aplastic anemia: pathogenesis, diagnosis, treatment, and prognosis. *N Engl J Med.,* 306: 645-718

Erdem F, Giindogdu M, Kiziltunc A. 2006. Serum Vascular Endothelial Growth Factor Level in Patients with Haematological Malignancies. *Eur J Gen Med.,* 3:116-20.

Fureder W, Krauth MT, Sperr WR, Sonneck K, Klupp IS, Mullauer L et al. 2006. Evaluation of angiogenesis and vascular endothelial growth factor expression in the bone marrow of patients with Aplastic Anemia. *AJP,* 168:123-29.

Gupta P, Khurana N, Singh T,Gupta D, Dhingra KK. 2009. Bone Marrow angiogenesis in Aplastic Anemia- a study of CD 34 and VEGF Expressions in Bone Marrow Biopsies. *Haematology*, 14:16-21.

Hamilton PW, Allen DC. 1995. Morphometry in histopathology. *J Pathol.,* 175:369-79.

Jackson P, Blythe D. 2008. Immunohistochemical techniques. In: Bancroft JD, Gamble M, editors. Theory and practice of histological techniques. 6th ed. Philadelphia: Churchil Livingstone., P.433-72.

Ji JL, Liu H, Sun C, Jiang SH, Ding RS. 2006. Expression of Vascular Endothelial Growth Factor in Patients with Aplastic Anemia and its Significance. Zhongguo Shi Yan Xue Ye XueZaZhi,14: 285-8.

Khunger JM, Arylselvi S, Sharma U. *et al.* 2002. Pancytopenia - a clinicohematological study of 200 cases. *Indian J Pathol Microbiol.,* 3:375-9.

Marsh JC, Ball SE, Cavenagh J. *et al.* 2009. Guidelines for the diagnosis and management of aplastic anaemia. *Br J Haematol.,* 147: 43-70.

Nishida N, Yano H, Nishida T. *et al.* 2006. Angiogenesis in cancer. *Vas Health Risk Manag.,* 2:213- 9.

Nissen DC. 1989. Pathophysiology of Aplastic Anemia. *Baillliersclin haematol.,* 2:37-49.

Somasundaram V, Tevatia MS, Purohit A, Ahuja A,Mahapatra M, Tyagi S et al. 2017. Evaluation of Bone Marrow Microvessel Density in Patients with Aplastic Anemia. *Indian J Hematol blood transfuse,* 33: 169-74.

Tuzuner N, Bennett JM. 1994. Reference standards for bone marrow cellularity. *Leuk Res.,* 18: 645–47.

Wang D, Stockard CR, Harkins L, Lott P, Salih C, Yuan K. *et al.* 2008. Immunohistochemistry in the evaluation of neovascularization in tumor xenografts. *Biotech histochem,* 83:179-89.

Weibel ER. Stereological methods. In: Weibel ER, editor. Practical methods for biological morphometry. Vol 1. London: Academic Press. 1979; 44-5.

Wu L, Mo W, Zhang W, Deng H, Li Y,Zhou R. *et al.* 2015. Impairment of hematopoietic stem cell niches in patients with aplastic anemia; *Int J Hematol.,* 102:645–53.

Yoon HH, Huh SJ, Lee JH, Lee S, Kim SH, Kwon HC, Kim HJ. 2012. Should we still use Camitta's criteria for severe aplastic anemia?, *Korean J Hematol.,* 47(2):126-30.

Young, NS. and Maciejewski, J. 1997. The Pathophysiology of Acquired Aplastic Anemia. *N Engl J Med.,* 1997; 336: 1365-72.

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