

# Hepcidin testing; establishing reference values for the Namibian blood donor population

Martin Gonzo, Aaron Maramba, Gordon Taylor

## ABSTRACT

**Aims:** Anemia is a global health concern affecting billions of people worldwide. The diagnostic approach to iron deficiency anemia could be streamlined in an attempt to increase diagnostic sensitivity and to reduce uncertainties in interpreting laboratory generated results. In doing so the introduction of hepcidin testing may prove to be useful but this requires the establishment of reference normal ranges. The main aim of this study was to establish reference values of hepcidin in the Namibian blood donor population which can be used as baseline or reference point in future hepcidin studies to be carried out in the same population. **Methods:** In establishing the reference range, a total of 40 healthy adult participants were randomly selected from eligible blood donors in Namibia. Venous blood samples from qualifying consenting donors were collected shortly before the blood donation session. The samples were refrigerated before being spun down for storage in a  $-70^{\circ}\text{C}$  freezer to maintain sample integrity. On the day of testing samples were prepared for testing using an enzyme linked immunosorbent

assay (ELISA) based hepcidin kit. The samples used to determine the reference values were also used to determine the serum iron levels. Full blood count results were also obtained from the same participants. Results for full blood count and serum iron levels were used to confirm the absence of iron deficiency anemia. **Results:** In this study, serum Hepcidin reference range values for adults were established to be 17.186–91.237 ng/mL for females and 18.227–81.541 ng/mL for males Namibia blood donor population. For the entire study population, the mean hepcidin level was 51.99 ng/mL ( $\pm 1\text{SD}$  17.44 ng/mL). Mean hemoglobin and serum iron values were 143.56 g/L ( $\pm 1\text{SD}$  12.92g/L) and 91.32 mg/dL ( $\pm 1\text{SD}$  16.77 mg/dL) respectively indicating iron adequacy in this non-anemic study population. **Conclusion:** The established reference ranges are comparable to those determined elsewhere. These values can, therefore, be used as baseline values for studies on hepcidin in Namibia.

**Keywords:** Anemia, Blood donors, Hepcidin, Iron deficiency

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## INTRODUCTION

Hepcidin is a polypeptide that is involved in iron regulation through its effect on ferroportin. Ferroportin is an iron exporter and the action of hepcidin can result in its internalization and subsequent degradation making it unavailable for its function. Testing for hepcidin can therefore give an indication of iron bioavailability. Hepcidin testing is not currently being performed in routine diagnostic laboratories. Introducing this biomarker into the diagnostic arena requires the establishment of reference normal ranges. Pasricha et al. [1] established reference range values for hepcidin as 5.4 ng/mL to 174.6 ng/mL in healthy premenopausal women in Australia. Galesloot et al. [2] also established normal ranges for hepcidin in the Netherlands. The latter were divided according to gender and age in strata of five years each.

The more recent work on hepcidin reference ranges was performed in Greece by Sdogou et al. [3] and it provided useful information relevant to the Greek population albeit for the pediatric populace. Appropriate reference range values are required for accurate diagnosis and prognosis of disease. However, for hepcidin all of this work has been performed outside Namibia a country that is in Africa South of Sahara. It is, therefore, justifiable to carry out the same work in the Namibian context given the differing geographical locations and ethnicity compositions.

Work on the present study to establish reference range values among blood donors in Namibia was the first of its kind. The established reference values in Namibia could potentially form the baseline and reference point for studying quantitative characteristics of hepcidin. Establishing these ranges in the Namibian blood donor population also provides an opportunity to compare them with already established data on reference values elsewhere. The other importance of locally reputable reference range values is the possibility of establishing hepcidin-hemoglobin correlations of which the data can also be compared to the same correlations found elsewhere.

Hepcidin is affected by iron levels and sampling for normal healthy adults has to be cognisant of that fact. Sampling in this study was therefore done among the blood donor population. The profile of a blood donor in general is consistent with a healthy individual and it suffices to say voluntary non-remunerated blood donors are a good representative of a healthy population.

## MATERIALS AND METHODS

Normal healthy blood donors were recruited to establish reference ranges. Healthy individuals were defined according to the donation criteria as set out by the Namibia blood transfusion service. The donation criteria states that individuals should be between the age

of 16 and 65 and in good health for them to be considered for blood donation [4]. It also states that they should have the following attributes:

- No recent alcohol intake
- No active bacterial infection
- Weigh more than 50 kg with a hemoglobin level above 125 g/L
- Lead a sexually safe lifestyle
- Enjoy general good health and
- Committed to helping others and can donate for the right reasons [4].

The initiative by the NaMBTS to recruit voluntary non-remunerated donors (VNRD) makes it possible to use this population to establish reference ranges. These VNRDs are conscious of their contribution to society and they generally have good healthy habits making them a better target than randomly selecting individuals outside of a healthcare establishment. The VNRDs provide blood freely and voluntarily without expecting anything in return [5]. Research by Kalargirou et al. [6] established that the majority of donors donate blood out of their unselfish concern for the welfare of others. These attributes are good features that contribute to the general wellbeing of an individual. They also demonstrate the appreciation of health for both self and others.

In their work, Sdogou et al. [3] carried out physical examinations on their participants to help ascertain their health status. In this study, NaMBTS guidelines on donor selection [4] were used. The procedures include checking for vital signs such as blood pressure, weight as well as asking questions about donors' general health using a standard donor questionnaire. The donor questionnaire elicits for information on an individual's health and medical history of which the certain criteria has to be met before being allowed to donate.

The approach on using donor questionnaire is similar to what Galesloot et al. [2] research used where they employed a health and lifestyle questionnaire as one of their participant selection tools. These approaches ensure the participants' health status is assessed based on clinical presentation on the day of blood sample collection, as well as relevant medical history. This holistic approach minimises confounding variables such as hidden medical conditions that interfere with the physiological mechanisms involved in hepcidin metabolism.

On that premise individuals who qualified to donate blood were therefore included in the study. Deferral, whether permanent or temporary, was one of the exclusion criteria the basis of which was its perceived deviation from how "normal healthy" adult was defined. Non-consenting participants were also excluded as it is ethically immoral to coerce anyone to participate in research studies. Donors with low hemoglobin levels as well as those with recent bacterial infections were also excluded. Bacterial infections are known to upregulate hepcidin expression and consequently reduce iron bioavailability [7]. Such participants invariably exhibit higher hepcidin levels. Excluding donors with recent infection was, therefore,

mandatory for normal references to be a true reflection of the normal healthy population.

After establishing the inclusion criteria, a sample size calculation was performed using a sample size calculation for quantitative variables obtainable from cross sectional studies. The formula for sample size calculation is shown below and has been described in Charan and Biswas [8].

$$\text{Sample size} = \frac{(Z_{1-\alpha/2})^2 SD^2}{d^2}$$

where  $Z_{1-\alpha/2}$  = standard normal variate

SD= standard deviation. The SD value is obtainable from previous studies by other researchers.

d= absolute error or precision.

The standard normal variate used in this study was 1.96 using 5% type 1 error ( $p < 0.05$ ) and the desired absolute error/precision (d) of 6 ng/mL. The performance characteristics of the CUSABIO ELISA kit were taken into consideration in selecting the absolute error. The kit has a detection range of 4.69–300 ng/mL and a minimum detectable dose of 1.17 ng/mL, the desired absolute error of 6 ng/mL was determined as reasonable.

The standard deviation was obtained by scanning through papers with similar studies such as the work of Sdogou et al. [3], Pasricha et al. [1] and Galesloot et al. [2]. An standard deviation of 18.94 ng/ml was used giving a sample size of 38.28. A sample size of 40 was, therefore, used to establish the normal range for hepcidin using the blood donor population in Namibia. A total of 19 female donors and 21 male donors were recruited into this study.

Approval was obtained from the University of Bath through the Research Ethics Approval Committee for Health (REACH) prior to commencement of this study. In Namibia, permission to carry out research work and ethical clearance was also granted by the Namibian Blood Transfusion Service and the Ministry of Health and Social Services in Namibia. Written informed consent was obtained from all blood donor participants before samples were collected.

Venous blood samples were collected from consenting healthy blood donors by the NaMBTS nursing staff. Before blood collection, participants' weight and vital signs such as blood pressure were determined to ensure they were safe to give blood. Normal phlebotomy procedures were followed as described in the WHO guidelines on phlebotomy procedures [9]. The blood samples were collected into vacutainer tubes which self-regulate volumes collected according to the amount of additives contained in the tubes. Samples collected were used to perform full blood count, Hepcidin and serum iron assays.

The participants then proceeded to donate blood after samples for the hepcidin study were collected. This order of events was important in order to avoid variation in hepcidin concentrations caused by changes in blood volumes post blood donation. Hepcidin values respond to decrease in blood volume because of the consequent reduction in iron content [10].

The samples were also collected from donors presenting at the NaMBTS for donation in the morning in order to minimise the effects of diurnal variation in hepcidin [11]. The timing of collections was also important in optimising sampling conditions and it also became pertinent to standardise preparation of samples for laboratory analysis.

In order to ensure sample validity samples for full blood count were processed on the same day. Samples for full blood count need to be tested as soon as possible because storage, regardless of conditions, affects the different parameters of full blood count results. This is in line with the recommendations and advice from different authors who have done some work on full blood count results and storage conditions [12, 13].

Samples for hepcidin and serum iron were centrifuged at 1000 g for 15 minutes according to the kit manufacturer's instructions [14]. The samples were then aliquoted and frozen at  $-70^{\circ}\text{C}$  to avoid loss of bioactivity of the hepcidin molecules. All samples were stored for at most two months before testing in line with the guidance from the hepcidin kit manufacturer [14].

On the days of the experiments samples were thawed and allowed to reach room temperature before being assayed. All the other reagents were also brought to room temperature as per the manufacturer's guidance. Once thawed the samples were spun down at 1000 g for five minutes. All reagents were prepared according to the assay procedure [14] and an ELISA was set-up.

Samples for the hepcidin assays were also used for assessing serum iron levels. Serum iron results were obtained to confirm the presence of normal iron levels in this group. On the day of analysis samples were thawed and working reagents were prepared immediately prior to use to ensure the validity of the assay was not compromised. The thawed serum samples were spun at 1000 g for five minutes to remove any insoluble particles that may interfere with spectrophotometric characteristics of the coloured complex when reading the absorbance of the final solution. According to the kit insert, samples needed to be separated as soon as possible. This was consistent with the requirements of the hepcidin assay protocol, therefore, the same sample preparation protocol was sufficient to ensure sample validity.

The hepcidin assays were run using an ELISA technique with a detection range of 4.69–300 ng/ml. The inter-assay and intra-assay precision for this assay was  $<10\%$  and  $<8\%$  respectively [14]. The intra-assay precision was performed by the kit manufacturer. This was carried out using three samples of known hepcidin concentration. The samples were tested twenty times on one ELISA plate and the results used to assess intra assay precision [14]. The inter-assay precision to determine precision between assays was also performed by CUSABIO® who were the kit manufacturers. On this instance three samples with a known concentration of hepcidin were tested in twenty assays and the results used to assess the inter-assay precision.

There are several methods to quantitate hepcidin which include the ELISA, mass spectrometry (MS), high performance liquid chromatography (HPLC), radio immunoassays (RIA), and other novel approaches such as surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) and liquid chromatography tandem mass spectrometry (LC/MS-MS). In their review, Macdougall et al. [15] discussed the different methodologies available for Hepcidin testing including the ELISA technique. In their review, a number of studies done on Hepcidin using the ELISA technique were discussed. In Macdougall et al. review [15], it appears Koliakari et al. used wells coated with polyclonal antibodies whereas in Ganz's work the wells were coated with monoclonal antibodies against hepcidin. They noted that Koliaraki et al. as well as Ganz et al. work on ELISA conferred that there is no cross reactivity between the hepcidin isoforms when using the ELISA technique [15]. An ELISA based immunoassay was chosen because its performance is comparable to other technologies. It is also relatively cheap in comparison to technologies such as radioimmunoassay and mass spectrometry. These two attributes makes ELISA the right choice for an assay in the setting in which hepcidin testing was being proposed.

Full blood count, serum iron and hepcidin assays were performed on the blood samples that were collected from the participants. All three tests were performed in the laboratory. Tests for full blood count were performed on the day of sample collection while assays for hepcidin and serum iron were performed at a later date. The full blood count tests were performed using a Pentra XL 80 full blood count analyser [16].

The samples for Hepcidin assaying were tested in duplicate using the Hepcidin ELISA kits supplied by CUSIBIO®. Antihepcidin antibody pre-coated wells were supplied in the kit. The wells came in strips with twelve wells on each strip. One hundred microliters of prepared standards and samples were added into the wells and incubated for two hours at 37°C. At the end of the incubation period the liquid contents of the wells were decanted. One hundred microlitres of biotin labeled antibody was added to each well and the mixture incubated for 1 hour at 37°C. After which the liquid from each well was removed by aspiration. The wells were washed three times using the wash solution provided by the kit manufacturer. Subsequently, 100 µl of avidin conjugated to horse radish peroxidase (HRP) was added to each well and incubates at 37°C for 1 hour. At the end of the incubation period the HRP-avidin mixture was aspirated before the wells were washed five times with the wash solution. Ninety microlitres of the tetramethylbenzidine (TMB), a peroxidase substrate was added in the wells and incubated for at 37°C for 30 minutes.

The plates were protected from light to avoid it interfering with the color development. The color reaction was stopped by the addition of 50 µl of stop solution before reading the optical densities for each well at 450 nm. The optical density was read using a microtiter plate reader.

The standards had the following hepcidin concentrations according to the manufacturer's instructions: 300 ng/ml, 150 ng/ml, 75 ng/ml, 37.5 ng/ml, 18.75 ng/ml, 9.375 ng/ml, and 4.6875 ng/ml. The logarithmic values of these concentrations were plotted against the logarithms of the optical densities obtained and this was the standard curve used to calculate sample results.

## RESULTS

A total of 40 donors were recruited using a multistage sampling method which comprised simple random sampling and stratified sampling. Donors were stratified according to their eligibility to donate on the day they presented at the blood transfusion service. Those able to donate were randomly selected and recruited into the study between October 2015 and June 2016 and hepcidin, full blood count and serum iron levels were tested. At total of 19 females mean age 4.63 years (+/-1SD=10.23) and 21 males mean age 38.26 years (+/-1SD = 10.50) were tested.

Serum iron values for this sample ranged from 69 µg/dL to 150 µg/dL with a median value of 91.5 µg/dL. The mean value for serum iron was 91.32 µg/dL (+/-1 SD = 16.78 µg/dL) giving a good indication of adequate iron bioavailability. The mean hepcidin concentration for the whole study population was 51.99 ng/ml (+/-1SD=17.44 ng/mL). The hemoglobin values ranged from 133–168 g/L for males and 125 g/L to 155 g/L for females all of which were normal for both genders.

The lowest and highest hepcidin values in this group were 29.8 ng/mL and 87.8 ng/mL respectively. Most of the values were above 40 ng/ml with 9 (~25%) of them having hepcidin values of at least 70 ng/mL.

For data analysis confidence intervals and reference ranges were calculated using a function as adopted from Altman [17]. The reference range values for hepcidin in this study was 17.186–91.237 ng/mL for females and 18.227–81.541 for males both values at 95% which is within 2SDs. The lower confidence intervals were 8.692–25.680 ng/mL and 11.148–25.306 ng/mL for females and males respectively. The upper confidence intervals were 82.743–99.731 ng/mL for females and 74.462–88.619 ng/mL for males. A range of 60.99 ng/mL was obtained from the entire study population. The standard deviations of hepcidin (18.891 females and 16.151 males) obtained in this study are lower but consistent with what was obtained in similar studies. These were used to calculate the sample size for this study.

For hemoglobin determinations the minimum values was 125 g/L which indicates that no anemic individuals were recruited in this study group. The reference values for hemoglobin were 120.42–156.11 g/L and 121.42–175.78 g/L for females and males respectively. The range for hemoglobin values for the entire study population was from 125 g/L to 168 g/L which is normal for the adult population. The mean, mode and median values for

hemoglobin were very close to each other. These values are measures of centrality which suggests the hemoglobin values in the study group are close to each other and this is supported by the low standard deviations for hemoglobin of 9.10 g/L (females) and 13.87 g/L (males).

## DISCUSSION

The reference ranges established for the Namibian blood donor population are consistent with those established elsewhere [1–3] albeit in different age groups. It is therefore reasonable to accept these as the true reflection of the hepcidin levels in the blood donation population which in this case was used as a representation of the entire blood donation community in Namibia. The range of hepcidin levels obtained in this study group was 29.8 ng/mL. From established ranges elsewhere [1, 2, 3], it is apparent that there is no suppression of hepcidin expression in this group. These results together with serum iron level values determined in the same group, confirms the eligibility of the study group as a genuine group for the establishment on reference normal ranges. It is also important to note that the  $\pm$  1SD values (18.891 ng/mL and 16.151 ng/mL) obtained for hepcidin was slightly lower than what was obtained elsewhere in studies by Sdogou et al. [3]. These standard deviations also indicate that the hepcidin values obtained are reasonably spread around the mean.

The serum iron values were determined in order to confirm the absence of iron deficiency in this group of participants. The serum iron levels ranged from 69  $\mu$ g/dL to 150  $\mu$ g/dL, results which showed that all the samples used had normal iron levels. These results are consistent with reference range values which are between 40–155  $\mu$ g/dL for females and 55–160  $\mu$ g/dL for males [18]. The modal value for the serum iron levels was 96  $\mu$ g/dL and together with other values obtained, indicates good iron bioavailability in the normal reference range group. Given the effect of iron levels on hepcidin values, it is reassuring to note that the hepcidin reference ranges were derived from a population with normal serum iron levels.

The comparability of the study results to reference ranges established elsewhere makes it possible for us to use them in this study without incurring prohibitive costs associated with establishing comprehensive age and gender stratified reference normal ranges. This activity would, however, be advised when incorporating hepcidin as a routine diagnostic test in practice. Alternatively, laboratories could opt to verify reference ranges established elsewhere with the intention of adopting the same values.

## CONCLUSION

Hepcidin reference range values are useful as baseline information for the elucidation of the properties of this molecule in iron deficient states. Establishment of these

reference range values makes it possible to assess the usefulness of hepcidin values in iron deficiency anemia in this study. The reference range values established in this study were 17.186–91.237 ng/mL for females and 18.227–81.541 for males. This reference range participant group provided sufficient data that is adequate to allow for the study on hepcidin in iron deficiency anemia to take place in the Namibian blood donor population. It is important to note that a larger sample would be needed to come up with reference values that can be generalized to the entire population in Namibia.

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## Author Contributions

Martin Gonzo – Substantial contributions to conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published

Gordon Taylor – Substantial contributions to conception and design, Revising it critically for important intellectual content, Final approval of the version to be published

Aaron Maramba – Analysis and interpretation of data, Revising it critically for important intellectual content, Final approval of the version to be published

## Guarantor

The corresponding author is the guarantor of submission.

## Conflict of Interest

Authors declare no conflict of interest.

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## SUGGESTED READING

- ABX Technical manual (nd) part number RAA022B. ABX Horiba Diagnostics. [Available at: <http://www.yeec.com/uploadimages1/forum/abx/abxpentra80%E6%8A%80%E6%9C%AF%E6%89%8B%E5%86%8C.pdf>]
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