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Manuscript title

Two measures of systemic inflammation are positively associated with haemoglobin levels in adolescent girls living in rural India: A cross-sectional study

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Short title

Systemic inflammation and haemoglobin

Abstract

Objective: This study tested the hypothesis that systemic inflammation is inversely associated with haemoglobin levels in adolescent girls in India.

Methods: The study population consisted of adolescent girls aged between 10 and 19 years living in a remote rural region in Maharashtra State, India. Data were collected on anthropometric measures, and a venous blood sample taken and tested for Complete Blood Count and C-reactive Protein (CRP).

Results: Of 679 individuals who were invited to the research site to participate, data were available from 401 participants giving a response rate of 59%. Median blood CRP was 1.26 mg/L (Range 0.00 to 26.33), and 167 (41.6%) participants had CRP level less than 1.0 mg/L. The mean haemoglobin was 12.24 g/dL (Standard deviation [SD] 1.51), and the mean total White Blood Cells (WBC) count was 9.02 $x10^3/\mu L$ (SD 2.00). With each g/dL increase in blood haemoglobin, the risk of having an elevated CRP of ≥1 mg/L increased with an odds ratio of 1.16 (95% CI 1.01 to 1.33, p=0.03). Total WBC count was also positively associated with blood haemoglobin, increasing by 0.24 $x10^3/\mu L$ (95% CI 0.11 to 0.37, p<0.001) per g/dL increase in haemoglobin. Both analyses were adjusted for age.

Conclusions: In this population, blood haemoglobin levels were positively associated with two measures of systemic inflammation, contrary to the primary hypothesis being tested. Other unmeasured environmental exposures may modify haemoglobin levels in this population. Understanding this observation may help design better public health interventions to improve the wellbeing of adolescent girls in India.

Keywords: Haemoglobin, CRP, Anaemia, Inflammation, Adolescent, India

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Introduction

Anaemia is a public health priority in India. Younger females in particular are at higher risk of anaemia compared to the rest of the population (1, 2). Iron deficiency is considered to be the primary cause of anaemia in girls and women in India (3), and this has resulted in a national supplementation programme, in which iron and folic acid are provided as a population-based intervention that is primarily targeted at adolescent girls and pregnant women (3).

Despite the national supplementation programme and recent economic growth, the anaemia prevalence in India remains very high, resulting in impaired growth with estimates of over 50% in women aged 15 to 49 years in 2015 (4-7). This raises the question as to whether other factors may be contributing to anaemia in females living in India in addition to micronutrient deficiency. One alternative cause of anaemia is chronic systemic inflammation, which results in anaemia by suppressing erythropoiesis as part of the biological process of mobilising host defences to counter infection or injury, at the expense of red-blood cell production (8). This may co-exist with nutritional deficiency (8), and if observed in Indian populations, may contribute to the sub-optimal response to the iron and folic acid supplementation programme. Such inflammation may start due to poor sanitation (9), lack of adequate nutrition (8, 10), indoor air pollution (11, 12), chronic infection (10) or chronic psychological stress (13).

We tested the hypothesis that systemic inflammation as measured by C-Reactive Protein (CRP), and total White Blood Cell (WBC) count is inversely associated with haemoglobin levels in a population of adolescent girls aged 10 to 19 years living in a rural, disadvantaged part of central India.

Methods

The Maharashtra Anaemia Study Phase 2 (MAS 2)

The Maharashtra Anaemia Study Phase 2 (MAS 2) was implemented by the Halo Medical Foundation (HMF), India in collaboration with the University of Nottingham, UK (14). The MAS 2 was a cross sectional study conducted in 20 villages of Osmanabad district of Maharashtra state of India covering approximately a total population of 40,000. The primary objective was to explore the association between systemic inflammation and blood haemoglobin levels. Eligibility criteria for study participants were being female, age 10 to 19, unmarried, and living in the project field area consisting of 20 villages. The study area is one of the marginalised regions in India with limited health and infrastructure facilities (14, 15). The study obtained ethical approvals from the Medical School Ethics Committee of the University of Nottingham, UK (Reference number: FMHS 145-1707), and the Institutional Ethics Committee of the Ashwini Rural Medical College, Hospital and Research Centre, Maharashtra, India (Reference number: ARMCH/IECHR/12/2017).

Recruitment of the study population and data collection

Each village selected for our research had one village health worker who had been appointed by the HMF to work with the organisation on several projects and was trained specifically for the procedures in this project. A research co-ordinator worked full-time over the study duration to plan and implement research activities with the support from the village health workers network. Community-level meetings were conducted from January to April 2018 across 20 villages primarily on Sundays, school holidays and evenings to identify all residents who were eligible for participation (unmarried adolescent girls).

The recruitment and data collection period were from the 24th of April 2018 to the 23rd of August 2018. Contact was made with eligible residents at community-level by village health workers with further support from the research co-ordinator during field visits to invite them to the HMF hospital to participate in the research. Those who were interested to participate in the study were asked to register with their village-based health worker once they had made a decision, who then informed the research co-ordinator over the telephone to plan the hospital visit for data collection purposes. The research co-ordinator or health workers involved in the study did not select participants directly, as participation was entirely

voluntary and dependent on self-registration. Participation was only possible, however, if at least one family member (parents, elder siblings >18 years, or a local guardian) could accompany the adolescent to the hospital. This was one of the ethical requirements as our target population included minors (those less than 18 years of age).

Information about the study was provided to each participant along with their accompanying adult at the HMF hospital verbally as well as in written format in local language. Written informed consents were then obtained from participants and accompanying adults, which were countersigned by the research co-ordinator. Due to logistical resource constraints, the MAS 2 was able to recruit up to 400 adolescent girls and no formal sample size calculations were conducted. No financial incentive was provided to participate in the study and study participation was voluntary. Blood investigations and health services such as consultation with a doctor for all participants were provided at no cost at the HMF hospital. Those with anaemia (Hb < 12.0 g/dL) received medications (IFA supplements) following a medical consultation and then had access to the HMF hospital for further healthcare services and advice up to the 23rd of December 2018. Those who provided consent were first involved in an interview where a validated questionnaire was administered to collect information on sociodemographic, anaemia history, and treatment. Physical measurements of height, weight, mid-upper arm circumference (MUAC) were taken followed by a venous blood withdrawal in a supine position by a phlebotomist and all laboratory investigations were conducted at the HMF hospital using routinely standardised equipment.

Blood sample analysis

Two investigations were conducted immediately on a fresh blood sample- Complete Blood Count (CBC) using Sysmex XP100 cell counter (Sysmex Corporation, Japan) which provided the haemoglobin and total white blood cell count (WBC), and C-reactive protein (CRP) test using a biochemistry analyser Erba Chem Touch (Erba Mannheim, Germany). Weight was recorded using an OMRON digital weighing machine. Height and MUAC were recorded using standardised measuring tapes. All study tools and equipment were checked and validated on the 1st working day of each month by the study co-ordinator across the data collection period. Monthly study equipment reports, data collection progress and overall project monitoring was done by the study lead (AA) in collaboration with project staff and

local co-investigator (PK) who also conducted site inspection visits to ensure good research practice in line with the study protocol.

Statistical analysis

The primary hypothesis of interest was the association between blood haemoglobin and the two measures of systemic inflammation, serum CRP and total WBC count. When the study was designed, the main outcome measure of systemic inflammation was serum CRP. However, when it was apparent that total WBC count was provided by the CBC analysis, and as the hypothesis of interest was systemic inflammation, this was added as a primary outcome measure. All collected data were entered into a computer, then checked and verified by two members of the study team independently. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in metres. BMI-for-age percentile were generated based on the WHO 2007 reference standards (16) using the Stata 13.1 (StataCorp, College Station, Texas, USA). Blood CRP levels were not normally distributed, and a binary variable was created with a cut-off of 1 mg/L, as this has been used previously and is associated with an increased risk of cardiovascular diseases (17). We selected a cut-off of 1mg/dL as this value was recommended by the Centers for Disease Control and Prevention, and the American Heart Association (17). The association of haemoglobin with CRP was analysed using logistic regression, and the association with total WBC count using linear regression. As this is a relatively unique study population, secondary analyses of measures of systemic inflammation with height, weight and MUAC were also performed to utilise all collected data efficiently. Age adjusted analysis were presented wherever permitted. Stata 13.1 was used for the analysis purposes (StataCorp, College Station, Texas, USA).

Results

Across the 20 villages, 679 adolescent girls were identified during community-level meetings as eligible for study participation. Four hundred and two (N=402, 59%) registered and attended the study hospital to provide questionnaire data, and blood investigations were conducted on 401 participants' samples, which constituted the final study population. Median CRP was 1.26 mg/L, and 25^{th} & 75^{th} percentile were 0.47 mg/L and 2.16 mg/L respectively. One hundred and sixty-seven participants (41.6%) had a serum CRP value of less than 1.0 mg/L (Figure 1). The mean haemoglobin was 12.24 g/dL (Standard deviation [SD] 1.51), and 124 (31%) participants had anaemia as defined by a haemoglobin of less than 12.0g/dL. Mean total WBC count was 9.02 $x10^3/\mu L$ (SD 2.00). The Spearman's rank correlation coefficient between serum CRP and total WBC count was 0.065 (p=0.18).

There was a positive association between blood haemoglobin levels and elevated CRP (Table 1). With each gram of increase in blood haemoglobin, the risk of having an elevated CRP increased with an odds ratio of 1.16 (95% Confidence interval (CI): 1.01 to 1.33, p=0.03) after adjusting for age. A positive association was also observed between total blood haemoglobin levels and WBC count. With each gram of increase in blood haemoglobin, total WBC count increased by 0.24 $x10^3/\mu L$ (95% CI: 0.11 to 0.37, p<0.001) after adjusting for age. No other anthropometric factors were associated with serum CRP (Table 1). In the secondary analyses, weight, MUAC and BMI-for-age percentile were also associated with total WBC count (Table 1).

Discussion

This is the first study to explore the association between systemic inflammation and haemoglobin in a population of adolescent girls living in remote rural India. The prevalence of anaemia as defined as a haemoglobin less than 12.0 g/dL was 31%, and the median serum CRP was relatively high at 1.26 mg/L. There was a positive association between two markers of systemic inflammation (serum CRP, total WBC count) and blood haemoglobin. This observation was unexpected and contrary to the primary hypothesis that was being tested. These positive associations from Indian adolescent girls living in a remote rural environment suggest that the association between systemic inflammation and anaemia is different in our study population compared to elsewhere.

The MAS 2 project has several strengths. Modern analytical techniques were used to measure haemoglobin, total WBC count and CRP values on blood samples that were collected near to the place of analysis. Laboratory devices were routinely tested for accuracy over the study duration. The data were collected by experienced research team who had access to laboratory facilities despite the remote location ensuring that all research procedures were followed as per the protocol. The study response rate of 59% was good considering the size of the field area with the nearest village being located 4 kilometres from the data collection site (HMF hospital), and the farthest 50 kilometres away. To our knowledge this is the first study investigating the association between any markers of systemic inflammation and haemoglobin in Indian adolescent girls. Our study population live in rural *difficult-to-reach areas* and can be regarded as relatively neglected from a public health research perspective. Sampling bias is unlikely in our study population as all adolescent girls within the pre-specified age range were eligible to participate in the study, and the decision to participate was made by the participant. Therefore, these data provide an opportunity to increase understanding of causes of anaemia and subsequently design public health programmes for adolescent anaemia prevention and control for this population where risk factors for anaemia may be different to elsewhere. However, our data have certain limitations. We did not have access to funding for laboratory equipment to estimate biomarkers such as alpha-1-acid glycoprotein (AGP), serum transferrin, hepcidin, ferritin, reticulocyte haemoglobin content, percentage hypochromic erythrocytes, serum transferrin receptor and vitamin levels. Importantly the α-1-acid glycoprotein (AGP) would have provided data on long term inflammation to supplement our existing CRP estimate, but due

to limited laboratory resources this was not possible. To obtain more than one blood measurement would have also been a significant additional burden on participants, travelling a long distance to the hospital to obtain samples on more than one occasion. Our data analysis plan was relatively simple and sample size precluded us from studying the data at the level of the village of residence.

The range of values for mean corpuscular volume in our study population (Figure 2) were wide, consistent with the explanation that a range of nutritional deficiencies were present (18-20). This distribution is relatively common in developing countries and may co-exist with elevated systemic inflammation. The medical history of our participants reported no active chronic disease, and none of them were on any medical treatment at the time of data collection. Nonetheless, our study population had a relatively high prevalence of increased systemic inflammation, with a median CRP value of 1.26 mg/L as opposed to a median value of 0.4 mg/L for a population-based sample of girls aged 3 to 17 years who lived in the USA (21). This may be due to a variety of possible environmental exposures such as the absence of clean water, poor sanitation and hygiene, limited access to healthcare and malnutrition (22), all of which are commonly observed in our study region.

There are no prior data available on the association between haemoglobin with serum CRP and total WBC count in adolescent girls living in rural Indian communities for comparison with our study population. Published studies have used variable cut-off values for serum CRP when categorising inflammation, and these populations include different groups such as pregnant women, children and elderly patients with chronic diseases making any direct comparison with our data challenging. Houghton and colleagues (23) analysed 75 young children aged 12 to 23 months old living in an urban slum in New Delhi, with a mean serum CRP value of 0.71 mg/L, which was much lower than the comparable value of 1.71 mg/L from our study population. Interestingly, there was an inverse association between CRP and haemoglobin levels in this population, which is consistent with our original hypothesis that systemic inflammation is inversely related to blood Haemoglobin levels. Similarly, another study from South India on 396 children aged 12 to 23 months reported comparable results with a mean CRP of 0.91 mg/L (95% CI: 0.77 to 1.06), and again an inverse association between CRP and blood haemoglobin levels (24). A study by George and his colleagues on children aged 6 to 59 months living in Cambodia reported that subclinical chronic inflammation as measured by a1-acid glycoprotein was an independent risk factor for

anaemia, but there was no association with the CRP in this population (25). It is important to note the outlined three studies involved young children who may have had different exposures than our study population having adolescent girls.

Arya et al (26) conducted a study on CRP involving healthy adolescent boys and girls living in a metropolitan area in north India. The mean CRP was 1.3 mg/L (SD 2.3, Range 0.02 to 17.5 mg/L) which was similar to that in our population and 9% of the total study participants (N=359) had very high serum CRP levels (> 3.0 mg/L) (21). A study from Nepal showed a mean CRP of 0.19 mg/L in 13 to 19 year-old girls (N=112), which is much lower than our population (27). A study by Htet and associates reported a much higher CRP levels in anaemic adolescent girls from Indonesia (28). Median CRP was 5.0 mg/L (95% CI 4.9 to 5.7, N=83), and 35% girls had higher AGP (> 1g/L) suggesting subclinical inflammation. Findings by Arya et al (26) and Htet et al (28) reported higher CRP levels in adolescents similar to our observations in the Maharashtra state of India.

Our population was relatively undernourished as assessed by BMI, with a mean BMI-for-age percentile of 28.7 (Range 1 to 99). As many of the observations of inverse associations between systemic inflammation and circulating haemoglobin levels have been in populations living in affluent developed countries, one possible explanation for the unexpected positive association between these factors in this population is a different body composition. Haemoglobin is associated with somatic measures of growth in a similar population (29), and body fat and weight increase is well recognised to have an inflammatory component (30), but in undernourished young populations, the relations between these factors may be different. Alternatively, chronic sub-clinical exposure to infection or other environmental inflammatory exposures may be important in this population. Understanding these associations is important as it may influence how adolescent girls respond to iron and folic acid supplementation treatment to prevent or treat anaemia in adolescents living in these environments.

Our secondary analysis also demonstrated that there were positive associations between all three anthropometric measures of MAUC, weight and BMI-for-age percentile and total WBC count, but not with height. No associations were observed with serum CRP. These observations are again novel, and propose that in this population, measures of somatic growth are positively associated with white blood cell production, possibly as a consequence

of the nutritional status or other life-course exposures. This may be clinically important, as it is well acknowledged that relatively malnourished individuals are at higher risk of infection (31), and these associations may contribute to this effect.

In summary, our data demonstrate that our population of Indian rural adolescent girls have a high prevalence of increased systemic inflammation as measured by serum CRP. Contrary to the original hypothesis, we did not observe an inverse association between systemic inflammation and prevalence of blood haemoglobin, and actually demonstrated that in this population two biomarkers for systemic inflammation (WBC and CRP) were positively associated with blood haemoglobin. Further research in similar populations on the causes of systemic inflammation and how this may modify blood haemoglobin levels, are required to understand how to modify interventions designed to promote optimal public health outcomes.

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Contributor statement: The Maharashtra Anaemia Study Phase 2 was designed by Dr Anand Ahankari, Dr Andrew Fogarty, and Dr Laila Tata. This specific study hypothesis was conceptualised by Dr Andrew Fogarty. Dr Ahankari obtained the MAS Phase 2 data with support from Ms Sandhya Rankhamb, and he conducted the analysis jointly with Dr Fogarty. Dr Kabra monitored the data collection, project progress and also conducted ethics inspections. Dr Tata and Prof Hayter contributed to this manuscript development along with all other authors.

Conflicts of interest statement: Authors have no conflicts of interest to disclose that are relevant to this study.

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436 **Figure 1: Histogram of C-reactive protein (CRP) in study population**
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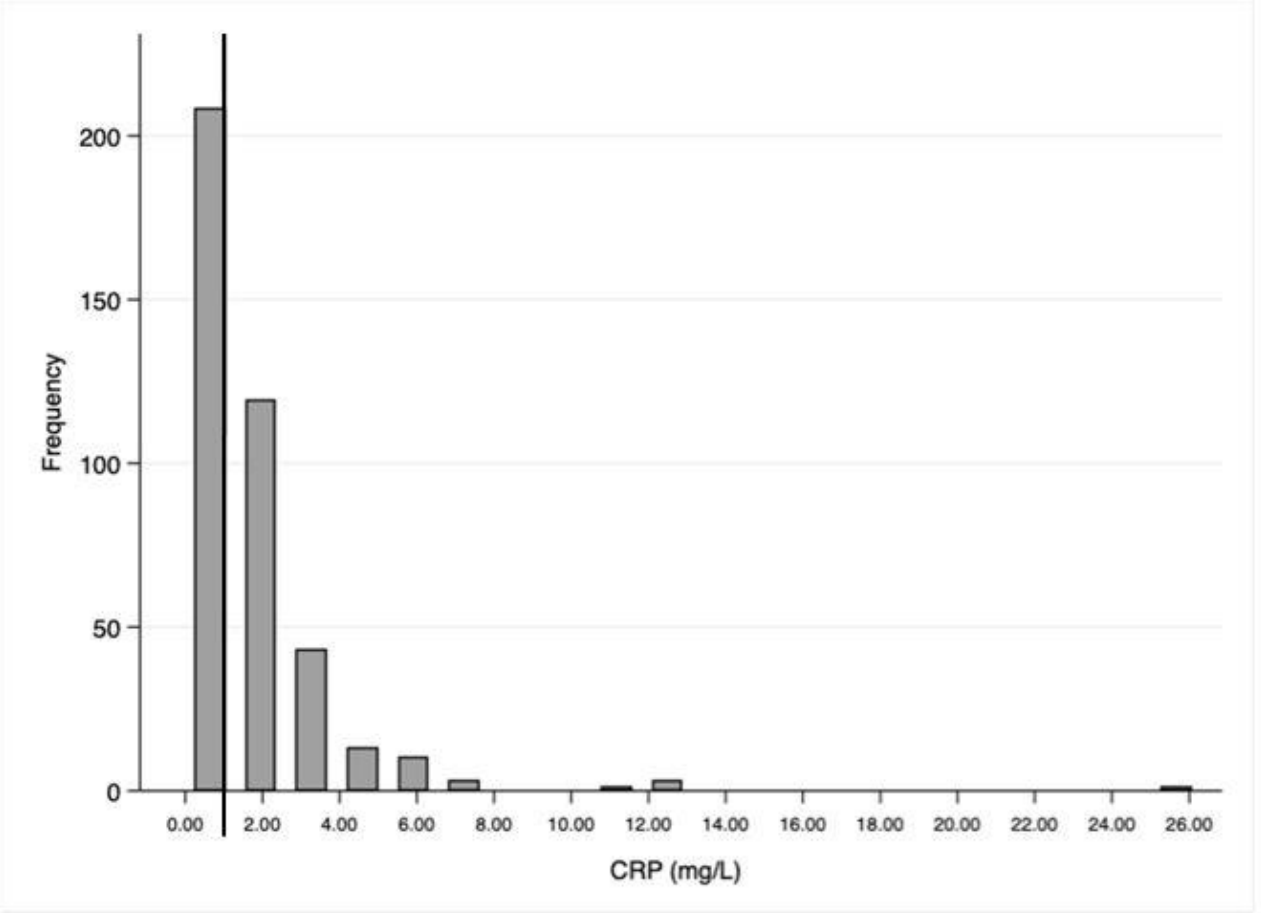

438
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440 Figure footnote: A vertical reference line on the X-axis indicates CRP value of 1 mg/L.

441 **Figure 2: Histogram of Mean Corpuscular Volume (MCV) in study population**
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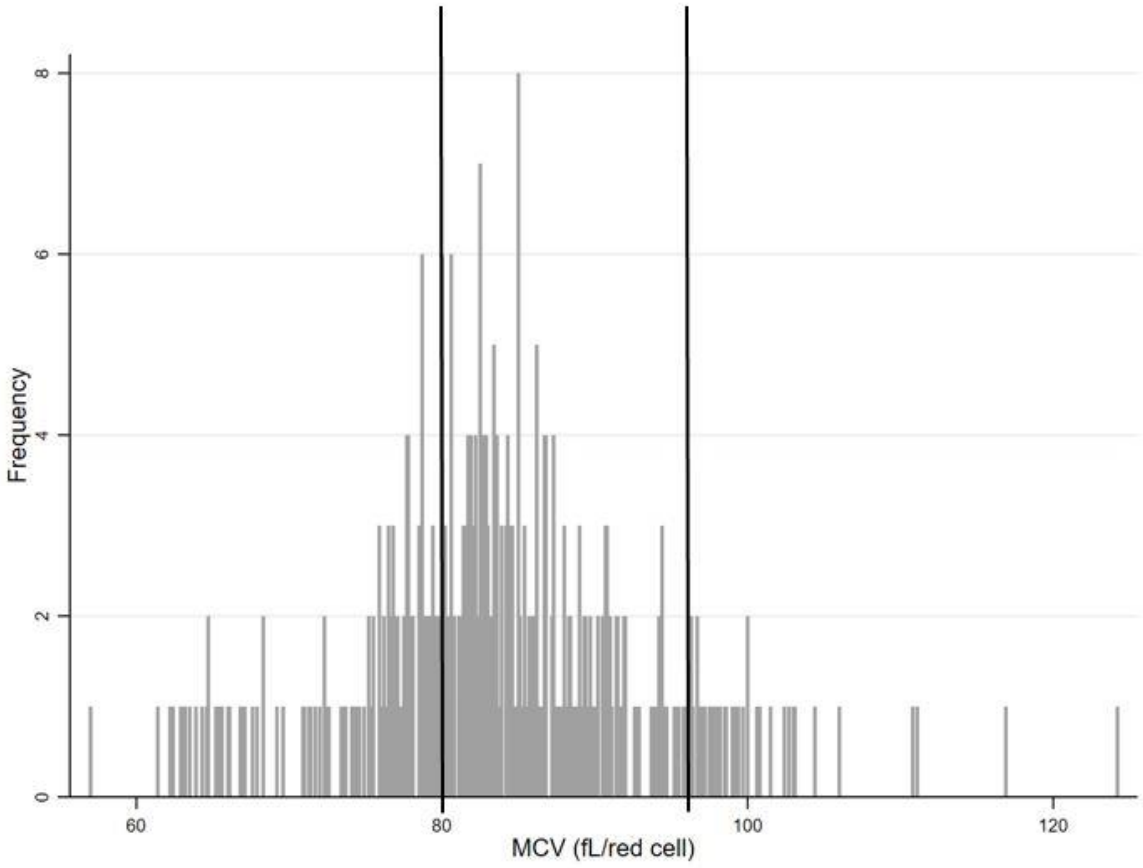

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445 Figure footnote: Two vertical reference lines on the X-axis indicate a normal MCV range
446 (80 to 96 fL/red cell).

Table 1: Characteristics of study population with regression analysis (N= 401 participants)

Characteristics	Summary statistics			CRP logistic regression analysis [1]		WBC linear regression analysis [3]	
	Mean (standard deviation)	Median	Range	Age adjusted analysis (95% CI)	p value	Age adjusted analysis (95% CI)	p value
CRP (mg/L)	1.71 (2.15)	1.26	0.00 to 26.33	NA	NA	NA	NA
White Blood Cells count (x10^3/μL)	9.02 (2.00)	8.8	3.5 to 16.5	NA	NA	NA	NA
Age (years)	14.02 (2.27)	14	10 to 18	NA	NA	NA	NA
Haemoglobin (g/dL)	12.24 (1.51)	12.5	3.9 to 14.8	1.16 (1.01 to 1.33)	**0.03**	0.24 (0.11 to 0.37)	**<0.001**
Height (cm)	148.28 (8.45)	150	120 to 166	0.99 (0.96 to 1.01)	0.51	-0.00 (-0.03 to 0.01)	0.49
Weight (kg)	39.38 (9.16)	39.9	16.9 to 69.8	1.00 (0.97 to 1.03)	0.80	0.05 (0.03 to 0.08)	**<0.001**
BMI-for-age percentile	28.68 (28.26)	17.37	1 to 99	1.00 (0.99 to 1.01) [2]	0.26	0.02 (0.01 to 0.02) [2]	**<0.001**
MUAC (cm)	22.27 (2.85)	22	15.5 to 32	1.04 (0.96 to 1.13)	0.30	0.19 (0.10 to 0.27)	**<0.001**

Table footnotes:

- [1] Odds ratio (OR) with confidence intervals (CI) for elevated CRP values of ≥1mg/L (n=234) compared with lower CRP values (n=167). Each OR is from a separate logistic regression model [Haemoglobin, Height, Weight and Mid-upper arm circumference (MUAC)] adjusted for age as a categorical variable.
- [2] BMI-for-age percentile were generated based on the WHO 2007 framework (11) using the Stata 13.1 (StataCorp, College Station, Texas, USA). BMI-for-age percentile were for age, thus the reported analysis is not age adjusted in the given two regression models.
- [3] β coefficient with confidence intervals (CI) for total WBC count as a continuous measure (primary outcome- WBC count). Each beta coefficient is from a separate logistic regression model [Haemoglobin, Height, Weight and Mid-upper arm circumference (MUAC)] adjusted for age as a categorical variable.
- NA: Not applicable