# Vitamin B12 reference intervals

Andersen, Stine Linding; Hansen, Annebirthe Bo; Hindersson, Peter; Andersen, Lærke; Christensen, Peter Astrup

*Published in:*
Danish Medical Journal



*Publication date:*
2023

*Document Version*
Publisher's PDF, also known as Version of record

DANISH MEDICAL JOURNAL

Original Article

Dan Med J 2023;70(6):A12220771

# Vitamin $B_{12}$ reference intervals

Stine Linding Andersen[1, 2], Annebirthe Bo Hansen[1], Peter Hindersson[3], Lærke Andersen[1] & Peter Astrup Christensen[1, 2]

1) Department of Clinical Biochemistry, Aalborg University Hospital, 2) Department of Clinical Medicine, Aalborg University, 3) Department of Clinical Biochemistry, North Denmark Regional Hospital, Hjørring, Danmark

Dan Med J 2023;70(6):A12220771

## ABSTRACT

**INTRODUCTION.** P-Vitamin B12 is a commonly used biochemical test. Evaluation of test results and diagnosis of vitamin $B_{12}$ deficiency are challenging, and the role of different biochemical methods remains unclear.

**METHODS.** The aim of this study was to establish reference intervals for plasma vitamin $B_{12}$ concentration using different immunoassays (method 1: Alinity, Abbott Laboratories; method 2: Cobas 6000, Roche Diagnostics; method 3: Atellica IM, Siemens Healthineers). Direct reference intervals were established among blood donors (n = 129) and indirect reference intervals among adult patient results of plasma vitamin $B_{12}$ concentration requested by general practitioners in the North Denmark Region from 15 August to 15 October 2022 (n = 34,181). Finally, the frequency of low vitamin $B_{12}$ concentration using different uniform cut-offs was evaluated.

**RESULTS.** Direct reference intervals (2.5-97.5 percentiles) were as follows for method 1: 168-553 pmol/l; method 2: 202-641 pmol/l; and method 3: 211-551 pmol/l. Indirect reference intervals were as follows for method 1: 133-541 pmol/l; method 2: 172-619 pmol/l; and method 3: 182-162-206 pmol/l. When different cut-offs were applied to patient results, the frequency of having a vitamin $B_{12}$ concentration below 250 pmol/l differed by biochemical method: 33% (method 1), 17% (method 2) and 14% (method 3).

**CONCLUSION.** Measurement of plasma vitamin $B_{12}$ concentration using different immunoassays revealed results and reference intervals that were not interchangeable. Clinical guidelines for the diagnosis of vitamin $B_{12}$ deficiency should consider the biochemical methods used.

**FUNDING.** None.

**TRIAL REGISTRATION.** None.

Vitamin $B_{12}$ deficiency warrants clinical awareness considering the potentially irreversible neurological symptoms that may arise if the deficiency is left untreated [1]. Thus, vitamin $B_{12}$ concentration is frequently measured in blood samples, and the clinical use extends beyond the testing of individuals with anaemia or obvious clinical signs of vitamin $B_{12}$ deficiency [2]. Uncertainties prevail regarding the diagnosis of vitamin $B_{12}$ deficiency including the choice of a biochemical test and the applied reference intervals or cut-offs. For vitamin $B_{12}$, a uniform grey zone is often described in which additional biochemical measurements are recommended to establish the diagnosis [3, 4]. Vitamin $B_{12}$ is measured using automatic immunoassays in clinical laboratories in Denmark. Thus, it is to be expected from the biochemical assay design that levels obtained with different methods are not necessarily interchangeable. In the North Denmark Region, different immunoassays are used in clinical laboratories. We aimed to establish reference intervals for each method. Furthermore, we assessed

patient results requested by general practitioners in the North Denmark Region and for each method, we evaluated the frequency of finding a vitamin $B_{12}$ level below uniform cut-offs.

## METHODS

The study was conducted in the Departments of Clinical Biochemistry, Aalborg University Hospital, and the North Denmark Regional Hospital in the North Denmark Region. The biochemical methods used for measurement of vitamin $B_{12}$ concentration were: B12 reagent kit, Alinity, Abbott Laboratories (method 1), Elecsys Vitamin B12 II, Cobas 6000, Roche Diagnostics (method 2) and Vitamin B12, Atellica IM, Siemens Healthineers (method 3). All measurements were performed in ISO15189-accredited laboratories. The intermediate precision of each method was evaluated at two quality control levels: Liquicheck Immunoassay Premium Control level 1 and level 3 (method 1 and method 2) and Liquicheck Immunoassay Plus Control level 1 and level 3 (method 3) from Bio-Rad Laboratories. The long-term coefficient of variation (CV) calculated across similar modules in each laboratory was: method 1 (CV = 9.8% (control mean: 204 pmol/l), 7.5% (534 pmol/l)); method 2 (4.8% (208 pmol/l), 3.3% (520 pmol/l)) and method 3 (12.8% (135 pmol/l), 8.4% (507 pmol/l)). Reference ranges for vitamin $B_{12}$ concentration in adults recommended by the manufacturer were: 138-652 pmol/l (method 1), 145-569 pmol/l (method 2) and 156-672 pmol/l (method 3).

We established direct reference intervals for each method using lithium heparin plasma samples from 129 adult blood donors (60 males and 69 females). All individuals gave informed consent for the use of residual blood samples for method validation purposes, and samples were analysed in anonymised form. Samples were handled according to standard preanalytical procedures and stored at –20 &;C until measurement. In addition, we obtained all vitamin $B_{12}$ results from adult patients in the North Denmark Region from August 15 to October 15, 2022 that were measured in the clinical hospital laboratories using methods 1 through 3 using lithium heparin plasma samples. These results served to indirectly establish reference intervals, and for this aim we included the first vitamin $B_{12}$ results from patients who only had blood samples requested by the general practitioner and for whom less than three requests had been made per decade [5, 6]. Finally, all patient results requested by general practitioners within the two-month period were assessed for classification of vitamin $B_{12}$ status using different cut-offs. The cut-offs evaluated were chosen to reflect the range of lower reference limits used in clinical laboratories in Denmark (150-200 pmol/l). Besides reference intervals, a grey zone is often used in clinical practice [4]. It is defined by a concentration range in which vitamin $B_{12}$ deficiency cannot be ruled in or out, and additional biochemical tests may therefore be required [4]. In Denmark, a commonly used limit for this grey zone is a vitamin $B_{12}$ concentration of 250 pmol/l [3] and we further evaluated this cut-off.

To establish reference intervals, outliers were identified using Tukey's outer fences (three times the interquartile range (IQR)) for the direct method, and Tukey's inner fences (1.5 times the IQR) for the indirect method. Reference limits (2.5 and 97.5 percentiles) were calculated by the non-parametric method and 90% confidence intervals were established by bootstrap resampling [7]. To evaluate if results would justify a partitioning of the established reference intervals, data from the direct assessment were pooled for calculation of the lower reference limit on the combined distribution [8]. Subsequently, the percentage of results below this limit was assessed for each biochemical method and evaluated according to the proposed criteria, i.e. that partitioning is recommended if ≥ 4.1% of subgroup results fall beyond the combined limit [8].

Statistical analyses were performed using Rstudio (version 2022.07.2 with R version 4.0.5 with the R packages plyr and dplyr installed) and STATA 17 (Stata Corp., USA).

*Trial registration:* not relevant.

## RESULTS

The established reference intervals differed by the biochemical method with a similar trend being observed in the direct and indirect cohort (**Table 1**). For each biochemical method, confidence intervals of the lower and upper reference limits were overlapping when comparing results of the direct and the indirect cohort (Table 1). When data from the direct assessment were pooled (n = 387), the combined distribution revealed a lower reference limit (2.5 percentile) of 181 pmol/l. The percentage of results below this combined limit varied: 5.4% (method 1), 0.8% (method 2) and 0.8% (method 3).

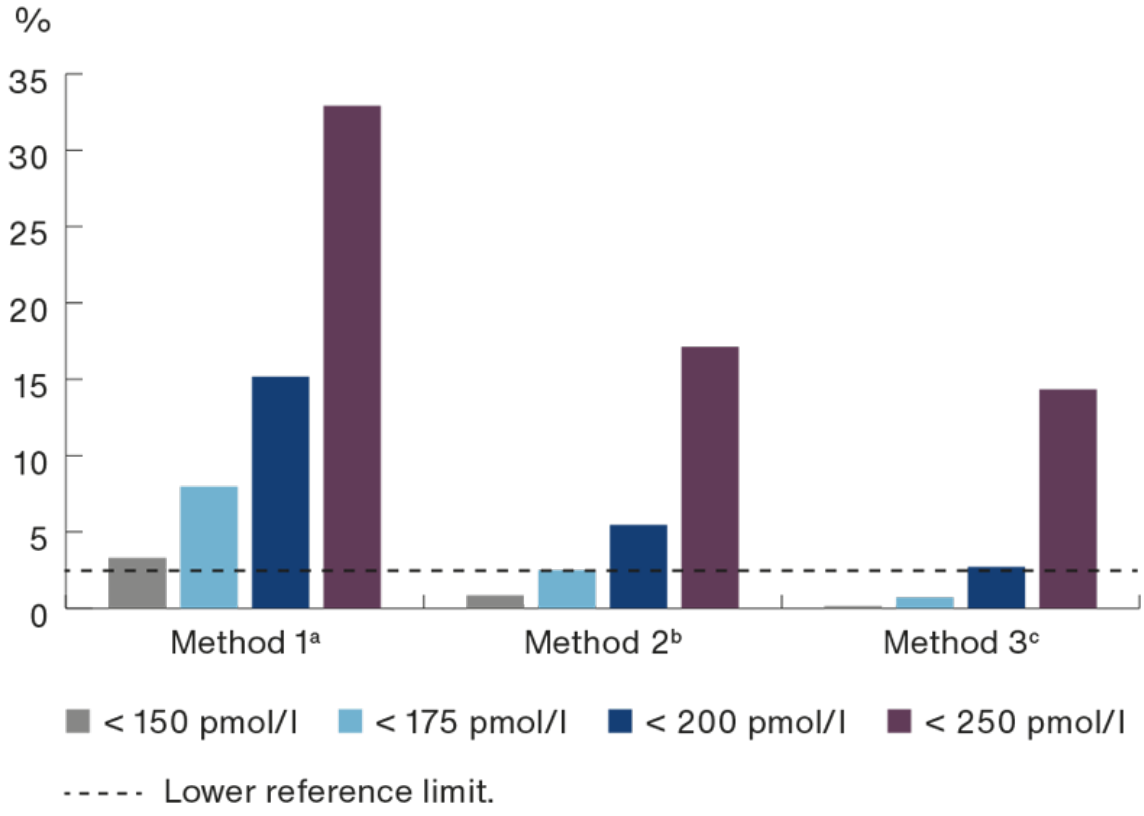
**TABLE 1** Directly and indirectly established reference intervals for P-Vitamin B12 across the different methods used in clinical laboratories in the North Denmark Region.

| | | | Percentiles (90% CI), pmol/l | |
|---|---|---|---|---|
| | All, n | Outliers, n[a] | 2.5 | 97.5 |
| *Direct* | | | | |
| Method 1[b] | 129 | 1 | 168 (123-181) | 553 (485-685) |
| Method 2[c] | 129 | 0 | 202 (160-213) | 641 (571-797) |
| Method 3[d] | 129 | 0 | 211 (186-225) | 551 (495-668) |
| *Indirect* | | | | |
| Method 1[b] | 1,096 | 42 | 133 (124-143) | 541 (533-554) |
| Method 2[c] | 129 | 5 | 172 (162-195) | 619 (536-542) |
| Method 3[d] | 304 | 13 | 182 (162-206) | 553 (519-598) |

CI = confidence interval.
a) Outliers identified using outer Tukey's fences (direct) and inner Tukey's fences (indirect).
b) Alinity, Abbott Laboratories.
c) Cobas 6000, Roche Diagnostics.
d) Atellica IM, Siemens Healthineers.

From August 15 to October 15, 2022, a total of 41,091 adult patient results were identified from 38,398 unique patients. A total of 12.3% of patients had vitamin $B_{12}$ concentration measured more than once during this period (10.2% twice, 1.6% three times and 0.5% four to eight times). Considering the first measurement in the study period among the 38,398 patients, 34,181 (89.0%) had the sample requested by their general practitioner. When the different uniform cut-offs chosen for evaluation were applied to identify low vitamin $B_{12}$ levels, the observed frequencies differed by biochemical method used (**Figure 1**). Thus, 33% of patients had a vitamin $B_{12}$ concentration below 250 pmol/l using method 1, whereas this share was 17% using method 2 and 14% using method 3. On the other hand, the identification of 2.5% of patients with low vitamin $B_{12}$ level would correspond to a cut-off of approximately 150 pmol/l using method 1; 175 pmol/l using method 2 and 200 pmol/l using method 3 (Figure 1).

**FIGURE 1** Frequency of low plasma vitamin $B_{12}$ concentration among 34,181 patients who had the biochemical test requested by their general practitioner in the North Denmark Region from 15 August to 15 October 2022. Results are illustrated when different uniform cut-offs for definition of low vitamin $B_{12}$ concentration were applied across the biochemical methods used in the North Denmark Region.




a) Alinity, Abbott Laboratories.
b) Cobas 6000, Roche Diagnostics.
c) Atellica IM, Siemens Healthineers.

## DISCUSSION

We established reference intervals for plasma vitamin $B_{12}$ concentration using three different immunoassays currently used in clinical laboratories in the North Denmark Region. Our findings consistently showed that results obtained with different immunoassays are not interchangeable across reference cohorts. When similar cut-offs were applied among patient results obtained with the different biochemical methods, the frequency of having a vitamin $B_{12}$ concentration below the cut-off varied considerably. These results emphasise the need to

focus on method-specific reference intervals and cut-offs for the diagnosis of vitamin $B_{12}$ deficiency to ensure uniform patient diagnosis and management independently of the local biochemical method employed.

Vitamin $B_{12}$ deficiency is a clinical concern, and the diagnosis may be challenging [2]. This has led to a widespread use of biochemical vitamin $B_{12}$ measurement as illustrated by our data. During a two-month period, we identified some 40,000 vitamin $B_{12}$ results from adult patients in the North Denmark Region roughly corresponding to 1,000 tests on a usual working day. Furthermore, we observed that more than 10% of patients managed in general practice had the measurement repeated within two months. These figures call for considerations regarding appropriate use of biochemical tests [9].

Another important finding was the reference intervals obtained for the different biochemical methods. We recently introduced a new method for measurement of vitamin $B_{12}$ concentration in our region (Alinity, Abbott Laboratories) and we implemented a method-specific reference range of 150-600 pmol/l in adults, whereas for the other methods used (Cobas, Roche Diagnostics, and Atellica, Siemens Healthineers), the implemented reference range is 200-600 pmol/l [10]. The lower reference range for the Abbott method was chosen according to this report as the value between the lower limit established in the direct and the indirect cohort. The choice of reference cohort is debatable, and blood donors included in our direct cohort may be too healthy, whereas the indirect cohort may potentially include ill individuals even if attempts were made to restrict the cohort to healthy individuals. *A priori*, we decided on a differential approach for the identification of outliers (inner or outer Tukey's fences), keeping in mind these population differences. However, the established lower reference limits were robust in both populations irrespective of the choice of outlier assessment. A lower reference range of 150 pmol/l for the Abbott method is in line with those presented in other reports [11] and was substantiated by the observed frequency of low vitamin $B_{12}$ levels across methods in our study cohort.

Overall, our results raise a concern about the use of uniform cut-offs across biochemical methods and call for considerations regarding method-specific strategies for the diagnosis of vitamin $B_{12}$ deficiency. This contrasts with the conclusions of a recent report from the Danish Institute for External Quality Assurance for healthcare laboratories [12]. In this national investigation, pooled serum samples (combined method mean of 153 and 247 pmol/l) were distributed for measurement in clinical biochemical laboratories across Denmark, and results were gathered for assessment of precision and bias across methods. The conclusion of this report was that reference intervals and grey zones could be harmonized across the biochemical methods used in clinical laboratories in Denmark [12]. We used a different methodology, and evaluated the clinical significance among patient results when uniform limits were applied. Furthermore, our data favour a partitioning of the established reference intervals according to previously proposed criteria [8].

It was a strength of our study that we established reference intervals using a direct and an indirect approach, but the preanalytical handling differed with the samples for direct assessment being kept frozen until analyses. All samples were from adults and were anonymous. Thus, any influence of patient age or other characteristics could not be evaluated. Our study was regional but evaluated a series of methods used accross Denmark for biochemical assessment of plasma vitamin $B_{12}$ concentration.

## CONCLUSION

The results of this study suggest that reference intervals and grey zone definitions are not interchangeable across methods and that a biochemical focus is needed in clinical guidelines regarding the diagnosis of vitamin $B_{12}$ deficiency.

**Correspondence** *Stine Linding Andersen*. E-mail: stine.a@rn.dk

**Accepted** 3 April 2023

**Conflicts of interest** none. Disclosure forms provided by the authors are available with the article at ugeskriftet.dk/dmj

**Cite this as** Dan Med J 2023;70(6):A12220771

## REFERENCES

1. Reynolds E. Vitamin B12, folic acid, and the nervous system. Lancet Neurol. 2006;5(11):949-60.
2. Nexø E, Buhl BB, Arendt JFH. Vitamin B12-mangel uden anæmi - en vigtig diagnose. Månedsskr Alm Prak. 2017;895-914.
3. Hvas AM, Ellegaard J, Nexø E. Diagnostik af vitamin B12-mangel - tid til eftertanke. Ugeskr Læger. 2003;165:1971-6.
4. Hvas AM, Nexo E. Diagnosis and treatment of vitamin B12 deficiency - an update. Haematologica. 2006;91(11):1506-12.
5. Lykkeboe S, Nielsen CG, Christensen PA. Indirect method for validating transference of reference intervals. Clin Chem Lab Med. 2018;56(3):463-70.
6. Lykkeboe S, Andersen SL, Nielsen CG et al. Blood sampling frequency as a proxy for comorbidity indices when identifying patient samples for review of reference intervals. Clin Chem Lab Med. 2022;60(2):252-60.
7. Horowitz GL, Altaie S, Boyd JC et al. EP28-A3C defining, establishing, and verifying reference intervals in the clinical laboratory; approved guideline - third edition. Clin Lab Stand Inst. 2010;28(30).
8. Lahti A, Petersen PH, Boyd JC et al. Objective criteria for partitioning Gaussian-distributed reference values into subgroups. Clin Chem. 2002;48(2):338-52.
9. Cadamuro J, Ibarz M, Cornes M et al. Managing inappropriate utilization of laboratory resources. Diagnosis. 2019;6(1):5-13.
10. Abildgaard A, Knudsen CS, Hoejskov CS et al. Reference intervals for plasma vitamin B12 and plasma/serum methylmalonic acid in Danish children, adults and elderly. Clin Chim Acta. 2022;525:62-8.
11. Sobczy&;ska-Malefora A, Katayev A, Steed D et al. Age- and ethnicity-related reference intervals for serum vitamin $B_{12}$. Clin Biochem. 2022;111:66-71.
12. National undersøgelse af cobalamin i lave koncentrationer 2022. Danish Institute for External Quality Assurance for Laboratories in the health sector, 2023.