# Proposing an accurate quantification method for myeloperoxidase staining on peripheral blood smears with varying hemoglobin and absolute neutrophil counts

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**Abstract**

**Introduction:** Myeloperoxidase (MPO), a heme-containing peroxidase is found mostly in the lysosomal azurophilic granules in neutrophils. Since the MPO directly associates with the neutrophil phagocytic system, a large number of diseases that associate with inflammation may directly be linked with MPO levels in neutrophils suggesting it as an active disease biomarker. As the present MPO estimation methods are extremely expensive, development of a slide based quantifiable staining method for MPO is a need.

**Aim:** To develop a simple quantifiable staining method for MPO in neutrophils on peripheral blood smear with varying hemoglobin concentrations.

**Method:** Total of 107 patients varying hemoglobin concentrations who attended Hematology Clinic at Colombo North Teaching Hospital, Ragama, Sri Lanka were selected and peripheral blood smears of them were initially fixed with cold buffered acetone, stained with 3-3 Di amino benzidine (3, 3 DAB) and, counterstained with Hematoxylin. Finally, Total MPO of each patient was calculated using a modified formula.

**Results:** The preliminary findings indicate that the higher intensity groups directly correlates with the higher values of the MPO score whereas the lower intensity groups inversely correlate with the Total MPO scores. The proposed reference range for MPO is 57.6 to 68.4. Total MPO: Absolute Neutrophil Count (ANC) Ratio too correlates with the intensities as the same. Accordingly, the proposed reference range for MPO is 16.4 to 31.4.

**Conclusion:** In this study, we proposed reference ranges for Total MPO estimation. Validation of this method to be performed by comparing with other MPO quantification methods.

Keywords: Myeloperoxidase staining; 3, 3-diaminobenzidine; Benzidine; Absolute Neutrophil Count; Iron Deficient; Quantification method.

## 1. Introduction

Myeloperoxidase (MPO) staining is one of the major cytochemical stains used in the identifications of the myeloid cells that present in peripheral blood films and bone marrow films in acute myeloid leukemia (AML).[1,2,3] This stain has been replaced by Sudan Black B due to the carcinogenic effect of the main reagent, benzidine used in MPO staining.[4,5] So, the benzidine has been replaced by 3-3 Di amino benzidine (3, 3 DAB) and MPO staining is still used to detect myeloperoxidase activity mainly in tissues in advanced laboratories as an immunocytochemical method.[6] However, the main disadvantage of immunocytochemical stain is the extremely high cost compared to the income-expenditure in developing and under-developed countries and continuation of the staining procedure is unworkable. Therefore, development of a suitable method that could address the aforesaid matters would be absolutely important.

Neutrophil is the principal cellular producer of Myeloperoxidase (MPO). With MPO levels in these cells ranging from 2–5% of total cellular protein in humans, or 2–4 mg per $10^6$ cells.[7] MPO is a heme-containing peroxidase, a crucial component of the innate immune system to provide defense against invading pathogens.[8,9,10] MPO is found mostly in the lysosomal azurophilic granules and accounts for around 5% of the dry mass of neutrophils.[11] MPO catalyzes the synthesis of reactive oxygen intermediates, such as hypochlorous acid, in the presence of hydrogen peroxide and halides (HOCl). Microbial death by neutrophils relies heavily on the MPO/HOCl system.[8,12,13] Additionally, studies have demonstrated that MPO is crucial for capping the respiratory burst since people with MPO-deficient neutrophils experience extended respiratory bursts and higher levels of hydrogen peroxide generation.[14,15] Since the MPO directly associates with the neutrophil phagocytic system large number of diseases which are associated with inflammation may possibly directly be linked with MPO levels in neutrophils and neutrophil burst conditions. By reviewing the literature, Khan et al. (2018)[16] stated that enhancement of the MPO levels direct and indirect involvement in various diseases or conditions such as CVD and atherosclerosis, Diabetes/diabetic retinopathy, Renal diseases, Liver diseases, Lung injury, Cystic fibrosis, Multiple sclerosis Alzheimer's disease, Parkinson's disease, Tuberculosis,, Rheumatoid arthritis Colitis and Pancreatitis suggesting it as an active disease biomarker.[17,18,19,20,21,22,23,24,25,26,27,28,29,30] Furthermore, a study carried out by Xiao et al. (2018)[31] revealed that MPO deficiency reduces systemic and dietary iron induced adverse effects. It has been suggested that MPO secondary deficiency (non-inherited) in polymorphonuclear (PMN) leukocytes can be occurred due to iron deficiency (ID) and iron deficiency anemia (IDA).[32] Therefore, targeting MPO levels in neutrophils may be an effective way to reduce the negative effects of iron supplementation.

In such a situation, it is an essential requirement to develop an accurate quantification method for the MPO present in neutrophils using basic staining methods. In this paper we propose such a method for iron deficient patients with varying absolute neutrophil counts aiming it to apply for other compatible diseases or conditions.

## 2. Materials & Methods

### 2.1. Method A

Peripheral blood smears were prepared from collected blood samples and stained with Benzidine. Staining procedure is discussed below in detail:

#### 2.1.1. Reagents

Fixative – 10% Formal Ethanol
Substrate – Benzidine
(Working substrate – add 30mg Benzidine in 60ml buffer, add 120µl H2O2)
Hydrogen Peroxide ($H_2O_2$)

Hematoxylin

2.1.2. Procedure

1. Use fresh smears of blood in EDTA.
2. Fix air dried smears for 60 seconds in 10% Formal Ethanol.
3. Rinse thoroughly in gently running tap water.
4. Incubate for 10 minutes in working substrate solution.
5. Rinse thoroughly in gently running tap water.
6. Counterstain with hematoxylin for 3 minutes.
7. Rinse in gently running tap water, air dry the smears and observe under microscope.
8. Changes were done after observing smears under microscope.
    - Time allocated for counter staining were reduced to 1 minute after several attempts done with changes of hematoxylin incubation time.
    - 10% Formal Ethanol fixative was replaced with Cold Buffered Formal Acetone as formal ethanol fixed smears showed fragmented RBC like background.

2.2. Method B (Improved)

The staining method was changed by introducing 3, 3 DAB instead of benzidine according to Mahjoub et al. (2015)[6] with a few modifications. Peripheral blood smears were prepared from collected blood samples and stained with 3, 3-diaminobenzidine (DAB) chromogen.

2.2.1. Reagents

Fixative – Cold Buffered Formal Acetone (BFA)
Substrate – 3, 3-diaminobenzidine (DAB)
(Working substrate – Add 6 drops of 1 in 10 diluted DAB, add1 drop of H2O2)
Hydrogen Peroxide (H2O2)
Haematoxylin

2.2.2. Procedure

1. Use fresh smears of blood in EDTA.
2. Fix air dried smears for 30 seconds in cold BFA.
3. Rinse thoroughly in gently running tap water.
4. Incubate for 3 minutes in working substrate solution.
5. Rinse thoroughly in gently running tap water.
6. Counterstain with hematoxylin for 1minute.
7. Rinse in gently running tap water, air dry the smears and observe under microscope

## 3. Results

Initially, by employing the staining method A, which consists of 7 steps provided the results with the dark black coloured neutrophils as shown in Fig 1.

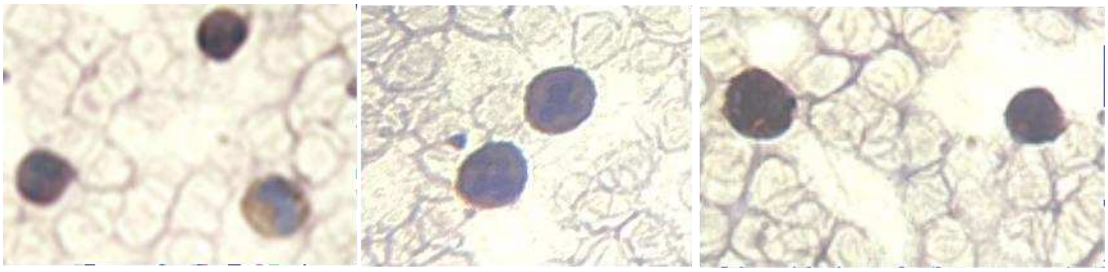

Fig 1: Darkly stained neutrophils filled with MPO with a clear background.

Even though the background staining is satisfactory, neutrophils with different intensities cannot be distinguished due to the dark coloured staining of their cytoplasm as well as the nucleus. So, the staining method was changed by introducing 3, 3 DAB instead of benzidine according to Mahjoub et al. (2015)[6] with a few modifications. To improve the nuclear stain into an optimum extent the counterstain time was reduced to 1 minute.

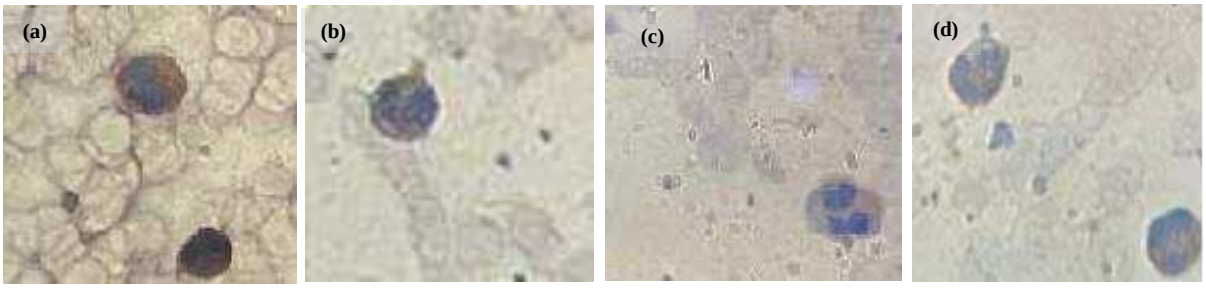


Fig 2: MPO staining performed in different counterstain timings with Hematoxylin. Counter stain was performed in a) 3 minutes; b) 2 minutes; c) 1 minute; d) One dip.

To improve the background, the fixation was performed by replacing 10% Formal Ethanol fixative with cold Buffered Formal Acetone as stated in Method B (Improved) the results are shown in Fig 3. The improved method B shows a clear nuclear visualization (in blue colour) with the presence of MPO in the cytoplasm (in brown colour).

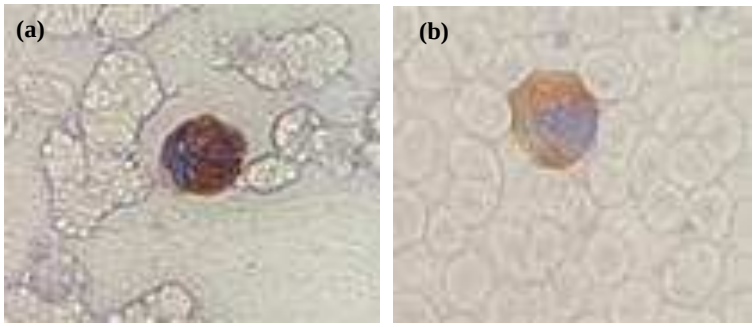


Fig 3**:** Improvement of the background. The blood smear fixed with (a) 10% formal ethanol; (b) cold buffered acetone.

### 3.1. Observations of colour and its arrangements

It has been observed that even after two weeks of preparation the blood smears can be stained successfully using the upgraded, new staining approach. However, the following discrepancies were observed among the neutrophils with respect to the colour and its distributions.

#### 3.1.1. Fulfilment of the colour in cells

It has been observed that the neutrophils are not entirely filled with the colour and, most of the cells show varying percentages of the colour arrangements. This feature is independent of the colour intensity.

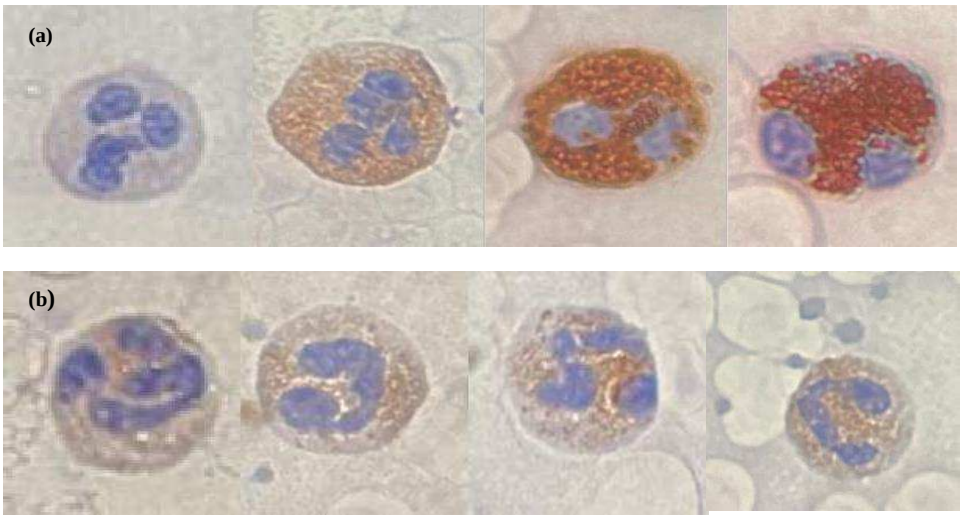


Fig 4: Neutrophils stained with modified MPO staining; a) Colour distributed equally in the entire cells; b) Colour distributed in the portion of the cells.

#### 3.1.2. Two colour intensities in a single neutrophil

The staining has not equally been distributed in certain neutrophils and we observed a single neutrophil with two colour intensities (Fig 5). This feature is prominent in the neutrophils with high MPO intensities.

**Fig 5:** Neutrophils stained with modified MPO staining. Two colour intensities are present in a single cell.

### 3.2. Quantification of modified MPO staining

This research paper suggests a quantification method for modified MPO staining considering all the aforesaid observations. The following features were considered to quantify the MPO in the neutrophils.

3..2.1. Development of a colour code for different intensities

Since the quantification is based on the colour intensities it is essential to develop a colour code that is proportionate to each other while varying them in colour intensities. Adobe Photoshop (Adobe Inc.) was used in the colour code development for 6 different increasing brown colour intensities and it is shown in (Fig 6). The colour intensity is set as proportionate to each other using the opacity value of Adobe Photoshop (Adobe Inc.). For example, the opacity value that represents the +1 intensity is double that of the intensity of +0.5. Likewise, the opacity value that is represented for the +4 intensity is 80% of the maximum value (100%) that is represented for the intensity of +5.

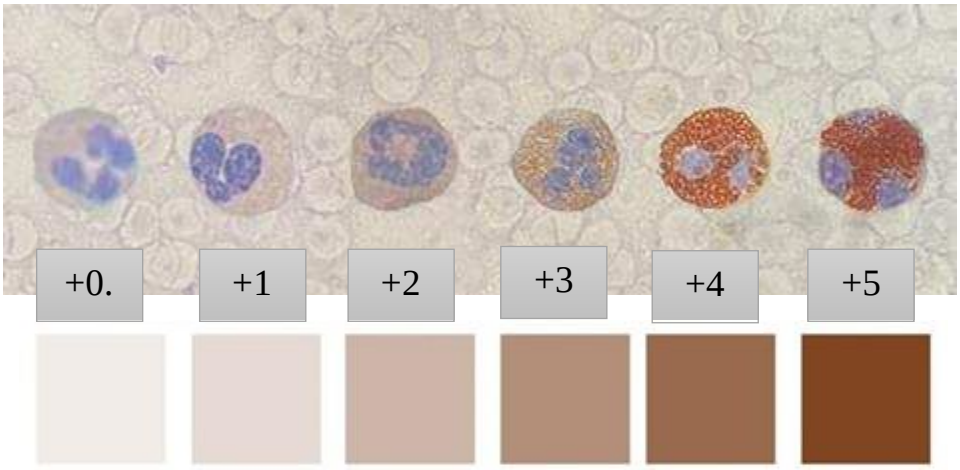


Fig 6: Neutrophils retained different colour intensities in the MPO staining (on top). A colour code (at the bottom) was developed with the well matched colour intensity using the opacity value of Adobe Photoshop (Adobe Inc.). The intensity proportionate is represented proportionally in numbers (+0.5 to +5).

3.2.2. Percentage volumes of the colour present in neutrophils

Since the initial observation shows the partially filled neutrophils in brown colour (Fig 4(b)) the percentage volumes from the total volumes of the neutrophils that filled with the brown colour are also considered while developing an accurate quantification method for modified MPO. Fig 7 shows the varying percentage of MPO in volumes with the different colour intensities.

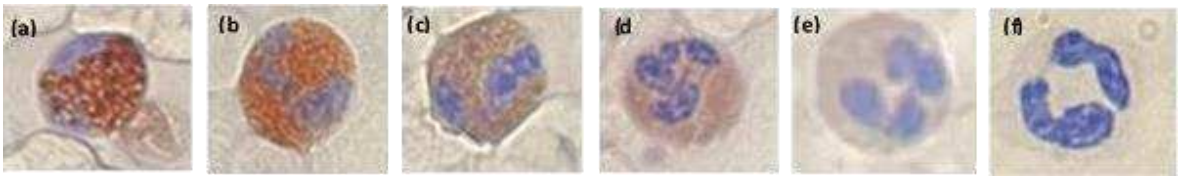


Fig 7**:** Different percentage volumes of MPO in neutrophils in varying brown colour intensities. a) 80% with +5; b) 70% with +4; c) 90% with +3; d) 65% with +2; e) 60% with +1; f) 55% with +0.5.

3.2.3 Two colours in in single neutrophil in different percentages

As observed in Fig 6 certain neutrophils have been stained in the modified MPO staining with two brown coloured intensities and these types of neutrophils are quantified by considering the both intensities and their

respective percentage volumes of MPO. Fig 8 shows an ideal example of it.

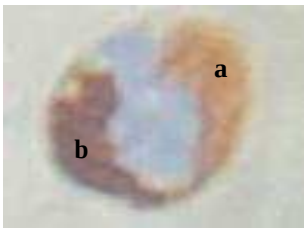


Fig 8: Varying percentage volumes of two different brown colours in a single neutrophil. It considers two events in a single cell in MPO calculation. a) Colour intensity +2 with a percentage volume 30%; b) Colour intensity +5 with a percentage volume 25%.

### 3.3. Proposing an equation to calculate MPO present in neutrophils

Considering the above quantifying steps the following equation is proposed for the accurate calculation of MPO orient in the neutrophils.

$$\text{Total MPO} = \Sigma [I \times (Pi/100) \times N]$$

Where, I = Brown colour Intensity (+0.5, +1, +2, +3. +4, +5)
Pi = Percentage (i = 1 to 100)
N = Number of Neutrophils

### 3.4. MPO calculations performed according to the modified method

Total of 107 patients who attended the Haematology Clinic at Colombo North Teaching Hospital, Ragama, Sri Lanka were selected for MPO calculation. Their Hemoglobin (HB) values ranged from 4.7 g/dl to 17.2 g/dl. Total MPO of each patient was calculated using the above mentioned formula. Maximum number of 50 neutrophils was used in each calculation. Furthermore, I x (Pi/100) x N was separately calculated for each patient and six I x (Pi/100) x N categories were defined based on the colour intensity (I). Non-parametric Spearman's rho Bivariate Correlations among the ANC, MPO, HB and I x (Pi/100) x N were established using SPSS (Version 21, IBM, USA). The patients were further categorized based on the ANC; i.e. ANC < 2.50 X $10^3/mm^3$, 2.50 X $10^3/mm^3$ =<ANC < 7.50 X $10^3/mm^3$ and ANC > = 7.50 X $10^3/mm^3$ and the same correlation analysis was performed for each group.

The Total MPO whole group showed the Mean of 62.9 with standard error of 2.79. The 95% Confidence Interval for Mean Lower bound and Upper Bound for the MPO are 57.6 to 68.4 respectively. The Correlation analysis (Pearson Bivariate) performed for the whole group reveals that none of the parameters, Total MPO, HB or ANC showed significant correlations among them. However, the correlation analysis (Non-parametric Spearman's rho Bivariate) of Total MPO and most of the I x (Pi/100) x N (I= +0.5 to +5) groups show statistical significance ($p<0.01$) with positive correlations with I =+3, I =+4 and I =+5 groups and negative correlations with I =+1 and I =+0.5 groups. I=+2 group shows weak positive correlation.

The correlation analysis (Non-parametric Spearman's rho Bivariate) performed among I x (Pi/100) x N (I= +0.5 to +5) groups showed positive significant ($p<0.01$) correlations in between I =+5 & I =+4; I =+4 & I =+3; I =+3 & I =+2; I =+2 & I =+1; I =+1 & I =+0.5 whereas, negative significant ($p<0.01$) correlations in between I =+5 with I =+0.5, I =+1; I =+4 with I =+0.5, I =+1, I =+2; I =+3 with I =+0.5, I =+1; I =+2 with I =+0.5 among them. Those findings indicate that when the intensity groups are closely in their colour intensities they always

showed a positive correlation among them whilst when they are in two ends in intensities, associated with negative correlations.

In the next step, the patients were categorized according to their Absolute Neutrophil Count (ANC); Neutropenia (ANC < 2.50 X $10^3/mm^3$), Normal neutrophil count (2.50 X $10^3/mm^3$ =<ANC < 7.50 X $10^3/mm^3$) and Neutrophilia (ANC > = 7.50 X $10^3/mm^3$) groups. The aforesaid similar findings were observed in the Total MPO in all 3 groups. During the MPO manual calculations, it has been observed that increased values of Total MPO with normal HB (>12.0 g/dl) for those who are in neutropenia group; Increased values of Total MPO with low HB (<12.0 g/dl) for those who are in normal neutrophil count group; Decreased values of Total MPO with low HB (<12.0 g/dl) for those who are in neutrophilia group. However, in the correlation bivariate analysis, none of the above findings were significantly correlated with each other.

Since there is no significant correlation between Total MPO and ANC in the whole group or the aforesaid sub ANC groups, Total MPO: ANC Ratio was calculated in order to normalize the Total MPO values with the total number of neutrophils. The Total MPO: ANC Ratio of the whole group showed the Mean of 23.9 with standard error of 3.76. The 95% Confidence Interval for Mean Lower bound and Upper Bound for the MPO are 16.4 to 31.4 respectively. The Correlation analysis (Pearson Bivariate) performed for the whole group of Total MPO: ANC Ratio reveals that none of the parameters, Total MPO, HB or ANC showed significant correlations among them. However, similar findings that were obtained for the Total MPO were observed in the Total MPO: ANC Ratio too.

## 4. Discussion

MPO that is present in the myeloid cells is estimated by flow cytometry, immunohistochemistry, or cytochemical staining using automated or manual methods.[1,33,16] Although several MPO assays have been described, no proper consistency for most standard assays has been proven. The main reason is that the substrates used in MPO staining are not MPO specific.[34] Also, HB also shows some peroxidase activity and other peroxidases or peroxidase inhibitors may interfere with the output results.[16,34] Since the flow cytometry or ELISA methods bear extreme cost, developing a simple manual staining method on a blood smear are still economical. On the other hand, the carcinogenetic effect of using benzidine has been overcome by replacing it with 3, 3-DAB.[6] Therefore, we made an attempt to develop a more improved manual quantitative method for MPO to stain neutrophils on the blood smear. To our knowledge this is the first time a manual staining method was used to quantify MPO in peripheral blood neutrophils.

In this study, a total of 107 patients who attended Haematology Clinic at Colombo North Teaching Hospital, Ragama, Sri Lanka with varying HB concentrations were selected for MPO calculation. Method described in Mahjoub et al (2015)[6] was modified by lowering the time allocation for the incubation of substrate to 3 minutes and hematoxylin to 1 minute. In addition, buffered formal acetone was used as the fixative instead of their formal ethanol fixative (Fig 3). The main observations of colour and its arrangements of the stained neutrophils are unorganized fulfilment of the colour in cells (Fig 4) and appearing two colour intensities in a single neutrophil (Fig 5). Considering all the aforesaid complications a quantitative method for Total MPO in blood neutrophils has been developed. A limited quantification methods are available using stained blood smears and the commonest one is Neutrophil Alkaline Phosphatase (NAP) that is mainly used to differentiate Chronic Myeloid Leukemia (CML) from leucomoid reactions.[35,36] In this method, the scoring system is based on colour intensity and the number of neutrophils only. The percentage of the colour represents in neutrophils has not been considered.[35] and it is the major drawback of this method. We were able to overcome this and also to quantify even two intensities present in one cell. The other shortcoming of the NAP score method is that the reporting of colour intensities may vary from the naked eye of person to person. We were successful in overcoming it by introducing an Adobe Photoshop (Adobe Inc.) based method for classifying the colour intensities (Fig 7) and therefore, it is irrespective of the person who quantifies this. So, the new method considers

the Brown colour Intensity (I), Percentage (Pi) and Number of Neutrophils (N) to provide an accurate quantification. Chand et al (2019)[37] used a method with similar parameters to develop a scoring system for oestrogen receptor/progesterone receptor in breast cancer tissues. Choudhury et al (2010)[38] too introduced a robust automated method for measuring average antibody staining in immunohistochemistry images and the quantification method has similar features to that of our method.

We were not only able to develop the method for MPO quantification in blood smears but also to apply it to the patients' community. Patients in varying HB concentrations (4.7 to 17.2 g/dl) were used, and the preliminary findings indicate that the higher intensity groups directly contributed to the higher values of the MPO score whereas the lower intensity groups negatively contributed to the Total MPO scores. It has been noticed that the presence of two colour intensities in narrow intensity margins, they tend to control the other directly and the presence of two colour intensities in wide intensity margins they tend to control the other inversely. Furthermore, we were able to propose a reference range (57.6 to 68.4) for the said quantification method. As the similar correlations were achieved in the sub groups; neutropenia, normal neutrophil count and neutrophilia the quantification method was further modified by dividing the Total MPO by the ANC and this too gave similar findings. Establishing a reference range using Total MPO: ANC Ratio would be more reliable than that of Total MPO as it provides the Total MPO estimation per neutrophil as it is irrespective of whether the person is in normal neutrophil count, neutropenia or neutrophilia. Accordingly, the proposed reference range for MPO is 16.4 to 31.4.

## 5. Future Scope

Since there is a need for a MPO assay, our scope is to develop it based on the blood film as it always comparatively low cost. In such situation, our group is planning to develop a blood film based assay kit for MPO in future. In addition, the association between Serum Ferritin and MPO is being studied. In a long run, a test kit method that could replace the serum ferritin by MPO will be developed.

## 6. Conclusion

In our study, we were able to develop a quantification method as well as to show the correlations among staining of different sets of neutrophils and the Total MPO. Furthermore, we proposed reference ranges for Total MPO estimation. However, before implementing this method as a quantification method it should be improved by applying with wider ranges of patients in varying HB concentrations. Meantime, validation of the methods to be performed by comparing with other MPO quantification methods. Even though a few MPO assay methods such as ELISA and Flow cytometry, Immunohistochemistry methods are being used in laboratories, to our knowledge no proper comparisons have been made in between them yet.

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