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Macro vitamin B12: an underestimated threat

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Abstract

Background: The correct identification of the macro-B12 interference (macroforms) is paramount to avoid potential erroneous clinical decisions. Our objectives were to determine whether immunoassays are affected by the presence of macro-B12 and to validate a polyethylene glycol (PEG) precipitation procedure to detect it.

Methods: Sixty-two serum samples obtained from healthy volunteers were analyzed to determine recovery and reference intervals (RIs) following PEG precipitation. Thereafter, 50 serum samples with very high levels of B12 (>1476 pmol/L) were randomly selected to search for macro-B12 interferences. Serum samples obtained from healthy volunteers and related PEG aliquots were analyzed on a Cobas® immunoassay. Patients' samples were analyzed on both Cobas® and Architect® immunoassays. Finally, samples suspected to contain macro-B12 were analyzed by size-exclusion chromatography (SEC) to confirm the presence of macro-B12.

Results: Recovery and post-PEG RIs determined on a Cobas 8000® in healthy volunteers ranged from 68.3% to 108.4% and from 122.1 to 514.4 pmol/L, respectively. Fifteen samples (30%) were found to show macro-B12 while using the recovery criteria, and nine samples (18%) while using the post-PEG RI. The other immunoassay ran on the Architect i2000® was also affected by the presence of macro-B12. Size-exclusion chromatography studies confirmed the presence of macro-B12 (immunoglobulin-B12 complexes).

Conclusions: The prevalence of macro-B12 in elevated B12 samples is high. We suggest to systematically screen for the presence of macro-B12 with PEG precipitation procedure in samples with elevated B12 levels to avoid potential misdiagnosis or harmful clinical consequences.

Keywords: chromatography; immunoassay; interference; macro-B12; polyethylene glycol; vitamin B12.

Introduction

Vitamin B12 (or cyanocobalamin) (B12) is a small watersoluble vitamin that plays a vital role as a coenzyme (methionine synthase and L-methylmalonyl-coenzyme A mutase), in erythropoiesis, and in methylation necessary for cell metabolism and deoxyribonucleic acid (DNA) synthesis [1–3].

In the blood stream, B12 is bound to two binding proteins, namely holo-haptocorrin (80%–95%) and holo-transcobalamin (5%–20%). Only the B12 bound to holotranscobalamin is bioactive. Free B12 is excreted in the urine. Therefore, B12 remains in circulation only if bound to its binding proteins [1–5].

The measurement of B12 is classically requested to identify B12 deficiencies classified in different categories such as severe malabsorption (i.e. pernicious anemia, total or partial gastrectomy), mild malabsorption (i.e. acid blocking drugs, mild atrophic gastritis), dietary deficiency, and abuse of nitrous oxide [1, 2]. B12 deficiency is classically associated with megaloblastic anemia, hemolysis, demyelinating neurological disease, abnormalities in infants and children (i.e. developmental delay) and infertility [1, 2].

On the other hand, elevated B12 levels have been observed in hematologic disorders (i.e. promyelocytic leukemia, polycythemia vera or hypereosinophilic syndrome), liver disease and in subjects taking supplementation or due to certain types of interference [3, 4, 6–8]. It also appears that Black people (African, Afro-American) and Latin-American people tend to have physiologically higher B12 levels than White people but there are no gender differences [9].

Interferences in clinical laboratory analyses still remain a challenging daily issue [10–12]. Given that we...
received several requests from clinicians who were not able to explain high levels of B12, we aimed at investigating the possible presence of macroforms in elevated B12 samples.

This is of particular importance because the presence of such interferences could lead to a misdiagnosis and harmful clinical consequences.

Materials and methods

Healthy volunteers

Serum samples from 62 healthy volunteers (HVs; 40 females and 22 males aged 20–60 years (mean age = 33 years; 95% confidence interval [CI] = 30–35 years) were collected. Blood was taken by venipuncture in the antecubital vein and collected into serum-gel tubes (5 Monovette® 7.5 mL tubes, Sarstedt, Nümbrecht, Germany). None of these HVs were taking any dietary supplementation nor did their laboratory results show any abnormality. Samples were directly stored at −20 °C and analyzed on the same batch.

Polyethylene glycol (PEG) precipitation procedure and macro-B12 definitions

All samples were then treated with the PEG precipitation procedure. For that purpose, 500 μL of PEG solution was added to 500 μL of serum samples, briefly vortexed-mixed, and centrifuged for 2 min at 19,744 × g (Eppendorf® 5424 benchtop centrifuge, Eppendorf AG) [13]. The PEG reagent consisted of 25% (weight/weight) PEG 6000 (Sigma product 4463). Two different macro-B12 definitions were considered. Firstly, the classic recovery (in %) was calculated using: \( \text{RI} = \frac{\text{B12 concentration after PEG treatment} \times 2}{\text{B12 concentration before PEG treatment}} \times 100 \), and the reference interval (RI) in HVs was then determined. The lower reference limit of the RI (including 90% CI) was used as the recovery cut-off to determine the presence of macro-B12 (first definition). Secondly, the post-PEG RI in HVs (in absolute values; pmol/L) was also determined. A post-PEG absolute value lower than the upper reference limit of the post-PEG RI (including 90% CI) was used to define the presence of macro-B12 (second definition).

Patient samples

Serum samples from 50 patients with elevated B12 (above the upper limit of measuring range >1476 pmol/L) were selected between November 2018 and January 2019 and subsequently treated with the PEG precipitation procedure described above. The study population displayed the following characteristics: 28 females and 22 males aged between 18 and 93 years (mean age = 59 years). According to their medical records, none of these patients were taking any dietary supplementation. The presence of macro-B12 was evaluated based on the two different definitions presented above.

Vitamin B12 measurement

In our laboratory, B12 was measured on the Cobas 8000® immunoassay automated platform (Elecsys® B12 assay, Roche Diagnostics®, Mannheim, Germany) before and after the PEG precipitation procedure. The method principle is based on the competition of serum B12 with exogenous biotinylated B12 for ruthenium-labeled intrinsic factor (IF) binding sites. The binding of the biotinylated complexes with streptavidin beads allows the generation of a signal inversely proportional to the B12 concentration. The limit of quantification (LOQ) was 111.0 pmol/L and the RI given by the manufacturer ranges from 138.0 to 569.0 pmol/L. Within-run precisions were 6.6% and 5.0% at concentrations of 331.0 and 677.0 pmol/L, respectively. Within-run precisions (patient pools; 10 replicates) were 4.3% and 4.2% at B12 concentrations of 133.0 and 992.7 pmol/L, respectively. Post-PEG within-run precisions (patient pools treated with PEG; 10 replicates) were 4.8% and 2.7% at B12 concentrations of 120.0 and 678.4 pmol/L, respectively.

In a second laboratory (Clinique St-Pierre, Ottignies, Belgium), 40 patients’ serum samples and related PEG aliquots (for which we had enough volume) were also measured using the Architect i2000® analyzer (Abbott®; IL, USA) to assess if the latter is also affected by the presence of macro-B12. The LOQ was 92.0 pmol/L and the RI given by the manufacturer ranges from 138.0 to 652.0 pmol/L. According to the manufacturer’s package insert (reference B7K610, revised November 2015), within-run precisions were 5.6% and 4.0% at B12 concentrations of 182.0 and 657.0 pmol/L. For the sake of simplicity, all B12 concentrations upper than 1476 pmol/L (upper limit of linearity on both analyzers) were rounded to this latter.

Size exclusion chromatography (SEC)

The samples that we suspected to contain macro-B12 and that had enough volume (n = 12) as well as three controls (no response after the PEG precipitation procedure) were also sent to the Regional Customer Support Center of Roche Diagnostics® (Penzberg, Germany) to analyze them by SEC in order to determine the clear nature of the interference. Roche was blind about the identification of these samples.

Questionnaires sent to clinicians

Lastly, we decided to evaluate the clinical impact of the macro-B12 cases that we observed in our study. For that purpose, we sent a questionnaire to the physicians that were in charge of the patients. We asked them four questions: (1) Is it a new discovery? (2) Was the patient taking dietary supplementation? (3) What did you do with the results? (bone marrow aspiration, confirmation with another blood sampling, referral to a specialist, drug prescription, …) and (4) Did you think about a possible interference?

Statistical analysis

Mean, percentiles, minimum and maximum B12 concentrations were determined in HVs and patients, before and after the PEG
precipitation procedure. Coefficients of variation (%) on 10 replicates 
([standard deviation/mean] × 100) were calculated to determine the 
within-run precisions before and after the PEG precipitation proce-
dure on patient pools. For the determination of RIs in HVs (95% CI; 
2.5th and 97.5th percentiles), the robust method as proposed by the 
CLSI EP28A3 document was used [16]. The Box-Cox transformation 
was used to transform data to normality [14, 15] and the normality of 
distribution was assessed by the Anderson-Darling’s test [16]. Dixon-
Reed and Tukey tests were used to detect potential outliers [16, 17]. 
90% CI of the RIs obtained with the robust method were derived 
using a Bootstrap resampling method [14, 15]. Lastly, the degree 
of agreement between the two methods for the search of macro-
B12 were determined by the weighed kappa index. Data analysis 
was performed using GraphPad Prism® software (Version 6.0e, San Diego, 
CA, USA). The Reference Value Advisor integrated on Microsoft Excel® 
2010 (Version 2.1) was specifically used for the calculation of RIs [15].

Ethical considerations

Our study fulfilled the Ethical principles provided by the Declaration 
of Helsinki and was approved by the local Medical Ethics Committee. 
All participants gave their informed consent.

Results

In HVs, the mean of B12 levels was 308.2 pmol/L (95% CI = 280.2–336.2 pmol/L). The recovery was determined to range from 68.3% (90% CI = 65.5–71.6%) to 108.4% (90% CI = 103.3–113.1%), with a mean of 86.5% (95% CI = 84.0–89.1%). The post-PEG RI was determined to

![Figure 1: Results obtained following the PEG precipitation procedure in highly elevated B12 samples, using recoveries (%). Reference intervals (95% CI) and their related CIs (90%) are presented with black and grey dotted lines, respectively.](image-url)
Based on the report we received from Roche Diagnostics®, it appeared that seven samples out of 12 contained immunoglobulin-B12 complexes. Among the three control samples sent, two did not contain interfering factors and one contained immunoglobulin-B12 complexes (Table 1).

Based on the questionnaire filled out by clinicians for the 15 patients suspected to have macro-B12, it appeared that three patients (20.0%) already had elevated B12 values in the past (two since 2011 and one since 2013). Because physicians could not explain such elevations, three patients (20.0%) were referred to hematologists. Two physicians asked internists for advice (13.3%). Six physicians programmed another blood sampling for their patients (40.0%) to confirm the first results. Patients were asked about possible B12 supplementation in 60% of cases, and none of them were taking any supplementations. Medical records did not show any supplementation for the remaining patients (n=6). Five physicians (33.3%) included the possible presence of an interference in their differential diagnosis. Neither drugs nor invasive procedures such as bone marrow aspiration or biopsy were prescribed (Table 1) although two physicians had thought about it.

**Discussion**

In the present study, we have clearly shown that the presence of macro-B12 in elevated B12 samples may be important and conduce to unnecessary additional actions. This issue will be important to address by clinical laboratories and in vitro diagnostic industries for the accuracy of the clinical decision and for patient safety.

Such macroforms are classically considered to be inactive and composed of immunoglobulin complexes. The real biological impact of macro-B12 still remains to be defined. Chromatography studies are the gold standard to identify the presence of such interferences. Nevertheless, these methods are costly, not widely available, and require a certain level of expertise [10, 13, 18, 19].

Hereby, we report the benefits of the PEG precipitation procedure to search for macro-B12. It is well known that the PEG precipitation procedure could be useful in the search for macroforms of prolactin (PRL) [13], thyroid-stimulating hormone (TSH) [10], parathyroid hormone, luteinizing hormone or follicle-stimulating hormone. Even though the PEG precipitation procedure is not specific and has to be interpreted with caution, it appeared to be cheap, fast and user-friendly to indirectly identify macroforms [10, 13, 18].

We decided to use two different macro-B12 definitions. The first one being based on the recovery calculation and the second on the post-PEG RI. As it has been shown for macro-PRL, the recovery calculation may be misleading given that the concomitant presence of inactive macro-PRL and of active monomeric PRL should be interpreted as a true increase in PRL and has to be treated as such [13, 20]. This situation also applied to B12. Indeed, in one control sample sent to Roche Diagnostics®, macro-B12 was

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**Figure 2:** Results obtained following the PEG precipitation procedure in highly elevated B12 samples, using post-PEG reference interval (pmol/L). RIs (95% CI) and their related CIs (90%) are presented with black and gray dotted lines, respectively. *Samples containing macro-B12 based on the recovery calculation, but not according to the post-PEG reference interval definition.
Table 1: Associations and discrepancies according to the macro-B12 definition used (recovery, post-PEG values and chromatography) and clinical information.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Recovery (Cobas®), %</th>
<th>Post-PEG (Cobas®), pmol/L</th>
<th>Post-PEG (Architect®), pmol/L</th>
<th>SEC</th>
<th>Clinical information</th>
<th>Clinical impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3</td>
<td>23.5</td>
<td>347.3</td>
<td>Insufficient volume</td>
<td>Insufficient volume</td>
<td>Hemochromatosis</td>
<td>Another blood testing and other appointments were planned</td>
</tr>
<tr>
<td>S5</td>
<td>23.2</td>
<td>343.0</td>
<td>Insufficient volume</td>
<td>Insufficient volume</td>
<td>RA, COPD, CKD</td>
<td>None</td>
</tr>
<tr>
<td>S7</td>
<td>51.9</td>
<td>765.6</td>
<td>Insufficient volume</td>
<td>Insufficient volume</td>
<td>JRA</td>
<td>Another blood testing was planned</td>
</tr>
<tr>
<td>S16</td>
<td>23.7</td>
<td>350.0</td>
<td>307.0</td>
<td>Immune complexes</td>
<td>Steatosis, temporal arteritis, HT, RA, T2DM</td>
<td>Patient known since 2013. Several other blood testings and appointments were planned. The patient was also referred to a hematologist</td>
</tr>
<tr>
<td>S18</td>
<td>36.0</td>
<td>530.8</td>
<td>522.5</td>
<td>Immune complexes</td>
<td>Aplastic anemia, COPD</td>
<td>Another blood testing and other appointments were planned. The patient was also referred to a hematologist</td>
</tr>
<tr>
<td>S20</td>
<td>64.2</td>
<td>946.9</td>
<td>909.2</td>
<td>No immune complexes</td>
<td>T-cell acute lymphoblastic leukemia</td>
<td>None</td>
</tr>
<tr>
<td>S26</td>
<td>5.6</td>
<td>83.1</td>
<td>187.5</td>
<td>Immune complexes</td>
<td>Steatosis, telangiectasia, epistaxis</td>
<td>Another blood testing and other appointments were planned. The patient was referred to our hospital to confirm the result</td>
</tr>
<tr>
<td>S28</td>
<td>17.9</td>
<td>263.9</td>
<td>277.5</td>
<td>Immune complexes</td>
<td>Hepatic steatosis, diverticulosis, asthma, allergies</td>
<td>None (patient known since 2011)</td>
</tr>
<tr>
<td>S29</td>
<td>27.1</td>
<td>399.3</td>
<td>405.9</td>
<td>Immune complexes</td>
<td>Ankylosing spondylitis, HT, gastritis, AD, depression, osteoporosis, T2DM, history of breast cancer</td>
<td>None</td>
</tr>
<tr>
<td>S32</td>
<td>57.0</td>
<td>840.6</td>
<td>764.6</td>
<td>No immune complexes</td>
<td>CKD, CHF, T2DM, polyneuropathy, inflammation of unknown origin</td>
<td>None</td>
</tr>
<tr>
<td>S36</td>
<td>63.6</td>
<td>938.1</td>
<td>888.6</td>
<td>No immune complexes</td>
<td>CHF, Parkinson’s disease</td>
<td>None</td>
</tr>
<tr>
<td>S44</td>
<td>55.2</td>
<td>815.2</td>
<td>606.6</td>
<td>No immune complexes</td>
<td>Alcoholic patient, without any particular pathology</td>
<td>The physician asked an internist for advice</td>
</tr>
<tr>
<td>S47</td>
<td>63.8</td>
<td>942.0</td>
<td>903.3</td>
<td>No immune complexes</td>
<td>Autoimmune hepatitis, cirrhosis and acute anemia (intestinal bleeding)</td>
<td>None</td>
</tr>
<tr>
<td>S48</td>
<td>33.1</td>
<td>487.8</td>
<td>433.9</td>
<td>Immune complexes</td>
<td>Without any particular pathology</td>
<td>The physician asked an internist for advice</td>
</tr>
<tr>
<td>S50</td>
<td>16.9</td>
<td>249.6</td>
<td>252.4</td>
<td>Immune complexes</td>
<td>CD, allergies</td>
<td>Patient known since 2011. Several other blood testings and appointments were planned</td>
</tr>
</tbody>
</table>

Samples classified as containing macro-B12 based on the three different definitions are indicated in blue. AD, Alzheimer’s disease; CD, Crohn’s disease; CHF, chronic heart failure; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; HT, Hashimoto’s thyroiditis; JRA, juvenile rheumatoid arthritis; PEG, polyethylene glycol; RA, rheumatoid arthritis; T2DM, type 2 diabetes mellitus; SEC, size-exclusion chromatography.
identified but the post-PEG value was 1051 pmol/L. A true increase in B12 should therefore be considered. Moreover, every samples suspected to contain macro-B12 based on the post-PEG RI were confirmed by the SEC. Thus, we showed that the identification of macro-B12 based on the post-PEG RI definition was highly correlated to the SEC in our study.

However, in five serum samples suspected to contain macro-B12 based on the recovery calculation definition, immunoglobulin complexes were not found by the SEC. All things considered, we suggest to renounce the classic recovery calculation and to use the post-PEG RI to search for macro-B12 instead, as it has already been endorsed for the search of macro-PRL.

The presence of such macro-B12 in highly elevated B12 samples has been rarely published in the literature and the overall awareness in the medical community is insufficient [6, 7, 19, 21]. Bowen et al. reported a case of macro-B12 on the Advia Centaur® system (Siemens®, Erlangen, Germany) [21]. Yang and Cook reported two macro-B12 cases in patients suffering from pernicious anemia on the Siemens Dimension Vista® system [7].

We also found that the Architect® analyzer (Abbott®, IL, USA) was sensitive to the presence of macro-B12. However, like macro-PRL [22] and macro-TSH [10], the intensity of the interference might be different across the different immunoassays. The interference is therefore not analyzer dependent, concerns most of the clinical laboratories and in vitro diagnostic suppliers and might represent a global issue.

Jeffery et al. have reported that macro-B12 are mostly formed by immune complexes (IgG) and that the prevalence in elevated B12 samples was at least 8%, as evaluated on the Roche E170® analyzer [19]. In our study, we confirm that the presence of macro-B12 in highly elevated B12 samples is important (18%). However, Jeffery et al. only used the recovery calculation criteria and the cut-off (50%) was arbitrary defined [19].

One strength of our study was that we determined cut-offs using 60 HVs after the PEG precipitation procedure, which is much more closely related to the SEC study. It is recommended to determine a specific post-PEG RI given that a fraction of the monomeric molecule may be co-precipitated, due to the matrix effect [20]. We also chose to perform a faster PEG precipitation method (faster centrifugation and no incubation) which is more compatible with the requirements and processes of core laboratories. In our study, we arbitrary decided to treat samples with the PEG precipitation procedure if B12 concentrations were >1476 pmol/L (upper limit of linearity). However, macro-B12 could also be present at lower concentrations (i.e. from 569 to 1476 pmol/L).

Physicians classically prescribed B12 tests to identify deficiencies. However, raised levels of B12 concentrations are more common in clinical practices (>8%) [5, 19, 23]. It has been shown that hematological malignancies (i.e. leukemias and myeloproliferative neoplasms), renal diseases, liver diseases, heart failure, autoimmune disorders (i.e. RA), infection diseases or some solid cancers may be associated to elevated B12 levels [4, 5, 9, 23, 24].

A proposal diagnostic strategy for interpreting unexpected high B12 concentrations did not take into account interferences such as macro-B12 [5]. However, the prevalence of macro-B12 is important (18% in our study). Further studies are necessary to harmonize clinical decisions in regard to highly elevated B12 samples [5]. It is also important to notice that we observed a patient with a very low post-PEG B12 concentration (83.1 pmol/L) that may correspond to a B12 deficiency as compared to the pre-PEG concentration (>1476 pmol/L), corresponding to a major increase in B12 concentration.

Fortunately, none of these patients underwent an aggressive procedure (i.e. bone marrow aspiration or biopsy) because of the wrong B12 results that physicians received. It appeared challenging for physicians to identify the cause of such B12 increases. The major consequences of the macro-B12 presence was the referral to hematologists and the realization of additional blood samplings to confirm the first results. If the interferences were promptly identified, such unneeded actions would have been immediately ceased. The magnitude of the problem may therefore be important.

In our laboratory, we analyzed 20,158 B12 samples in 2017 and 20,568 samples in 2018. In 2017, 427 samples showed B12 levels >1476 pmol/L and 443 samples did too in 2018. Given that we evaluated the prevalence of macro-B12 as being 18%, approximately 78 cases may be observed every year.

In our routine, and based on our results, we decided to perform the PEG precipitation procedure for each sample with B12 level ≥1476 pmol/L. If B12 levels are falsely increased (the presence of macro-B12 after the PEG treatment), a comment will be transferred to the laboratory information system to explain the false B12 elevation in order to avoid any potential erroneous clinical decisions.

It still remains unclear why some patients developed such immune complexes. These complexes are however not correlated to the presence of IF autoantibodies [19] and the Elecsys B12® assay prevents this interference from occurring [25].

Our study has some limitations. First, CLSI C28-A3 guidelines recommend using 120 HVs to establish RIs [14]. However, we were only able to include 60 HVs in our
study. Hence, our data could be considered as preliminary and further studies should be carried out on a larger dataset to confirm our data. Second, we did not determine the post-PEG RI on the Architect analyzer as we did on our Cobas analyzer, and therefore decided to use the same RIs for the search of macro-B12. This might introduce a bias. However, a high correlation between both analyzers has been reported (r = 0.99) with only a slight elevation for higher values (slope of 1.140; 1.095–1.184; n = 69) [26]. If post-PEG RIs become the standard method to evaluate the presence of macro-B12, specific post-PEG RIs for other analyzers (including the Architect) should be determined.

Conclusions

Our results have clearly shown that the prevalence of macro vitaminB12 in elevated vitamin B12 samples (>1476 pmol/L) may be high (i.e. 18%). As confirmed by the SEC, these interferences are most likely caused by immunoglobulin-B12 complexes. It is important to notice that the problem is not analyzer dependent, could be observed in platforms from different in vitro diagnostic manufacturers and will therefore concern most of the clinical laboratories testing vitamin B12. Based on our results, we suggest to systematically screen for the presence of macro-B12 with PEG precipitation procedure in samples with elevated B12 to avoid potential erroneous clinical decisions.

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