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A Study Protocol for Evaluation of Microcytic Hypochromic Anemia by High-Performance Liquid Chromatography (HPLC)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Study Protocol

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ABSTRACT

Background: One of the most regularassessments done in clinical practice is anemia. Different levels classify the anemiasbased onthe numerical value of hemoglobin, alteration in the morphology and chromium, underlying etiologies, red cell volumetric parameters and functionally depending on pathophysiologic processes of anemia. Microcytic hypochromic anemia (MCHC) has different underlying causes, including iron deficiency anemia, beta-thalassemia trait, and hemoglobinopathies. Before planning the treatment, assessing the hemoglobin for its variants and the detection of abnormal hemoglobinis mandatory. High-performance liquid chromatography (HPLC) has proved to be significant for the evaluation of MCHC because of its superior separation and quantification analytical powers.The present study has been undertakingthat anemia is the most prevailing and commonly treated clinical state managed by numerous nutritional supplements without much being done to know the underlying etiology or without evaluation of hemoglobin for its abnormalities.
Methods: This will bean observational (Prospective and Retrospective) study. Blood samples of

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100 patients will be evaluated by HPLCBio-Rad variant,and various hemoglobin patterns; associate dhemoglobinopathies will be evaluated.
Results: The observations will be made according to the objectives and tabulated, which will be subjected to statistical tests for their significance and conclusions.
Conclusion: This study would contribute to understanding and knowing the etiology of MCHC in the population that our tertiary care hospitals serve.

Keywords: High-performance liquid chromatography; anemia; microcytic hypochromic; bio-rad; hemoglobin.

1. INTRODUCTION

One of the commonest assessments done in the clinical practice is for the state of anemia. Different levels classify the anemiasbased onthe numerical value of hemoglobin, alteration in the morphology and chromium, underlying etiologies, red cell volumetric parameters, and functionally depending on pathophysiologic processes of anemia. Whatever the scheme of classification may be, the usual approach to classify anemia is morphological [1].

The morphological manifestation within the erythrocytes for its size and chromium has long been evaluated for underlying etiology. The appreciation for this morphological alteration is always manual by microscopy of a stained blood film and the electronic cell counters in modern hematology laboratories [2].

The clinicians commonly encounter a morphological class of anemia that of microcytic hypochromic anemia (MCHC) alternately allows cell volume anemia, which has plentiful underlying etiologies. The MCHCfor its etiologies includes the underlying causes of iron deficiency state, beta-thalassemia trait, hemoglobinopathies because of HB E, HB D, HB C, and many others. Such a situation of MCHC before being embarked for the treatment requires assessing the hemoglobin for its variants and detecting abnormal hemoglobin [3].

The traditional approach to evaluating MCHC was to differentiate the underlying etiologies of iron deficiency anemia or beta thalassemia trait by knowing the red blood cell indices such as Mentzer index, Green and King's index, and many others. However, these indices can only be used for screening the limited situation and etiologies. MCHC has far broad underlying etiologies. For this, hemoglobin studies are needed [4].

Hemoglobin mutation is one of the single most common "single gene disorders" that manifests uniquely as microcytic erythrocytosis (MCHC) [5].

The treatment of MCHC is the iron replacement therapy if the iron deficiency is suspected as its cause. However, the therapeutic iron can produce harmfuleffects if the underlying pathology for MCHC is otherwise like, beta-thalassemia trait, sickle cell disease, or the other hemoglobinopathies [1-5].

Therefore, an MCHC popularly called in pathology as microcytic erythrocytosis must undergo the evaluation of the hemoglobin for its variant and abnormality before the iron therapy is prescribed [6-11].

A few studies have evaluated Microcytic erythrocytosis (MCHC) for the result of hemoglobin variants and hemoglobinopathies [1-11].

The recent year's interest has been generated to analyze the hemoglobin variant and abnormal hemoglobinopathies in the situations of microcytic erythrocytosis enabled by the advent of technology. There is a shift of evaluation of hemoglobinopathies from simple solubility test to high definition electrophoresis to high-performance liquid chromatography (HPLC) with its superior separation and quantification analytical powers [5,11].

The present study has been undertaken as anemia is the most prevailing and commonly treated clinical state managed by numerous nutritional supplementswithout much being done to know the underlying etiology or without evaluation of hemoglobin for its abnormalities.

1.1 Research Gap

The ignorance about the abnormality of hemoglobin results in the unfair treatment of anemia. This is always observed with MCHC, which has numerous underlying hemoglobin-

related abnormalities. The overall treatment by iron is a common danger that can prove deteriorating and fatal if the status of beta-thalassemia trait and other hemoglobinopathies are unknown. Such studies that evaluated hemoglobin variants and hemoglobinopathies from India are infrequent. This study would contribute to understanding and knowing the etiology of MCHC in our tertiary care hospitals' population.

1.2 Research Questions

- Do the knowledge of hemoglobin variants and abnormal hemoglobinopathies is required to evaluate MCHC on HPLC.
- With this backdrop of the review of ed literature knowledge, the present study is organized for its aims and objectives below.

1.3 Aim and Objectives

1.3.1 Aim

The present study aims to assess MCHC by chromatography (HPLC) to know associated hemoglobin variants and hemoglobinopathies.

1.3.2 Objectives

1. To study the MCHC (Microcytic erythrocytosis) to know its association with hemoglobin variants and abnormal hemoglobins by HPLC.
2. To know the frequencies of the hemoglobin variants and abnormal hemoglobins with MCHC on evaluation by HPLC.
3. The role of HPLC in segregating MCHC of iron deficiency versus beta-thalassemia trait. In the cases where hemoglobin electrophoresis was ambiguous for results.

2. SHORT REVIEW

The literature search over the topic of assessment of MCHC by HPLC by electronic vial media has shown multiple studies related to single or multiple parameters about variants of hemoglobin and hemoglobinopathies. Two such studies are presented as a short review below for their inferential abstract.

Joneja et al. carried out a study to evaluate the hypothesis that the reasons for hemoglobin variant abnormality (HVA) performed in the patients revealed Microcytic erythrocytosis without accompanying anemia for pluralistic causes. The study comprised 137 patients in the hemoglobin range of 7.20 to 16.1 gm/dl with low or decreased MCV with a median value of 64 fl.

Those patients' blood samples were run for high-performance liquid chromatography bio-rad variant. The results revealed that 93 of 137,i.e. (67.9%) patients could be diagnosed as thalassemia trait and or hemoglobinopathy as a cause of microcytic erythrocytosis.

The common abnormalities that were underlined for MCHC were of beta-thalassemia trait, delta/ beta-thalassemia trait, hemoglobin E disease, hereditary persistence of fetal hemoglobin (HPFH), possible HPFH, HPFH with beta-thalassemia, delta/ beta-thalassemia iron deficiency anemia, hemoglobin C trait with beta-thalassemia, hemoglobin C trait with possible alpha thalassemia, hemoglobin C with HPFH, unidentified hemoglobinopathy with possible delta/beta-thalassemia, hemoglobin S trait combined with possible alpha and possible beta-thalassemia trait.

Seventeen patients showed regular hemoglobin patterns on HPLC with predetermined cutoffs. The study concluded that hemoglobin variant analysis provided a very high positive yield in determining the etiology of microcytic erythrocytosis. Therefore, the patients diagnosed with MCHC should regularly analyze hemoglobin abnormality. In their study of MCHC by HPLC have identified the objectives of knowing the underlying hemoglobinopathies. The study mainly focusedon antenatal cases along with patients with anemia. The study conducted overfour years was performed, and the instrument high-performance liquid chromatography BIO RAD variant analyzer. The blood samples were collected in the quantity of 2 ml in an EDTA vial. The samples went under the complete blood count (CBC), iron stores, high-performance liquid chromatography. As part of population statistics, the primary abnormality of hemoglobin observed as a cause for MCHC was of beta-thalassemia trait (high HB A2). 15.8 cases displayed abnormal hemoglobin pattern of which 20.3 were of the other abnormalities like beta-thalassemia major, beta-thalassemia intermedia, sickle cell trait, Hb S/ beta-thalassemia, sickle cell disease, HPFH, HbE/ beta-thalassemia, homozygous HbE ds, HbD-Punjab trait, homozygous HbD- Punjab HBD/beta-thalassemia, HbQIndia trait.

The study concluded a high prevalence of hemoglobinopathies amongst the patients

revealing MCHC, especially in antenatal care patients.

The high-performance-performance liquid chromatography helped prevent unnecessary iron loading and unwanted blood transfusions. The study recommended the regular use of high-performance liquid chromatography to evaluate MCHC in avoidance of inappropriate treatment.

3. MATERIALS AND METHODS

The following material and methods will be adopted for the present study:-

Recording of preliminary data in proforma with following details:-

a. Name
b. Age
c. Gender
d. Ward
e. OPD
f. Unit in charge
g. MRD
h. Complains
i. Comorbid conditions

Place of study: Department of Pathology JNMC, SawangiMeghe, Wardha.

Duration of study: Two years.

Study Design: Observational study design.

Sample size: Ninety-seven cases by formula rounded to 100 cases.

$$n = (Z^{a/2}) 2 \times p \times (1- p) / d2$$

Where

$Z^{a/2}$ is the level of significance at 5 %, i.e., 95% confidence interval.
p = Prevalence of breast carcinoma
d = desired error of margin
n = sample size

Subject characteristics: Described below for inclusion and exclusion.

Inclusion criteria:-

a. Patients with microcytic hypochromic anemia (MCHC) as determined by values of cell counter and blood film microscopy.
b. The patients are refractory to the conventional treatment of MCHC by iron.
c. The patients with microcytic hypochromic anemia (MCHC) on two consecutive laboratory evaluations spanned over one month.

Exclusion criteria:-

a. The patients with known comorbid causes of MCHC.
b. The patients who have received the blood transfusion as a treatment of MCHC.
c. Patients under the age of one year.

Investigations:-

a. The complete blood count to be performed on automated electronic cell counters by standard methods.
b. Microscopic evaluation of RBCs on the stained preparation of peripheral smear.
c. The investigation of high-performance liquid chromatography (HPLC) to be carried out on a whole blood sample

Technical Method of HPLC in the Detection of Hemoglobin Variant and Abnormal Hemoglobin [12]:-

1. Switch on the HPLC, and look for the screen on HPLC for Min 0 and Max 380. After switching on the computer, double click on the LC solution software on the desktop.
2. Then double click on PDA (1) and click OK There is a sound of the beep, which means the instrument is connected to a computer (LC solution software) system.
3. Completed window with one or two graphs will open (click on instrument parameter if the window does not appear)
4. Click the Advanced button, which is below the graph
5. Click on the pump. Change the “Total pump A flow” to 3 ml/min
6. Then click on PDA to check that the lamp is on OFF mode.
7. Then click on file – save method file as – click our method name – click on save – yes
8. Press download, then half turn the knob on the pump carefully and press the purge button. Purging will start automatically, which is already set by the system for 5 min.
9. During purging, “purging line” appear on the screen of the pump.
10. After the purging, Min 0- Max 380 pressure limits appear on the pump screen. Return the knob as it is.

11. Click on the pump and change the “Total pump A flow” to 1 ml/min on the computer. Press download- Yes
12. Then press the “oven” key on the oven and “pump” key onthe pump and look for the increasing pressure on the screen of HPLC
13. Washing of column with distilled water starts. Wash the HPLC column with filtered and sonicated DW for 30 min. at 1 ml/min. Flow rate. The 30 min. Time is to be maintained by the researcher.
14. After that, wash the column with filtered and sonicated ACN:DW (70:30) for valproate assay and MeOH:DW (70:30) for PBT, PHT, CBZ, and LTG assay 30 min. at 1ml/min. By placing the filter above HPLC into the beaker containing the desired solution. The 30 min. time is to be maintained by the researcher
15. Prepare the mobile phase as per the assay given in SOP.
16. Filter the mobile phase and sonicate for 15 min.
17. Saturate the column with the mobile phase for 10 min. At flow rate 1ml/min. The 30 min. Time is to be maintained by the researcher.
18. After 10 min. Change the flow rate (total pump flow) as per test protocol on the computer.
19. Click PDA. Change the lamp to D2. Then right-click on chromatogram- display setting- PDA – change the wavelength as per test protocol- OK – Apply- Ok- download- Ok.
20. Check the D2 lamp (on) on the PDA instrument (Detector)
21. Wait for 10 min. Click on plot (on the upper right side corner) to check the baseline.
22. After that, click acquisition- single run- sample name – sample ID- OK
23. Then put the sample syringe into injector- turn the injector to load position - inject the sample- turn the injector knob to inject position (downward)
24. Graph will be plotted automatically. Repeat the steps v and w for the following sample
25. After the last sample,put the oven off and pump off- change the flow rate to 3 ml/min- put the (PDA) lamp off and download- OK.
26. Repeat the procedure 8,9,10,11,12,13.

Statistics:-

Statistical Tools: The correlation will be carried out by statistical tests and values of significance compatible with said objectives. (p-value), Fischer exact test, Univariate comparisons.

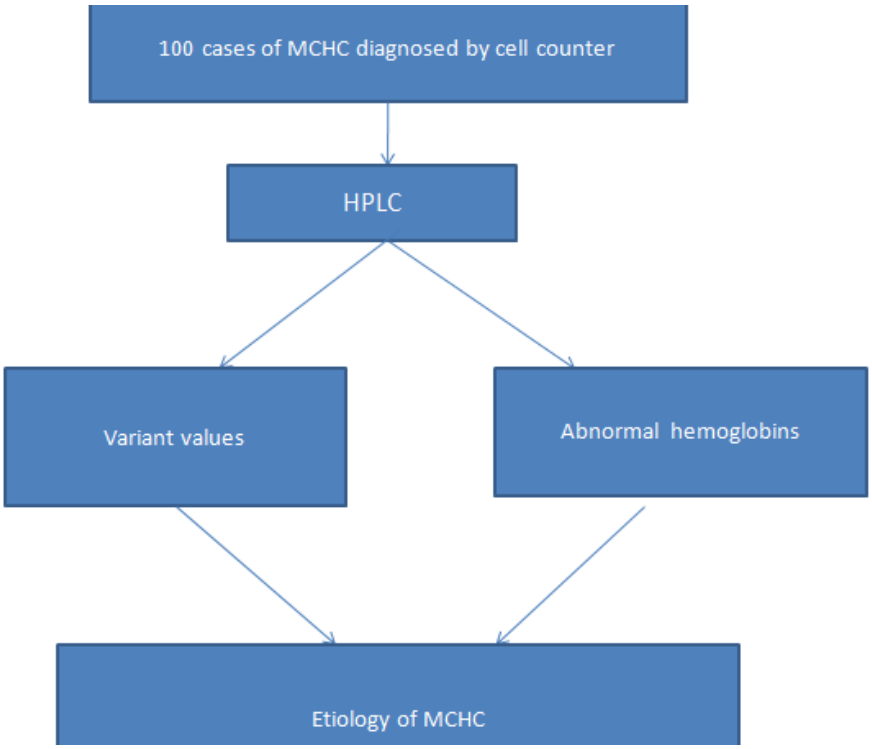

Fig. 1. Scheme of methodology

4. RESULTS

The observations will be made about the objectives and will be tabulated. These observations will be subjected to statistical tests for their significance and conclusions.

5. DISCUSSION AND CONCLUSION

The present study results will be compared with the studies published in the literature with similar objectives.

Joneja et al. carried out a study to evaluate the hypothesis that the reasons for hemoglobin variant abnormality (HVA) were performed in the patients revealing Microcytic erythrocytosis without accompanying anemia for pluralistic causes. The study comprised 137 patients in the hemoglobin range of 7.20 to 16.1 gm/dl with low or decreased MCV with a median value of 64 fl.

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The common abnormalities that were underlined for MCHC were of beta-thalassemia trait, delta/ beta-thalassemia trait, hemoglobin E disease, hereditary persistence of fetal hemoglobin (HPFH), possible HPFH, HPFH with beta-thalassemia, delta/ beta-thalassemiawith iron deficiency anemia, hemoglobin C trait with beta-thalassemia, hemoglobin C trait with possible alpha thalassemia, hemoglobin C with HPFH, unidentified hemoglobinopathy with possible delta/beta-thalassemia, hemoglobin S trait combined with possible alpha and possible beta-thalassemia trait [12,13].

Seventeen patients showed regular hemoglobin patterns on HPLC with predetermined cutoffs. The study concluded that hemoglobin variant analysis provided a very high positive yield in determining the etiology of microcytic erythrocytosis. Therefore, the patients diagnosed with MCHC should regularly analyze hemoglobin abnormality.

In their study of MCHC by HPLC have identified the objectives of knowing the underlying hemoglobinopathies. The study mainly focused the antenatal cases along with patients with anemia. The study conducted overfour years was performed, and the high-performance liquid chromatography BIO-RAD variant analyzer was used. The blood samples were collected in the quantity of 2ml in an EDTA vial. The samples went under the complete blood count (CBC), iron stores, high-performance liquid chromatography. As part of population statistics, the primary abnormality of hemoglobin observed as a cause for MCHC was of beta-thalassemia trait (high HB A2). 15.8 cases displayed abnormal hemoglobin pattern of which 20.3 were of the other abnormalities like beta-thalassemia major, beta-thalassemia intermedia, sickle cell trait, Hb S/ beta-thalassemia, sickle cell disease, HPFH, HbE/ beta-thalassemia, homozygous HbE ds, HbD- Punjab trait, homozygous HbD- Punjab HBD/beta-thalassemia, HbQIndia trait.The study concluded that there exists a high prevalence of hemoglobinopathies amongst the patients revealing MCHC, especially so in patients in antenatal care. Evidence of different hemoglobinopathies is available from GBD Studies [14-16]. Sain et al. reported discriminant indices for distinguishing beta thalassemia trait from iron-deficiency anemia [18]. Few other studies related to anemia were reported [19-22].

The diagnosis of high-performance liquid chromatography helped prevent unnecessary iron loading and unwanted blood transfusions. The study recommended the regular use ofhigh-performance liquid chromatography to the evaluate MCHC in avoidance of inappropriate treatment [23-26].

CONSENT

The investigations over the blood sample in AVBRH are carried out by informal consent. The investigations specified in this work do not involve infringement and harm to human subjects participating as a patient in the present study.

ETHICAL APPROVAL

The study doesn't involve major or minor issues offending human subjects.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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