In this issue

Laboratory investigation of vitamin D metabolites and bone metabolism markers
Harjit Pal Bhattoa

Vitamin D and its metabolites: from now and beyond
Etienne Cavalier, Jean-Claude Souberbielle

Vitamin D and endocrine disorders: routine laboratory diagnostic implications
Giovanna Muscogiuri, Harjit Pal Bhattoa

Laboratory aspects and clinical utility of bone turnover markers
Harjit Pal Bhattoa

Practical guide for identifying unmet clinical needs for biomarkers
Phillip J. Monaghan, Sarah Robinson, Daniel Rajdl, Patrick M.M. Bossuyt, Sverre Sandberg, Andrew St John, Maurice O’Kane, Lieselotte Lennartz, Ralf Röddiger, Sarah J. Lord, Christa M. Cobbaert, Andrea R. Horvath

Prevalence of anemia and its associated factors among children in Ethiopia: a protocol for systematic review and meta-analysis
Zegeye Abebe, Wubet Worku Takele, Degefaye Zelalem Anlay, Daniel Tekelia Ekubagewargies, Zegeye Getaneh, Molla Abebe, Mulugeta Melku

Acute myeloid leukemia with severe coagulation disorder and concomitant central nervous system bleeding – a clinical diagnostic case report
S. Hatzl, B. Uhl, M. Hinterramskogler, S. Leber, F. Eisner, M. Haring, P. Jud

Vitamin B12 immunoassay interference in a patient with multiple myeloma – troubleshooting in a two step reagent kit based on enhanced chemiluminescence testing
V. Pant, A. Tumbapo, B. Kumar Yadav
In this issue: Laboratory investigation of vitamin D metabolites and bone metabolism markers

Laboratory investigation of vitamin D metabolites and bone metabolism markers

Guest Editor: Harjit Pal Bhattoa
Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Hungary

ARTICLE INFO

Corresponding author:
Harjit Pal Bhattoa
Department of Laboratory Medicine
Faculty of Medicine
University of Debrecen
Hungary
E-mail: harjit@med.unideb.hu

EDITORIAL

It may well be stated that routine laboratories have been overwhelmed with requests for determination of 25-hydroxyvitamin D, the analyte reflecting vitamin D status. The number of test requests is increasing pretty much from year to year in essentially all known laboratory settings.

Given the pandemic of vitamin D insufficiency experienced on a global scale it is important to understand the laboratory implications pertaining to the determination of this not so easy analyte.

Similarly to quite a few other hormone determinations, harmonization and standardization is a valid issue and professional bodies have set up task forces to resolve the discrepancies experienced between the different currently available methodologies.

In the first article of three from this thematic issue, Cavalier et al. have comprehensively reviewed all data implicated in vitamin D testing that is relevant to the practicing laboratory professional.

Professor Etienne Cavalier is Head of the Department of Clinical Chemistry, University of Liege, Belgium. He is currently the president of the Belgian Royal Society of Laboratory Medicine. He is member of the Editorial Board of leading Journals in the field of Laboratory Medicine and has over 230 publications with an h-index of 28. He is member of numerous National and International Working Groups. Being a professor of
Clinical Chemistry, he has published extensively on groundbreaking research in routine laboratory practice and has dealt not only with research issues but also the practical implication of novel biomarkers particularly in the field of musculoskeletal endocrinology and metabolism. His current research interests include Bones markers, vitamin D, PTH, vascular calcification markers, markers of acute kidney diseases, glomerular filtration rate (estimation, biomarkers), markers of frailty and sarcopenia, LC-MS/MS methods for steroids and peptides quantification. Given their professional achievements, Professor Cavalier and colleague are one of the most authentic to address this very important and relevant topic of vitamin D determinations.

Vitamin D is known to have various non-skeletal effects including its role in endocrinological conditions. The second article summarizes the importance of vitamin D in common endocrinological diseases penned by Muscogiuri et al.

Dr. Giovanna Muscogiuri graduated in Medicine from the Catholic University, Rome in 2006 and then carried out postgraduate residency in Endocrinology and Metabolic Disease at the same university, obtaining her degree of specialization in 2012. She attended the post-doctoral fellowship at University of Texas Health Science Center at San Antonio, TX – USA. She is currently focused on the study of vitamin D and related diseases. She has received several awards for her scientific achievements in the metabolic research field. She has written or co-authored over 100 papers in peer-reviewed scientific journals. Muscogiuri et al place in perspective what our correct knowledge on vitamin D is with relevance to endocrinology. Furthermore, given the argument supporting achievement of optimal vitamin D status in the common endocrinological diseases dedicately discussed, the laboratory issues that may arise are briefly enumerated.

In the third article, Bhattoa summarizes the importance of determination of biochemical markers of bone turnover in metabolic bone diseases. Given the sensitive nature of their determination, pre-analytical, analytical and post-analytical issues are extensively discussed and their clinical utility is placed in perspective.
Vitamin D and its metabolites: from now and beyond
Etienne Cavalier¹, Jean-Claude Souberbielle²

¹ Department of Clinical Chemistry, University of Liège, CHU de Liège, Belgium
² Laboratoire d’Explorations fonctionnelles, Hôpital Necker-Enfants malades, Paris, France

ARTICLE INFO

Corresponding author:
Etienne Cavalier
Department of Clinical Chemistry
University of Liège
4000 Liège
Belgium
E-mail: Etienne.cavalier@chuliege.be

Key words:
25 hydroxyvitamin D,
1,25 dihydroxyvitamin D,
standardization,
Vitamin D Metabolism Ratio (VMR)

ABSTRACT

Total 25-hydroxyvitamin D is currently considered as the most representative metabolite of vitamin D status. There are a multitude of challenges that still deserve to be addressed and despite recent technological advances its determination remains complicated. This current review gives an abbreviated overview of the phases of development that vitamin D metabolite determination has gone through and discusses the difficulties that still require resolving. Furthermore, given the different platforms and methodologies available, the critical issue of standardization and all efforts made as far towards its realization have been discussed. And last but not least, the concepts of ‘free’ and ‘bioavailable’ vitamin D along with the ‘Vitamin D Metabolism Ratio’ have been discussed.
INTRODUCTION

Until recently, 25(OH)-vitamin D (25-OHD) was merely the only vitamin D (VTD) metabolite of interest to explore vitamin D status and metabolism. Unfortunately, the determination of this VTD metabolite, as well as the levels that need to be achieved in healthy or diseased individuals are quite problematic and remain an important matter of debate [1,2].

Recently, other VTD metabolites, like 24,25-OH2D, “bioavailable” or “free” vitamin D, cholecalciferol itself and 1,25-OH2D, have emerged as potential new players to better understand the important vitamin D pathway. In this paper, we provide a brief overview on the issues regarding 25-OHD assays and standardization and we will evoke 24,25(OH)2D and Vitamin D ratio (VMR) as potential metabolites of choice to explore vitamin D deficiency.

25-HYDROXY VITAMIN D DETERMINATION

25-OHD is still currently considered as the most representative metabolite of vitamin D status. Unfortunately, its determination remains complicated despite recent technological advances [3]. The reasons why this metabolite is so complicated to be correctly assessed are multiple. First, 25-OHD assays need to recognize 25-OHD2 and 25-OHD3. Second, 25-OHD is a very hydrophobic molecule that circulates bound to vitamin D binding protein (DBP), albumin (ALB) and lipoproteins and a thorough dissociation of the analyte from its ligands is mandatory prior to measurement. This step is particularly complicated for automated immunoassays where, in contrast to radio-immunoassays, binding-protein or chromatographic assays, organic solvents cannot be used for extraction. Hence, automated immunoassays need alternative releasing agents, which do not always achieve total dissociation of 25-OHD. In particular physiological or pathological conditions such as pregnancy, estrogen therapy or renal failure, automated immunoassays often fail to correctly quantify 25-OHD [4-7]. Third, 25-OHD2 and 25-OHD3 have different affinity constants for the carriers and the dissociation step must be highly efficient to obtain an accurate quantification of both forms. Forth, in-vitro recovery experiments with the molecule give spurious results with immunoassays since it is not clear whether exogenous metabolites bind to all the different carriers in the same proportions as endogenous metabolites. Under-recovery of exogenous 25-OHD has been reported in automated immunoassays [8-9] and even liquid chromatography tandem mass spectrometry (LC-MS/MS) methods [10].

The different methods available for the quantification of 25-OHD use chromatographic separation (HPLC with UV or LC-MS/MS detectors), antibodies or binding-proteins. Binding protein assays have been used in the early eighties and presented clinically acceptable analytical sensitivity and imprecision. They were based on the displacement of H3-labelled 25-OHD and necessitated a chromatographic purification after organic extraction. These home-made methods were very time-consuming and performed in some reference laboratories only. Hence, they have been superseded by radio-immunoassays (RIA) methods. The first commercially available RIA was based on a method described by Hollis et al. in 1993 [11] and the DiaSorin RIA method has been the most widely used method for both routine diagnostic testing as well as for clinical studies until recently. Traditional 25-OHD cutoffs in use today for vitamin D deficiency (either 20 or 30 ng/ml) have been defined on the basis of studies (and meta-analyses of studies) that predominantly used this assay. However, due to the logarithmic increase in 25-OHD requests observed during the last decade, laboratories have opted for more automated immunoassays and less than 1% of laboratories participating in the DEQAS still use this RIA assays nowadays.
The first automated immunoassay for 25-OHD determination was launched in 2001 by Nichols Diagnostics on the Advantage platform. This assay used a competitive ligand binding technique with acridinium-ester labelled anti-DBP. Nowadays, most of the major in-vitro diagnostic companies have launched their own methods for 25-OHD determination. These methods use a competition design, except the one from Fujirebio on the Lumipulse, which is a non-competitive (sandwich) method based on antimetatype monoclonal antibodies against a hapten–antibody immunocomplex using an ex vivo antibody development system, namely the Autonomously Diversifying Library system, a process which has recently been validated [12]. A large number of studies have evaluated the different automated assays by comparison with RIA, HPLC or, more recently, with LC-MS/MS methods. Conclusions regarding the accuracy of the assays have also been based on the results of large external proficiency testing programs, such as DEQAS or CAP which now use a reference method to measure the samples sent to the participants, allowing a true calculation of the bias. In a recent study coordinated by the Vitamin D Standardization Program (VDSP) group [13], a set of 50 healthy individuals donor samples were analyzed by 15 different laboratories to provide results for total 25-OHD using both immunoassays and LC-MS/MS methods. The results were compared with those obtained by two reference methods, namely the Ghent University and the U.S. National Institute of Standards and Technology (NIST) methods. Results showed that all but 2 LC-MS/MS achieved VDSP criteria of performance (namely CV ≤ 10% and mean bias ≤ 5%), whereas only 50% of immunoassays met the criteria. These results can be regarded as optimistic for immunoassays. First, it is obvious from these results that standard deviation around the bias is much more important for immunoassays than LC-MS/MS. As an example laboratory 2a that used an immunoassay and laboratory 10 used a LC-MS/MS method which both presented an excellent mean bias of -1%. But the standard deviation around this bias was 14% for the immunoassay vs. 5% for the LC-MS/MS method. As a consequence, the LC-MS/MS will have 75% of its value within the 5% boundaries whereas the immunoassay will only have 29%. Second, this study has been performed on serum obtained in healthy donors and not in patients. Indeed, patients with chronic kidney disease, dialysis patients, pregnant women, different ethnic groups, patients in intensive care with fluid shifts present differences in their serum matrix compared to healthy individuals and this can impact the performance of automated 25-OHD immunoassays. Recently, we have shown good clinical concordance between 4 different immunoassays and a VDSP-traceable LC-MS/MS method in healthy subjects. However, significantly poorer agreement with the same LC-MS/MS method has been found in other clinical populations [4, 14].

In the past years, the IFCC has made great efforts to promote standardization of laboratory assays. Indeed, standardization is important to achieve comparable results across different methods and manufacturers. For 25-OHD assays, clinical cut-offs are generally used as target values, and applying common cut-offs on results generated with poorly standardized assays will inevitably lead to inconsistent patient classification and inappropriate therapeutic decisions. Hence, in 2010, the Vitamin D Standardization Program (VDSP) was established to improve the standardisation of 25-OHD assays. The aim of VDSP is that 25-OHD measurements are accurate and comparable over time, location, and laboratory procedure to the values obtained using reference measurement procedures (RMPs) developed at the NIST [15] and Ghent University [16]. As mentioned earlier, a method is considered as standardized if the CV is <10% and the bias <5% [17]. Each candidate receives a set of
10 samples 4 times a year and has to run these samples in duplicate on 2 consecutive days. In January 2018, 27 methods, coming either from IVD companies or clinical laboratories were considered as standardized against the RMPs. However, the proportion of the 40 samples that met the bias criterion (<5%) in 2017 was quite different from one method to the other and ranged from 23 to 85%, with LC-MS/MS methods presenting better results than immunoassays. The list of these standardized methods can be found on the CDC website (http://www.cdc.gov/labstandards/pdf/hs/CDC_Certified_Vitamin_D_Procedures.pdf).

Although substantial progress has been made, a range of important issues like standardization of 25-OHD2 and 24,25-(OH)2D as well as improvement of (immuno)assays performance on samples from diseased patients or subjects from different ethnic groups still needs to be achieved. It may thus be tempting to think that immunoassays are outdated and that LC-MS/MS should replace these methods. There are clear limitations to this simplistic view. Indeed, performing a LC-MS/MS is complex and needs experienced and very well trained people. Notably, extensive validation of the LC-MS/MS and sample preparation are of extreme importance. To run a LC-MS/MS is much more complicated than “crash the proteins, inject and obtain the results in 2 minutes”. A detailed review on how complex running a LC-MS is out of the scope of this present paper, but can be found in a previous report [18]. Finally, laboratories that run LC-MS/MS do not run the reference method, even if their method is certified by the VDSP. As an illustration of this assertion, one can see that some VDSP-certified LC-MS/MS methods present a percentage of samples out of the bias criterion that is lower than immunoassays and much lower than other LC-MS/MS. Also DEQAS results show that LC-MS/MS methods present CVs that are as high as immunoassays.

24,25-(OH)2D DETERMINATION AND THE VITAMIN D METABOLITE RATIO

One advantage of LC-MS/MS methods over immunoassays is the possibility to simultaneously quantify 25-OHD and 24,25-(OH)2D allowing to calculate the 25-OHD/24,25-(OH)2D ratio, also known as the Vitamin D Metabolism Ratio (VMR). Indeed, some light has recently been shed on the potential interest of this vitamin D metabolite to better reflect vitamin D deficiency [19]. In summary, CYP24A1, the enzyme allowing the degradation of 25-OHD and 1,25-(OH)2D into 24,25-(OH)2D and 1,24,25-(OH)3D sees its expression increased when there is an increased binding and activation of the VDR in response to 1,25-(OH)2D [20]. Hence 24,25-(OH)2D concentration may thus reflect VDR activity which is not really the case with 25-OHD. It has recently been demonstrated that lower 24,25-(OH)2D concentration and lower VMR were associated with increased hip fracture risk in community-living older men and women, whereas 25-OHD was not associated with hip fracture risk. Another point of interest with 24,25-(OH)2D and VMR is that, although concentrations of 25-OHD and 24,25-(OH)2D strongly correlate with each other and are both lower in black Americans than in whites, blacks and whites have equivalent median VMR values [21]. In CKD patients, Bosworth et al have shown that 24,25-(OH)2D was better associated with PTH than 25-OHD or 1,25-(OH)2D [22]. These findings are of great interest but still need to be confirmed by other studies. On the other hand, it is clearly demonstrated that biallelic mutations in CYP24A1 led to idiopathic infantile hypercalcemia [23], a phenotype characterized by profound hypercalcemia, suppressed intact parathyroid hormone, hypercalciuria and nephrocalcinosis. Many heterozygous mutations of CYP24A1 have recently been described [24]. If they are associated with a less dramatic phenotype than homozygous mutations, patients suffering from these mutations...
often present with hypercalcemia, suppressed PTH and renal stones [25]. Hence, in patients presenting with a non-parathyroid hypercalcemia (without evident clinical cause), CYP24A1 mutations should be investigated and simultaneous 24,25-(OH)2D and VMR ratio should be measured. A ratio higher than 50, or even 80 should lead to a genetic research of a CYP24A1 mutation. Again, this measurement should be standardized. Fortunately, one candidate reference measurement procedure (RMP) has been published [26] and NIST standard reference material (SRM) 2972a includes 4 standards with certified values (unfortunately, these 4 values are very close to each other) [27]. DEQAS data report that about 10 laboratories provide 24,25-(OH)2D results. These data show quite a large variability, which can partially be attributed to the low concentration of the analyte, but also to the lack of ongoing standardization program. This latter will be (probably) even more important than the 25-OHD itself since small variations in 24,25-(OH)2D have a dramatic impact on the VMR.

CONCLUSION

The assessment of vitamin D status is a changing landscape [19]. Although 25-OHD is still recommended as the marker of choice by virtually all scientific bodies growing evidence indicates significant limitations that hamper the utility of this analyte in clinical practice. Issues related to the use of 25-OHD include analytical aspects and the interpretation of results. While in normal individuals the agreement of results generated with automated assays is improving, comparability of results in distinct populations, such as children, pregnant women, hemodialysis patients or intensive care patients, remains problematic. The relationships between 25-OHD and various clinical indices are also rather weak and not consistent across races. Recent studies have provided new insights in physiological and analytical aspects of vitamin D that may change the way how we will assess vitamin D status in the future. The VMR (25-OHD/24,25-OH2D ratio), but also ‘free’ and ‘bioavailable’ vitamin D are all interesting markers that have expanded our knowledge about vitamin D metabolism and some of these analytes may now be considered for routine use (at least in specialized centers).

REFERENCES

Vitamin D and its metabolites: from now and beyond


Vitamin D and endocrine disorders: routine laboratory diagnostic implications

Giovanna Muscogiuri¹, Harjit Pal Bhattoa²

¹ Department of Clinical Medicine and Surgery, Endocrinology Unit, Federico II University, Medical School of Naples, Italy
² Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Hungary

ARTICLE INFO

Corresponding author:
Giovanna Muscogiuri
Department of Clinical Medicine and Surgery
Endocrinology Unit
Federico II University
Medical School of Naples
Italy
E-mail: Giovanna.muscogiuri@gmail.com

Key words:
Vitamin D, Hashimoto’s thyroiditis, Grave’s disease, Addison’s disease, primary hyperthyroidism

ABSTRACT

The role of vitamin D in calcium-phosphorus metabolism regulation is the most highlighted, nonetheless there is enormous literature on the extra-skeletal effects of vitamin D, and lately new insight into the role of vitamin D in endocrine disease mechanisms has seen light of day. The present narrative review gives an overview of the proposed roles of vitamin D in the etiology of Hashimoto’s thyroiditis, Grave’s disease, Addison’s disease and primary hyperthyroidism. The implications as pertaining to the routine laboratory practice are readily applicable to this patient group as well, and do not pose any additional challenge.
INTRODUCTION
The role of vitamin D in calcium-phosphorus metabolism regulation is the most highlighted, nonetheless there is enormous literature on the extraskeletal effects of vitamin D [1]. It is known that non-skeletal cells, including those of the endocrine system, too express vitamin D receptor and as such interact with the active form of vitamin D, i.e., 1,25-dihydroxyvitamin D, furthermore, most of these cells express extra-renal forms of 25-hydroxyvitamin D-1α-hydroxylase enzyme and, eventually the active form of vitamin D is known to regulate quite a number of genes, including those implicated in proliferation, differentiation and apoptosis [2-5]. Recently, it has been described that the pathophysiology of autoimmune thyroid disease, adrenal disease and hyperparathyroidism has a Vitamin D component [6-12]. The present review discusses the association between vitamin D status and the aforementioned endocrine conditions and delineates the routine laboratory assessment of vitamin D metabolites.

VITAMIN D AND HASHIMOTO’S THYROIDITIS
Hashimoto’s thyroiditis is an autoimmune disease with defective suppressor T-cell function. Thyrocytes in this disease condition express major histocompatibility complex class II (MHC II) surface HLA-DR antigens, as a result of cytokines produced by T helper 1 (Th1) cells, making them prone to immunologic attack as such triggering the autoimmune process. Subsequently the T cell activated B lymphocytes produce autoantibodies reacting with thyroid antigens [9,13,14].

The active form of vitamin D suppresses the autoimmunity in Hashimoto’s thyroiditis at a number of stages. Initially it may suppress dendrocyte mediated T lymphocyte activation, may cause blunting of Th1 cell proliferation and its interferone gamma secretion. By the inhibition of cytokine production, it may block MHCII surface HLA-DR expression on thyrocytes. Additionally, B cell proliferation, as a result of activation by T cells, may be seduced and B cell apoptosis may be accelerated by 1,25-hydroxyvitamin D. Hence, active vitamin D may decrease the autoantibody load [8,13,14].

Lately, studies have reported increased risk of Hashimoto’s thyroiditis where vitamin D levels may be low, and in conditions where vitamin D functionality may be compromised, as in certain gene polymorphisms of the vitamin D receptor (VDR) and abnormality in vitamin D-binding protein [15,16]. Nonetheless, solid data are necessary to clarify the link between vitamin D status and Hashimoto’s thyroiditis.

VITAMIN D AND GRAVE’S DISEASE
In Grave’s disease TSH receptor autoantibodies cause hyperthyroidism. Reflected in the increasing interest in the role of vitamin D in susceptibility to autoimmune conditions, potentially this autoimmune disease may also be linked to vitamin D. This may be candidly assumed since it is known that vitamin D suppresses activated T cell proliferation and encourages macrophage phagocytic ability [17,18].

Grave’s diseases’ etiology has been reported to be associated with VDR and vitamin D binding protein gene polymorphism, presumably by vitamin D function reduction, that may consequently have inhibitory effects on immune regulation [19,20].

These effects appear to be ethnicity specific, Asians with Apal, Bsml and FokI polymorphisms seem to have a higher susceptibility as compared to their Caucasian counterparts [21]. Additionally, it has also been reported that autoimmune thyroiditis is associated with Bsml and TaqI polymorphism, whereas Apal and FokI are not [22].

Those with increased ultrasonography measured thyroid volume and early-onset disease
are reported to have low vitamin D levels [23]. Additionally, those in remission were reported to have higher vitamin D levels as compared to their non-remittent counterparts [24]. Although data at present is meager, further studies on the role of vitamin in Grave’s disease are worthwhile.

**VITAMIN D AND ADDISON’S DISEASE**

Addison’s disease results from destruction of the adrenal cortex due to an autoimmune process, and may present solitarily or as a polyendocrine syndrome. Although elusive, the etiology of the disease currently suggests that environmental factors along with a genetic component may be responsible for the destruction of the adrenal cortex mediated by CD8 lymphocytic infiltration and autoantibodies against the 21 hydroxylase enzyme [25]. Genetic susceptibility is identified primarily at the HLA locus, but VDR and CYP27B1 genes have also been implicated [26,27].

Given the genetic background it may be assumed that vitamin D is involved in major pathophysiological pathways, as active vitamin D may downregulate CYP21A2 and upregulate CYP17A1 and CYP11A1, as such disrupt steroidogenesis. Additionally, vitamin D also acts on the adrenal tissue along with the immune system in adrenal cell models [28].

Although vitamin D levels comparing controls and Addison’s disease patients are not available, the association between vitamin D status and susceptible gene loci may allow one to presume the vitamin’s disease modifying role. Nonetheless, there is a need for studies investigating the function and relevance of vitamin D.

**VITAMIN D AND PRIMARY HYPERPARATHYROIDISM**

The tight regulation of serum ionized calcium level is a result of orchestrated regulation by the parathyroid hormone (PTH), calcitonin and active vitamin D. The biosynthesis of active vitamin D is primarily achieved by the renal CYP27B1, which is stimulated by PTH. Conversely, active vitamin D and ionized calcium downregulate PTH.

The inactive, 25-hydroxyvitamin D is a potent regulator of parathyroid tissue. In contrast to other tissue, parathyroid cells readily take up vitamin D binding protein along with its 25-hydroxyvitamin D, as such guaranteeing better hold of the circulating 25-hydroxyvitamin D. Additionally, the gland possesses CYP27B1 ensuring 1,25 dihydroxyvitamin D production for paracrine action. This proficient access to both circulating 25-hydroxyvitamin D and 1,25 dihydroxyvitamin D, in addition to its inherent production of 1,25 hydroxyvitamin D induces suppression of PTH secretion and proliferation of parathyroid cells [29].

It is known that sizable parathyroid adenomas have poor feedback by calcium and active vitamin D, as a result in primary hyperthyroidism, 1,25 dihydroxyvitamin D correlate positively with 25 hydroxyvitamin D [30]. Primary hyperthyroidism remains latent, i.e., normocalcemic primary hyperparathyroidism, when circulating vitamin levels are low [31]. Increased 25 hydroxyvitamin D levels and elevated PTH activate renal CYP27B1 accelerating 1,25 dihydroxyvitamin D production in approximation to the 25 hydroxyvitamin D and consequently causing hypercalcemia. This relationship highlights a fundamental aspect of the vitamin D system: its operation under first-order reaction kinetics, namely, the yield of the product (1,25 dihydroxyvitamin D) is proportional to the supply of the substrate (25 hydroxyvitamin D).

As such, the yield of 1,25 dihydroxyvitamin D is proportional to the supply of 25 hydroxyvitamin D and hence qualifying the kinetics of the vitamin D system as a first-order reaction. Consequently, the vitamin D system enzymes modify their function depending on the supply
of 25 hydroxyvitamin D. Depending on severity, primary hyperparathyroidism can disrupt the adaptation, resulting in elevated 1,25 dihydroxyvitamin D and increased intestinal calcium absorption. In primary hyperthyroidism this functional adaptation may be disrupted causing increased 1,25 dihydroxyvitamin D production and as such increased intestinal absorption of calcium. Given the relatively high prevalence of parathyroid adenomas, the hypercalcemia is not a manifestation of overt vitamin D toxicity rather a hypersensitivity to the high dose of vitamin D [32].

In the healthy, increasing 25 hydroxyvitamin D levels are known to decrease serum PTH concentrations. As such, PTH levels may serve as determinants for vitamin D level adequacy. This association does not hold in primary hyperparathyroidism, where the high PTH levels cause unregulated overproduction of 1,25 dihydroxyvitamin D, a potent hypercalcemic hormone. Nonetheless, the role of vitamin D in primary hyperthyroidism needs clarification.

ROUTINE LABORATORY DIAGNOSTIC IMPLICATIONS
Given the implication of vitamin D, as summarized in the above sections, the number of samples sent for measurement of total 25-hydroxyvitamin D to the routine laboratory may increase from patients suspected or diagnosed with the above mentioned common endocrine disorders. Apart from the laboratory tests routinely requested from patients suffering from endocrine conditions, 25-hydroxyvitamin D measurements may well become part of the routine diagnostic and follow-up panel for this patient group.

An additional sampling tube need not be drawn for the procedure, since the primary tube sent for the routine endocrine laboratory tests would suffice for total 25-hydroxyvitamin D measurement as well. Furthermore, no extraordinary preanalytical requirements need to be fulfilled apart from those already expected when drawing samples for routine endocrine testing. Total 25-hydroxyvitamin D measurement methodology has long enjoyed the boons of automatization and test results would ideally be delivered within a short turnaround time.

Lately, the technological advancements in the measurement of 1,25 dihydroxyvitamin D have made the methodology commercially available allowing application to platforms in use at the routine albeit specialized laboratory setting, although the gold-standard 1,25-dihydroxyvitamin D liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay may not be readily available at the basic laboratory level, a recently introduced automated chemiluminescence immunoassay may increase the popularity of this testing [33-36].

Nonetheless, data on 1,25 dihydroxyvitamin D and its implications in various disease contexts, including endocrine disorders, is limited and there will probably be a lapse before mass requests for 1,25 dihydroxyvitamin D measurements overwhelm the routine diagnostic laboratory. Technically, the short half-life of about 4 hours and its minute concentrations in the pmol/L range have limited its utility in a routine setting.

Given the data presented in literature, the 75 nmol/L rather than the 50 nmol/L would plausibly suffice as the desired target value to reap all benefits of vitamin D as implied in its various non-skeletal effects [37].

REFERENCES


Laboratory aspects and clinical utility of bone turnover markers

Harjit Pal Bhattoa

Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Hungary

ARTICLE INFO

Corresponding author:
Harjit Pal Bhattoa
Department of Laboratory Medicine
Faculty of Medicine
University of Debrecen
Hungary
E-mail: harjit@med.unideb.hu

Key words:
bone turnover, bone formation, bone resorption, bone biomarkers, biochemical markers of bone turnover

ABSTRACT

With an aging population, there is a marked increase in prevalence of metabolic bone diseases, especially osteoporosis. Perhaps the most dreaded complication of metabolic bone disease, fractures typically impose a huge burden on the ailing body and are associated with high co-morbidity and mortality. The consequent public health and socioeconomic burden warrant timely diagnosis, treatment and follow-up of these disorders. Knowing the limitations of radiological techniques, biochemical markers of bone turnover measurements come handy since the changes in their levels readily reflect bone physiology. Bone biomarkers typically analyzed in high throughput automated routine laboratories are collagen degradation products, reflecting osteoclast activity, and the collagenous or non-collagenous proteins produced by the osteoblasts. Since bone biomarker levels vary considerably due to quite a few endogenous and exogenous pre-analytical factors, knowledge of these limitations is mandatory prior to clinical utilization since these variabilities complicate test result interpretation. Standardization to harmonize different assay methodologies is desired, and the primary aims of the IFCC/IOF bone marker standards working group are also presented. Current literature data advocate bone markers as best used in monitoring anti-osteoporosis therapy.
efficacy and compliance, nonetheless, there is abundant data supporting their role in predicting bone loss and fracture risk. Furthermore, they have widespread clinical utility in osteoporosis, renal osteodystrophy, and certain oncological conditions and rheumatic diseases.

INTRODUCTION
The interplay of the bone cells, namely, the osteoclasts and the osteoblasts is generally termed as bone turnover. Knowingly, the macrophage-lineage derived osteoclasts are destined to carry out bone resorption and the mesenchyme-lineage derived osteoblasts are responsible for bone formation ideally in a coupled fashion (1,2). The imbalance in their functioning ultimately disrupts bone turnover and is characteristically noticed in metabolic bone disease (3). Per se diagnosis, monitoring, disease severity and treatment efficacy is generally challenging due to the silent, symptomless nature of these disorders, usually at onset, and primarily because the radiological features do not promptly reflect changes in bone metabolism. The high morbidity and mortality associated with these conditions mandate diagnostic procedures that would ideally reflect the actual state of the bone. The present narrative mini-review presents the biomarkers characteristic of osteoblast and osteoclast functioning, namely the markers of bone formation and those of bone resorption, respectively. Ideally changes in levels of the biochemical markers of bone turnover coupled with radiological findings and fracture risk assessment in individual patients ideally identify the patient most susceptible to suffer a non-traumatic fracture. Although dual energy x-ray absorptiometry (DEXA) till date is the gold standard methodology to measure bone mineral density (BMD), it is known that decrease in bone mass does not solely account for fracture risk (4,5). Realizing this limitation, the University of Sheffield, UK launched the fracture risk assessment tool (FRAX) in 2008 under the guidance of Professor John A. Kanis (6), where apart from the femur neck BMD 11 other easily assessable risk factors are included. The FRAX algorithms give the 10-year probability of hip fracture and the 10-year probability of a major osteoporotic fracture (clinical spine, forearm, hip or shoulder fracture) (7). Lately, the addition of bone turnover marker levels to the FRAX algorithms has also been advocated (8). Nonetheless, it is realized that bone mass changes measurable by radiographical techniques, including DEXA, are detectable almost a year following change at the cellular level (9).

Apart from mineralization, which is rather a physicochemical affair, bone turnover reflects bone cellular activity and is a dynamic biological process (10). Given the difficulties of assessing dynamic processes at a static interval, bone histomorphometry using tetracycline double-labeling is the gold standard in determining this feature of bone biology (11,12). Although considered the gold standard, bone histomorphometry has its own limitations, including sampling error, invasiveness, costs, and lack of availability at the primary level. As such, due to limitations of both radiographic techniques and bone histomorphometry, measurement of biomarkers readily assessable from blood samples is an attractive alternative to evaluate bone turnover.

Since all metabolic bone diseases usually present with alterations in osteoblast and osteoclast activity, biochemical markers of bone turnover reflecting these activities mirror real time bone turnover. Furthermore, bone biomarker levels also provide an index of disease activity in certain tumorous and rheumatological diseases affecting bone. The present review summarizes the pre-analytic, analytic and post-analytic implications of bone turnover biomarkers, since knowledge of these limitations is mandatory in correct test result interpretation. Furthermore,
the clinical utility of these biomarkers has been summarized in renal osteodystrophy, certain oncological conditions, rheumatic diseases and Paget’s Disease of the bone.

MARKERS OF BONE TURNOVER

Bone biomarkers typically analyzed in high throughput automated routine laboratories are collagen degradation products, reflecting osteoclast activity, and collagenous or non-collagenous proteins produced by the osteoblasts (table 1). All these markers can be quantitated well from blood samples, serum being the preferred sample of choice. Although assays for urine examination were developed for quite a few markers, blood sampling generally detours the pre-analytic issues usually involving urine sampling (13). The most commonly used bone resorption and bone formation markers are discussed below.

BIOMARKERS OF BONE RESORPTION

C- and N-terminal telopeptide of type I collagen

During bone degradation, osteoclast derived tartrate-resistant acid phosphatase (TRAP) and cathepsin K breakdown the bone matrix, including the triple helices of the mature type I collagen, to release carboxy- and nitrogen telopeptide containing fragments (CTx and NTx). The assay designed determines specific amino acid sequence of the telopeptide of Type I collagen termed as crosslaps, and those with β-aspartic acid as βCTx (14). Although its counterpart the N-terminal telopeptide (NTx) can also be measured from urine samples, CTX has gained increased popularity as it can be measured from blood samples on automated platforms, and given the increasing body of literature dealing with this biomarker it may perhaps be stated that it has turned out to be the biomarker of choice to examine osteoclastic bone resorption activity (15). CTx and NTx are both cleared by the kidneys, as such its clinical usefulness in CKD is significantly limited.

At the dawn of bone turnover biomarker development tartrate-resistant acid phosphatase, collagen cross-link molecules pyridinoline and deoxypyridinoline, hydroxyproline and bone sialoprotein saw light of day and were examined to measure osteoclast activity (16). All the aforementioned markers have since been superseded by the more sensitive and specific telopeptides of type I collagen, namely the C-terminal telopeptide (CTx).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Biochemical markers of bone turnover</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Bone formation markers</th>
<th>Bone resorption markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteocalcin</td>
<td>C-Telopeptide of Collagen Cross-links (CTx)</td>
</tr>
<tr>
<td>Bone Specific Alkaline Phosphatase (BSAP)</td>
<td>N-Telopeptide of Collagen Cross-links (NTx)</td>
</tr>
<tr>
<td>Carboxyterminal propeptide of Type I Collagen (P1CP)</td>
<td>Pyridinolines</td>
</tr>
<tr>
<td>Aminoterminal propeptide of Type I Collagen (P1NP)</td>
<td>Deoxypyridinoline</td>
</tr>
<tr>
<td>Tartrate-Resistant Acid Phosphatase (TRAP)</td>
<td></td>
</tr>
</tbody>
</table>
BIOMARKERS OF BONE FORMATION

N- and C-terminal propeptides of type I collagen

Osteoblasts secrete type I collagen as a pro-collagen which forms a triple helix (containing two α- and β-chain), and contains the N- and C-terminal propeptides (P1NP and P1CP), these propeptides are immediately cleaved in its extracellular vicinity eventually entering the blood circulation (17). As such, the N- and C-terminal propeptides qualify themselves as being biochemical markers of bone formation (18). The cleaved products are initially in the trimeric form that are eventually broken down to the monomeric form in the circulation the trimeric P1NP is cleared by hepatic uptake, while the monomeric form is cleared via the kidneys. Assays measure the monomeric and trimeric forms (total P1NP) or only the trimeric form (intact P1NP) (19). Being dependent on renal clearance, the monomeric form of P1NP accumulates in chronic kidney disease (20,21).

Osteocalcin

Osteocalcin is the most abundant non-collagenous protein in the bone composed of 49 amino acids and is secreted by the mature bone formation cells the osteoblasts. It is also known as bone gamma-carboxyglutamic acid-containing protein since it contains 3 glutamic acids at positions 13, 17 and 20 that undergo gamma-carboxylation in a vitamin-K dependent fashion (22). It is noteworthy here that patients on vitamin-K antagonists (e.g., warfarin) show decreased osteocalcin concentrations. Osteocalcin has appeared again recently in the reflector light, following the identification of its role as a bone derived hormone influencing male fertility, glucose metabolism, and its actions on the central nervous system and muscle in animal experiments (23-25). Although primarily identified as a marker of bone formation, due to its tight correlation with bone formation measurements by bone histomorphometry, it may well be considered a marker reflecting both formation and resorption, i.e, a marker of bone turnover since it is also liberated during osteoclastic bone resorption (19, 26).

Due to its labile 6-amino acid C-terminal sequence samples for osteocalcin determination have traditionally required special collection and transportation requirements, this has been overcome by development of assays that determine the more stable N-MID fragment (27,28). Nonetheless, osteocalcin measurement has limited value in patients with reduced renal function since its mainly cleared by the kidneys (29).

Bone-specific alkaline phosphatase

In the healthy adult, almost half of the circulation total alkaline phosphatase is derived from the bone, i.e., produced by the osteoblasts and the remainder is constituted by the fraction produced by the hepatocytes (30,31). Bone-specific alkaline phosphatase (BSAP) primarily inactivates the mineralization inhibitor pyrophosphate (32). Although commercial assays are available to measure BSAP, they show cross-reactivity with liver alkaline phosphate, as such in patients with liver disease BSAP measurements have limited applicability (19). BSAP show good correlation with fracture risk in CKD populations (33). Although a disease of the osteoclasts, it has been reported that BSAP proved to be sensitive in monitoring disease progress in patients suffering from Paget’s disease (34).

In summary, as compared to the bone resorption biomarkers, there is a larger repertoire of biomarkers of bone formation, reflecting osteoblast activity, that can be used in automated high throughput laboratories, namely serum bone-specific alkaline phosphatase, osteocalcin and procollagen type I N-terminal propeptide (PINP). Although produced by the osteoblasts,
osteocalcin may be defined as a bone turnover marker reflecting both bone formation and bone resorption, since it is also released from the bone matrix during bone resorption. P1NP is more extensively described in literature as compared to the other bone formation biomarkers (35).

**PRE-ANALYTICAL AND ANALYTICAL CONSIDERATIONS IN ROUTINE LABORATORY DETERMINATION OF BIOMARKERS OF BONE TURNOVER**

Biomarkers of bone turnover are quite sensitive to a number of pre-analytical and analytical issues.

Technical pre-analytical issues pertaining to sample collection are implicated mainly in urinary sample collection (13). As mentioned earlier, due to the cumbersomeness of spot or 24 hr urine sample collection and the need for correction for creatinine, urine sampling has started to go out of fashion and blood sampling is the preferred mode of sample collection.

Perhaps the major challenge is the over-coming of biological factors that cause variability in test results. Although trivial for the professional at home with the markers of bone turnover, one needs to be reminded of a number of endogenous and exogenous factors that should be mandatorily considered before interpreting test results.

Normal blood levels of bone turnover markers is usually higher in children, depending on the biomarker these elevations may well be a multifold of those expected in the adult population (16,36). The levels in the elderly usually show a decline, but there usually is an elevation in women following menopause (16,37,38). Levels are albeit usually higher in men as compared to women (16,38). The ethnic background of the patient is also to be considered since data suggest that the Caucasians usually have lower levels as compared to their age and sex matched adult counterparts (16,39).

Marker levels are elevated during pregnancy and lactation and tend to normalize after a few months following weaning (40,41). Marked elevations have been reported in marker levels in those immobilized or bedridden for any reason (42). Marker levels may be significantly elevated even at 6 months following a bone fracture (43-45). Patient with concomitant comorbidities such as primary hyperparathyroidism, Paget’s disease, multiple myeloma and metastatic prostate and breast cancer usual present with higher levels (46-52). Abnormal kidney function results in elevated marker levels, particularly monomeric P1NP, CTx and osteocalcin, these mainly undergo renal clearance (53).

Biomarker levels vary considerable due to quite a few endogenous factors, as such one needs to take into consideration the circadian rhythm, the phase of the menstrual period, seasonal variation, physical exercise and diet.

Bone markers, particularly the resorption markers, follow a circadian rhythm where the peak levels are typically observed in the early morning hours and the levels taper off during the day (16,37).

Biomarkers of bone formation are characteristically elevated following ovulation, i.e., during the luteal phase (54). On the other hand, resorption markers are elevated during the follicular phase of the menstrual period (55). Biomarker levels of both resorption and formation reflect vitamin D status in the winter months; this usually translates into these levels being higher during this time of the year (56). Given the literature till date, there is no clear consensus on the effect of exercise on bone turnover biomarker levels (57,58). A meat or gelatin rich diet usual results in elevated marker levels (59). Patients may be advised to have an overnight fast before
the examination. High basal bone biomarkers levels are usually observed in current smokers and those with low body mass index (60). Since most bone turnover markers are also present in other non-skeletal organs with type I collagen, cardiac conditions and systemic sclerosis, e.g., have also shown to present with elevated bone biomarker concentrations (61-63).

There is marked analytical variability of bone biomarkers. The methodology is not standardized and using different assays from various manufactures generally present a huge difference in test results from the same sample. Task forces or working groups like the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) bone marker standards in affiliation with the International Osteoporosis Foundation (IOF) are working arduously to harmonize various assay methodologies (64-66). Additionally, participation in external quality control schemes could help minimize inter-laboratory variations (15).

IFCC WORKING GROUP ON STANDARDISATION OF BONE MARKER ASSAYS (WG-SBMA) IN COLLABORATION WITH IOF

The WG-SBMA is currently headed by Professor Etienne Cavalier, and the following are the terms of reference and projects for the period 2018-2020, as presented in the IFCC Handbook 2018-2020 (67).

Terms of reference

- To standardise or harmonise clinical assays available for routine and research use, for the following two bone turnover markers; the serum assay for CTx and P1NP.

Current projects

- Review literature and current status of available assays in order to develop and undertake a project to establish a reference measurement system for serum β-CTx or harmonisation of the assays for serum β-CTx as appropriate.
- Review literature and current status of available assays in order to develop and undertake a project to establish a reference measurement system for serum P1NP or harmonisation of the assays for serum P1NP as appropriate.
- Review and identify data required for the regulatory authorisation of these modified assays.
- Review literature and consider the critical decision limits and potential target levels of serum β-CTx and serum P1NP for treatment of postmenopausal osteoporosis and other causes of osteoporosis as appropriate
- IOF-IFCC study to summarize fracture prediction strength of reference bone turnover markers.

The above is in line with their position paper published in 2001, where they advocate use of P1NP and CTx (i.e., one formation and one resorption marker) in clinical trials and other studies to improve our understanding of bone biomarkers and their application in everyday clinical practice (65).

CLINICAL UTILITY OF BONE BIOCHEMICAL MARKERS

Monitoring anti-osteoporosis therapeutic efficacy and compliance

Perhaps the greatest part of our knowledge on the clinical utility of bone markers is based on the results achieved in various pharmaceutical studies on novel anti-osteoporotic drugs (68-78). The nature of their change in response to treatment is well characterized, and can be utilized to forecast increases in bone mineral density and therapeutic efficacy in reducing fracture risk.
Anti-resorptive drugs sabotage bone resorption by driving osteoclasts into apoptosis and hence resulting in rapid decrease in bone resorption marker levels. The dose and mechanism of action dictate the degree of inhibition of bone resorption, as such the resorption marker levels. Since bone resorption and formation are coupled processes, inhibition of bone resorption results in decrease of bone formation, as such decrease in bone formation marker levels.

It is now evident that following patients every 3-6 months with their bone marker levels can monitor drug adherence and efficacy. This is an advantage over bone mineral density examinations where a follow-up of within 1-year period is not productive, since changes in BMD take longer to happen than bone markers, follow-up DEXA scans are limited by their estimation of least significant change (LSC) and BMD changes explain only a part of fracture reduction. Table 2 summarizes the changes in bone marker levels during different anti-osteoporosis therapeutic regimes. Treatment with anti-resorptive drugs, e.g., estrogen, selective estrogen receptor modulators, like raloxifene, bisphosphonates, like alendronate and risendronate, and denosumab are associated with decrease in markers of bone resorption and formation. Human recombinant PTH treatment is associated with increase in bone formation markers followed by increase in bone resorption marker levels.

Both baseline bone marker levels and changes following initiation of therapy predict BMD changes. Early decrease in bone marker levels with bisphosphonates and denosumab are known to correlate with 2-3-year increases in BMD. Fracture risk reduction at the spine and hip has been reported where early marker level reduction (P1NP, BSAP, CTx) was demonstrated upon commencing alendronate therapy. Raloxifene induced changes in osteocalcin predicted spine fracture risk reduction better that changes in BMD. Similar association was observed also between P1NP and zolendronate.

**Predicting bone loss and fracture risk**

It has been recognized, based on population-based studies, that elevated marker levels predict accelerated bone loss and increased non-traumatic fracture risk independent of underlying co-morbidities, age or sex. Nonetheless, the implementation of these population-based observations has been difficult on the individual patient level. Prospective randomized clinical trials designed to assess the efficacy and cost-effectiveness of screening programs are

<table>
<thead>
<tr>
<th>Bone marker</th>
<th>Type</th>
<th>Therapy</th>
<th>Target levels</th>
<th>Follow-up period</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Crosslaps</td>
<td>Resorption marker</td>
<td>Anti-resorptive</td>
<td>min. 35% ↓</td>
<td>Baseline and every 6 months</td>
</tr>
<tr>
<td>Total P1NP</td>
<td>Formation marker</td>
<td>Anti-resorptive</td>
<td>min. 40% ↓</td>
<td>Baseline and every 6 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anabolic</td>
<td>min. 40% ↑</td>
<td>Baseline and every 6 months</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>Turnover marker</td>
<td>Anti-resorptive</td>
<td>min. 20% ↓</td>
<td>Baseline and every 6 months</td>
</tr>
</tbody>
</table>
missing, and currently use of bone marker measurements are not recommended to identify patients at increased risk of bone loss as a public health measure (84).

**Use in Nephrology**

End-stage renal failure is usually associated with renal osteodystrophy. The hallmarks of renal osteodystrophy include low serum calcium levels and elevated PTH (secondary hyperparathyroidism). The high bone turnover characteristic of the condition results in high bone turnover marker levels. TRAP and BSAP are the only biomarkers not cleared by the kidney and as such reflect the state of bone turnover. Monomeric P1NP, osteocalcin and CTx typically are elevated, and these elevations do not reflect the true nature of bone turnover. PTH levels are known to show good association with bone turnover (85). Practically speaking serum BSAP ad PTH are the only reliable marker in patients suffering from kidney disease.

**Use in Oncology**

Solid tumors like prostate, lung and breast cancer typically metastasize to the bone. Furthermore, primary involvement of bone is characteristic of multiple myeloma. Depending on the tumor, the bone involvement may be osteolytic or osteoblastic. This potentially suggests that resorption marker elevations dominate during osteolytic presentation and formation markers are elevated when osteoblastic bone lesions are manifested (86). Furthermore, bone biomarkers function as tumor markers when secreted directly by primary bone tumor. Osteoid osteoma secretes osteocalcin (87). BSAP can be secreted by osteosarcoma (88).

**Use in Rheumatology**

The inflammatory pathogenesis of most rheumatological disorders promotes bone resorption and suppresses bone formation. This uncoupled constellation in marker levels has been reported in rheumatoid arthritis, polymyalgia rheumatic, psoriatic arthritis, ankylosing spondylitis, and re-active arthritis (89,90). Furthermore, the resorption marker levels usually show good correlation with disease activity indices of different rheumatological conditions (91-93).

**Use in Paget’s disease of the bone**

Paget’s disease of the bone is bone metabolic disorder characterized by extremely high bone turnover causing expansion and deformation of affected bones. Patients usually present with marked increase in all bone turnover marker levels (94). P1NP concentrations have been demonstrated to correlate with disease activity and to anti-resorptive therapeutic response, as such bone turnover markers present traits of both diagnostic and disease monitoring in Paget’s disease (95).

**CONCLUSION**

Biochemical markers of bone turnover reflect bone homeostasis, i.e., the activity of osteoblasts and osteoclasts in both physiological and pathophysiological conditions. Although quite sensitive to a multitude of exogenous and endogenous pre-analytical factors, bone markers are best used in monitoring anti-osteoporosis therapy efficacy and compliance. Combination of BMD measurement by DEXA with biochemical markers of bone turnover levels, at least one bone resorption and one bone formation marker, may potentially improve early detection of individuals at increased risk for bone loss and eventually non-traumatic bone fracture. Furthermore, they have widespread clinical utility in osteoporosis, renal osteodystrophy, certain oncological conditions and rheumatic diseases.
REFERENCES


Practical guide for identifying unmet clinical needs for biomarkers

Phillip J. Monaghan\textsuperscript{1}, Sarah Robinson\textsuperscript{2}, Daniel Rajdl\textsuperscript{3}, Patrick M.M. Bossuyt\textsuperscript{4}, Sverre Sandberg\textsuperscript{5}, Andrew St John\textsuperscript{6}, Maurice O’Kane\textsuperscript{7}, Lieselotte Lennartz\textsuperscript{8}, Ralf Röddiger\textsuperscript{9}, Sarah J. Lord\textsuperscript{10,11}, Christa M. Cobbaert\textsuperscript{12,15}, Andrea R. Horvath\textsuperscript{13,14,15}

\textsuperscript{1} The Christie Pathology Partnership, The Christie NHS Foundation Trust, Manchester, United Kingdom
\textsuperscript{2} Thermo Fisher Scientific. Clinical Mass Spectrometry Clinical Diagnostics Systems, United Kingdom
\textsuperscript{3} Department of Clinical Biochemistry and Hematology, Medical Faculty in Pilsen, Charles University, Czech Republic
\textsuperscript{4} Department of Clinical Epidemiology, Biostatistics & Bioinformatics, Academic Medical Center, University of Amsterdam, The Netherlands
\textsuperscript{5} Laboratory of Clinical Biochemistry, Haukeland University Hospital, Helse Bergen HF, Bergen, Norway, Norwegian Quality Improvement of Laboratory examinations (Noklus), Haraldsplass Deaconess Hospital, Bergen, Norway and Department of Global Health and Primary Health Care, University of Bergen, Norway
\textsuperscript{6} ARC Consulting, Perth, Australia
\textsuperscript{7} Clinical Chemistry Department, Altnagelvin Hospital, Western Health and Social Care Trust, Londonderry, N. Ireland, United Kingdom
\textsuperscript{8} Abbott Diagnostics, Wiesbaden, Germany
\textsuperscript{9} Medical and Scientific Affairs, CPS, Roche Diagnostics GmbH, Mannheim, Germany
\textsuperscript{10} School of Medicine, University of Notre Dame, Sydney, Australia
\textsuperscript{11} National Health and Medical Research Council Clinical Trials Centre, University of Sydney, Australia
\textsuperscript{12} Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Centre, The Netherlands
\textsuperscript{13} New South Wales Health Pathology, Department of Clinical Chemistry and Endocrinology, Prince of Wales Hospital and School of Medical Sciences, University of New South Wales, Australia
\textsuperscript{14} School of Public Health, University of Sydney, Australia
\textsuperscript{15} For the Test Evaluation Working Group of the European Federation of Clinical Chemistry and Laboratory Medicine
ARTICLE INFO

Corresponding author:
Phillip J. Monaghan
The Christie Pathology Partnership
The Christie NHS Foundation Trust
Manchester
United Kingdom
Phone: (+44) 161 918 2466
E-mail: phillip.monaghan@nhs.net

Key words:
unmet clinical need, clinical pathway, test evaluation

Author contributions:
All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Financial support:
The European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) provided financial support to the Test Evaluation Working Group for the preparation of this paper through payment of travel expenses to attend Working Group meetings. Abbott, Roche Diagnostics, and Thermo Fisher Scientific have provided independent educational grant to EFLM (EFLM grant reference numbers 2017/01, 2017/02 and 2017/04 respectively).

Role of sponsor:
Neither the funding organization (EFLM) nor the independent educational support by Roche Diagnostics, Abbott and Thermo Fisher Scientific influenced the content of this paper. This publication reflects the collective view of the Working Group. The authors include representatives from Roche Diagnostics and Abbott Diagnostics as they provided important insights from the IVD industry, a key stakeholder in the research and development of new biomarker assays.

Employment or leadership:
None declared.

Honorarium:
None declared.

Competing interests:
The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

ABSTRACT

The development and evaluation of novel biomarkers and testing strategies requires a close examination of existing clinical pathways, including mapping of current pathways and identifying areas of unmet need. This approach enables early recognition of analytical and clinical performance criteria to guide evaluation studies, in a cyclical and iterative manner, all the time keeping the clinical pathway and patient health outcomes as the key drivers in the process.

The Test Evaluation Working Group of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM TE-WG) https://www.eflm.eu/site/page/a/1158 has published a conceptual framework of the test evaluation cycle which is driven by the clinical pathway, inherent to which is the test purpose and role within the pathway that are defined by clinical need.

To supplement this framework, the EFLM TE-WG has also published an interactive checklist for identifying unmet clinical needs for new biomarkers; a practical tool that laboratories, clinicians, researchers and industry can equally use in a consistent manner when new tests are developed and before they are released to the market. It is hoped that these practical tools will provide consistent and appropriate terminology in this diverse field and offer a platform that facilitates greater consultation and collaboration between all stakeholders. The checklist should assist the work of all colleagues involved in the discovery of novel biomarkers and implementation of new medical tests. The tool is aligned with the IOM recommendations and the FDA and CE regulating body’s requirements.
INTRODUCTION

Clinical laboratory scientists and pathologists, responsible for the provision of in vitro medical tests, are regularly approached by industry colleagues about the availability of new tests. This late notification sometimes poses problems; e.g. the new test does not seem to fulfil an unmet clinical need, the evidence on the clinical effectiveness of the biomarker is not yet available or controversial and therefore the new biomarker may not be commissioned or get on the reimbursement schedule.

Unmet clinical needs for new biomarkers are often discussed at clinical meetings within the health care setting, yet these perceived needs are rarely communicated to R&D and industry colleagues as key stakeholders in the biomarker development process.

Furthermore, laboratory professionals are more likely to experience pressure from the hospital board to reduce the costs of pathology testing and to rationalize test requesting rather than adding new tests to the laboratory’s repertoire; the new test should be shown to improve patient care and outcomes or the cost-effectiveness of care.

To support laboratories in evidencing the value of tests, the Test Evaluation Working Group of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM TE-WG) has published a conceptual framework of the test evaluation cycle which is driven by clinical need and the clinical pathway of managing patients [1].

The goal of the test evaluation framework and the unmet clinical needs checklist is to provide consistent and appropriate terminology in this diverse field and to offer a platform that facilitates greater consultation and collaboration between all stakeholders.

THE CYCLICAL FRAMEWORK OF TEST EVALUATION

The test evaluation framework of the EFLM TE-WG [1] is intended to be applied after a potential biomarker has been discovered in basic research (so-called ‘proof of concept’) studies and is ready for further development and evaluation in clinical settings. The framework describes biomarker evaluation as a cycle, where key elements of the process, i.e. assessment of the analytical performance, clinical performance, clinical effectiveness, cost-effectiveness, and overall impact of the new test, are primarily driven by clinical needs and the clinical pathway that should lead to improved health outcomes or greater health care efficiency.

This dynamic framework reflects well-known steps in test evaluation but unlike most other linearly staged test evaluation models, it places the clinical pathway and thus testing-related patient outcomes into the centre (Figure 1).

This is a critical component of the cyclical test evaluation framework, since the relationship between laboratory testing and subsequent patient outcomes is, more often than not, indirect. In most cases, only if test results are utilised to inform and guide effective downstream clinical decisions can patient outcomes be improved.

Thus, clear identification of the test purpose (i.e. intended clinical application and how the test information will be used to improve clinical management; e.g. diagnosis, prognosis, monitoring, screening, treatment selection, etc.) and test role within the clinical pathway (i.e. how the test will be positioned to alter the existing clinical
pathway; e.g. replacement, triage, add-on test) are essential.

**NOVEL APPROACH TO TEST EVALUATION IN PRACTICE**

The problem with many frameworks published so far is that they describe what needs to be done, but they do not offer clear explanation of how each of the key steps should be undertaken. The EFLM TE-WG therefore aims to provide practical tools for each step of this framework to help operationalize the theory and the key principles described. The working group found that clinical pathway mapping is a useful method for identifying clinical needs and management decisions and to link information from testing to health outcomes.

Unmet need for medical tests is relatively vaguely defined and its assessment, in general, is a complex process that could be very subjective depending on the background, practice, experience and interest of stakeholders. For example, a representative from a reimbursement organization with strict funding and under government pressure for cost-effectiveness of health care services could see the need for a new biomarker from a very different perspective, compared to a researcher who has just discovered

**Figure 1** Framework for the evaluation of *in vitro* medical tests

The cyclical framework illustrates the interplay between the key elements of the test evaluation process and that all are dynamically linked to one another in a cycle driven by the intended use of a test in the clinical pathway. Adapted from Horvath, et al [1].
a promising new biomarker of potential clinical effectiveness; or indeed a clinician who is struggling to manage patient treatment in the absence of a reliable biomarker of treatment effectiveness.

To ensure that the differing perspectives of various stakeholders involved in the biomarker translation pipeline are captured, we concluded that a checklist of specific questions, with checkpoints, would be a practical and informative tool, rather than a set of generic recommendations. The checkpoint could also act as a catalyst for open dialogue between various stakeholders to identify and assess unmet needs in view of the clinical pathway.

The TE-WG used a 4-step process to develop the unmet clinical needs checklist:

1. scoping literature review;
2. face-to-face meetings to discuss scope, strategy and checklist items;
3. iterative process of feedback and consensus to develop the checklist;
4. testing and refinement of checklist items using case scenarios.

The checklist is intended to achieve more efficient biomarker development and translation into practice [2].
Clinical pathway mapping was utilised to identify clinical management decisions linking testing to health outcomes and the 14-item checklist was set around 4 key domains:

1. **Identifying the unmet need in the current clinical pathway**;

2. **Verifying the unmet need**;

3. **Validating the intended use**; and

4. **Assessing the feasibility of the new biomarker to influence clinical practice and health outcomes**.

The checklist presents an outcome-focused approach that can be used by multiple stakeholders for any medical test, irrespective of the purpose and role of testing (Figure 2). In each main domain there are more specific questions that need to be discussed and answered by stakeholders in order to facilitate a structured, considered judgment process. The checklist is built with checkpoints in such a way that if the answers to certain key questions are unfavorable, then the whole process should stop and the medical need for the biomarker and further evaluation of the test are not justified.

Based on the working group’s experience, a checklist such as the AGREE checklist to assess the methodological quality of guidelines [3], or the STARD checklist that guides researchers on how to design and report diagnostic accuracy studies [4], is a very effective tool in providing clear guidance and a standardized way of handling complex evidence-based clinical decisions. The central strength of the checklist is that whilst it takes into consideration the perspectives of all stakeholders, it prioritizes the clinical pathway and health outcomes of the patient at the centre of the needs evaluation process.

In collaboration with the EFLM Working Group for Distance Education and e-Learning, we have developed an interactive version of this checklist, now openly available through the EFLM e-Learning platform: [https://elearning.eflm.eu/course/view.php?id=11](https://elearning.eflm.eu/course/view.php?id=11). The platform also contains a short video showing how to use the interactive checklist, including worked examples.

### SYNERGY WITH THE NATIONAL ACADEMY OF MEDICINE BIOMARKER REPORT

The National Academy of Medicine (formerly the Institute of Medicine [IOM]) has issued several very useful documents over the years which influenced the development of the unmet clinical needs checklist of the EFLM TE-WG. These include the 2011 IOM report on the “Evaluation of Biomarkers and Surrogate Endpoints in Chronic Disease” [5] and recommendations for:

- effective biomarker evaluation and improving evidence-based regulation;
- development of biomarker-based tools for cancer (2006) [6];
- improving diagnosis in health care (2015) [7]; and most recently,
- biomarker tests for molecularly targeted therapies (2016) [8], a document that was issued after the EFLM checklist had already been completed.

Equally the TE-WG was very strongly influenced by the new *in vitro* diagnostic (IVD) regulatory changes in Europe [9] and the US [10], which demand more clinical evidence before new tests enter the market. Significant consideration was also given to the work of the Global Harmonization Task Force (GHTF) [11], which issued a number of valuable documents that have set the definitions, principles and key elements of and proposed processes for more effective biomarker evaluation before market approval.

The EFLM checklist provides a practical ‘how to’ tool that addresses the goals and principles set out by the above groups and regulatory bodies of the United States Food and Drug Administration (FDA) and Conformité Européenne (CE) in the
European economic area. It is anticipated, that the checklist will facilitate inter-disciplinary, multi-stakeholder collaboration for efficient biomarker development and pre- and post-market evaluation. Critically, the checklist will help to verify and validate the purpose and role of a biomarker in the context of the clinical pathway, thus providing the necessary evidence for the proposed intended use of the test. These important clinical considerations should then guide the analytical and clinical performance requirements and the generation of evidence of clinical effectiveness and value, as promulgated in IOM recommendation 3 [8].

**PROFESSIONAL UPTAKE OF THE TOOLS**

Initially, it is expected that the checklist will be used by a number of stakeholders such as clinical laboratory professionals, and that it will be pilot-tested with other colleagues, including those staff providing direct clinical care and industry representatives when reviewing the need for new biomarkers or new intended uses of existing medical tests.

Good communication with stakeholders in research and development is needed so that the biomarker development pipeline is aligned with evolving unmet clinical needs. The checklist will ideally drive multidisciplinary collaboration to break down the conventional working silos, and contribute to making biomarker evaluation a more efficient and targeted process, thus becoming an enabler for the adoption of innovative tests. Such collaboration will promote robust implementation planning proportionate to the clinical pathway, so test results are available and acted upon in an appropriate and timely manner, with a strong link to clinical intervention and outcomes.

There are numerous publications reporting biomarker failures and even harm caused by poorly performing biomarkers [12-18]. It is known that only a very tiny fraction of the many newly discovered ‘omics’ markers find their way ‘from bench to the bedside’. Laboratory professionals are under increasing pressure from clinicians and health care administration to prove the value of existing tests in terms of impact on various health, organisational and financial outcomes.

It is also envisaged that IVD companies involved in research and biomarker development will use this checklist before investing in setting up major work for releasing new tests for novel biomarkers to the market.

Due to stricter regulations both by the FDA and CE marking authorities in Europe, IVD companies are under increasing pressure to provide data on the clinical performance of biomarkers before regulatory approval. Such studies are complex, costly and time-consuming. Notwithstanding the complexities of translational research, it has been stated to take on average 17 years for research evidence to reach clinical practice [19]. Therefore, it is in the interest of the IVD industry that the unmet clinical need and the purpose and role of new biomarkers in a clinical pathway and the potential impact of testing on various outcomes are thoroughly considered. This would reduce research waste and prevent the release of useless or even harmful tests to the market.

**REGIONAL IMPLEMENTATION, DIFFERENCES AND EXAMPLES OF THOSE USING THE CHECKLIST**

The strength of the working group’s checklist is that, instead of providing recommendations which may match the healthcare setting of one country or region but not that of another, it asks open questions that can be answered with full consideration given to the local health care setting.
For example, the checklist asks the user of a new test to consider their current local practice, the limitations of current practice and to map out the current clinical pathway to see where the new test would fit in and what value it would add to current practice.

Obviously this clinical pathway can be very different even for the same medical condition in Europe, the US, or Africa or Australia.

Even within one country the care pathway and the utility of or the need for a test may depend on whether the relevant health care is provided in a metropolitan or in a rural care setting.

For example a point-of-care (POC) Troponin test may not fit well into the clinical pathway of a metropolitan hospital, which has 24/7 access to a higher sensitivity and more reliable Troponin assay with a <1hr turn-around-time in its central laboratory. Nevertheless, it may do so in a rural setting where there is limited access to laboratory testing and where a POC Troponin test may save lives by selecting patients who need urgent transport to a hospital, where appropriate care for an acute myocardial injury can be provided.

The checklist also asks whether the new biomarker is feasible in practice technically, commercially, economically, and organizationally, and what other local, cultural, social, etc. barriers may exist to its implementation. These again can be locally determined issues and the answers tailored to each setting may define medical need for the same test completely differently in various countries.

Indeed, unmet clinical need is a crucial primary component of the wider value proposition framework of laboratory medicine [20], taking into account the impact on clinical, operational and economic outcomes to assure feasibility of implementation.

**SUMMARY**

The unmet clinical need checklist produced by the EFLM TE-WG is a practical tool that should assist the work of all stakeholders involved in the discovery or implementation of new biomarkers and testing strategies.

We encourage pilot testing and regular use of this new interactive tool. The checklist can be used before new biomarkers are developed or fully validated for clinical use as well as when assessing the clinical need for and the clinical utility of existing tests. The TE-WG would appreciate feedback to inform future refinements of the checklist based on user experience.

**REFERENCES**


Prevalence of anemia and its associated factors among children in Ethiopia: a protocol for systematic review and meta-analysis

Zegeye Abebe1,*, Wubet Worku Takele2, Degefaye Zelalem Anlay2, Daniale Tekelia Ekubagewargies2, Zegeye Getaneh3, Molla Abebe4, Mulugeta Melku3,*

1 Department of Human Nutrition, Institute of Public Health, College of Medicine and Health Sciences, University of Gondar, Ethiopia
2 School of Nursing, College of Medicine and Health Sciences, University of Gondar, Ethiopia
3 Department of Hematology and Immunohematology, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Ethiopia
4 Department of Clinical Chemistry, School of Biomedical and Laboratory Sciences, College of Medicine and Health Sciences, University of Gondar, Ethiopia

*Contributed equally to this protocol for systematic review and meta-analysis

ARTICLE INFO

Corresponding author:
Mulugeta Melku
Department of Hematology
and Immunohematology
School of Biomedical and Laboratory Sciences
College of Medicine and Health Sciences
University of Gondar
P.O. Box 196, Gondar
Ethiopia
E-mail: mulugeta.melku@gmail.com

Key words:
anemia, children, Ethiopia, meta-analysis, protocol, systematic review

ABSTRACT

Background

Anemia is one of the global public health problems that affect more than one third of the world population. It has been strongly associated with poor growth and development, limited psychomotor development, and poor long-term performance in cognitive, social, and emotional functioning in children. There is not a single national figure about childhood anemia in Ethiopia. There is also inconclusive evidence about factors associated with anemia. Moreover, the present meta-analysis will generate concrete evidence in which the result may urge policy makers and program managers to design appropriate intervention to control childhood anemia. Thus, the aim of this review is to estimate the pooled prevalence and to identify associated factors of anemia among children in Ethiopia.
**Method**

Published relevant cross-sectional studies will be searched using comprehensive search strings through PubMed/Medline, SCOPUS, HINARI, EMBASE, Web of Science databases. In addition, Google Scholar and Google will be searched for grey literature. Reference lists and communication with content experts will be used to get additional relevant studies. Two groups of review authors will independently appraise the studies for scientific quality and extract the data using the Joanna Briggs Institute (JBI) tools. The pooled estimate will be determined using random effect model. Heterogeneity between the studies will be assessed using the I2 statistics. Sensitivity and subgroup analysis will be employed in the case of heterogeneity. Publication bias will be assessed by visual inspection of the funnel plot, and using Egger’s and Begg’s statistical tests.

**Discussion**

Childhood anemia is known to have negative consequences on mental, physical and social development of children. The burden and its associated factors are greatly varied as to the social, economic, and geographical differences of the target population.

Therefore, the proposed systematic review will generate evidence about the pooled prevalence of anemia and its associated factors among children in Ethiopia.

**Protocol registration**

The protocol was registered at PROSPERO International Prospective Register of Systematic Reviews.

(Registration number: CRD42018088223).

**BACKGROUND**

Anemia is recognized as a major public health problem globally, mostly affecting children, women of childbearing age and pregnant women [1]. In developing countries, anemia can be a result of a number of causes; but nutritional anemia, particularly iron deficiency, is the most common cause. It is primarily caused by insufficient dietary intake and/or insufficient absorption, and suboptimal utilization of micro-nutrients like vitamin or mineral and/or due to frequent infections [1, 2].

The health implication of anemia is numerous. It is associated with impaired mental, physical, motor and language development; and poor coordination, scholastic achievement and immune function [3-5]. In addition, the outcomes of anemia can be considered from a variety of perspectives, including detrimental impacts on economic and social development [6].

According to World Health organization (WHO) 2015 report, about 43% of under five children were anemic, with regional variations of 62.3% in African, 53.8% in South-East Asia and 21.9% in Western Pacific Region [7].

In Ethiopia, though the prevalence of severe to moderate anemia in the last fifteen years has significantly declined, children and pregnant women are still suffering from the consequences of anemia due to high iron requirements, low intake of iron from foods, and frequent episodes of infection [8, 9]. As many as six in ten under five children in Ethiopia are anemic. Moreover, according to the local conditions, the proportion varies across the regions of the country due to feeding, wealth and cultural differences. As an illustration, the highest level of childhood anemia was found in Somali Region (83%), followed by Affar (75%) and Dire Dawa (72%), but the lowest was found in the Amhara Region (42%) [10].
The government of Ethiopia has been working to reduce childhood anemia. Accordingly, it endorsed the national nutrition program, bimannual school deworming, developed micronutrient deficiency prevention and control guideline and implemented micronutrient fortification. But studies from different corners of the country have shown that childhood anemia is still a major public health problem. In addition, there is not a single national figure about childhood anemia and also inconclusive evidence about factors associated with it. Systematic review and meta-analysis generates concrete evidence in which the evidence may urge policy makers and program managers to design appropriate intervention to control and minimize the negative consequences of childhood anemia. As far as the authors are aware, there is no published systematic review and meta-analysis that generated the pooled estimated prevalence of anemia and the pooled effect size of factors associated with childhood anemia in Ethiopia. Therefore, the aim of this systematic review and meta-analysis is to estimate the pooled prevalence of childhood anemia and its associated factors among children in Ethiopia.

OBJECTIVES
The objectives of this systematic review and meta-analysis are:

- To estimate the pooled prevalence of anemia among children in Ethiopia;
- To identify factors associated with childhood anemia in Ethiopia.

METHODOLOGY

Design and protocol registration
This protocol is designed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Protocols (PRISMA-P 2015 Guidelines) [11]. The protocol of this systematic Review and Meta-analysis has been registered in the PROSPERO, international prospective register of systematic reviews, with registration number of CRD42018088223.

Search strategy
Relevant published articles will be searched in PubMed/Medline, HINARI, SCOPUS, EMBASE and Web of Sciences electronic databases. Google scholar, and Google will be searched for grey literature. In addition, conference paper and thesis or research final report will be accessed, professional annual research conference log book, and research and publication directorate from each University in Ethiopia upon request.

The search terms will be developed in accordance with the Medical Subject Headings (MeSH) thesaurus using a combination of key terms. Hand searching of articles published in Ethiopian journal of health sciences, Ethiopian Medical journal, Ethiopian journal of Health and Development, and Ethiopian Journal of Health and Biomedical Sciences will be conducted.

Reference lists of retrieved articles will be probed to identify any studies that are not retrieved from electronic databases. Content experts will be contacted to get additional studies that are not retrieved by electronic database searching and reference list scrutinizing. Two author groups: group one (MM, ZA) and group two (DTE, WWT), will independently search the articles. In the case of lack of necessary data from studies, we will contact the corresponding author through email.

Search Strategy:

- anemia OR iron deficiency anemia OR nutritional anemia OR hemoglobin OR nutritional status OR hematological parameters OR determinant factors of anemia OR associated factors of anemia AND children OR adolescent OR preschool AND Ethiopia
Study selection and quality appraisal

All articles retrieved through search strategy will be imported to EndNote X7 (Thomson Reuters, New York, USA). After excluding the duplicate articles, titles and abstracts will be independently screened for inclusion in full text appraisal by two groups of review authors: group one (MM, ZA) and group two (DTE, WWT). Differences between two groups will be resolved through thorough discussion. In case of disagreement between the two groups of review authors’ reports, the decision will be determined by third group of review authors (ZG, MA, DZA).

For articles deemed to appear relevant, the full text will be appraised for inclusion in systematic review and meta-analysis.

The quality of articles will be assessed using Joana Brigg’s Institute (JBI) critical appraisal checklist for simple prevalence [12] and analytical cross-sectional studies [13] having nine and eight checklist items, respectively. Studies with an overall quality assessment score greater than 50% will be included. The discrepancies during critical appraisal will be solved as similar manner for title/abstract screening phase.

Participants

Studies reporting the prevalence and/or factors associated of anemia among children in Ethiopia.

Eligibly criteria

Inclusion criteria

Studies will be included in this systematic review if they met all the below inclusion criteria:

• Studies reporting the outcome of interest among children (up to 18 years old) in Ethiopia
• Observational studies like cross-sectional, prospective cohort studies and repeated cross-sectional studies. For prospective cohort and repeated cross-sectional studies that reported the outcomes of interest, the baseline data will be used for our systematic review and meta-analysis.

Exclusion Criteria

Studies will be excluded if they have met at least one of the exclusion criteria below:

• Studies conducted in healthcare facilities
• Studies that used Visual comparative method (Sahli-hellige method, and MBS hemoglobinometer color scale) and Copper Sulphate densitometer to ascertain the outcome (anemia)
• Studies conducted in children living with HIV/AIDS

Outcomes of the study

The primary outcome of this systematic review is to determine the prevalence of anemia among children in Ethiopia. It will also identify factors associated with the burden of anemia among children in Ethiopia. Anemia is defined as hemoglobin value below the established reference interval for the sex and age of the population.

Data extraction

The JBI tool will be adopted for data extraction. Two groups of review authors, group one (MM, ZA) and group two (DTE, WWT), will extract the data independently. In case where there are differences between the two authors’ groups with regard to the extracted data, the difference will be solved via discussion.

Unless resolved via discussion, the decision will rely on the third review authors’ group (ZG, MA, DZA) report. Information such as name of first author, year of publication, age group of children, study year, study area/region, study design, total number of children, number of anemic children, and number of anemic cases and non-anemic cases for the reported associated factors will be extracted.
The data will be recorded in a Microsoft excel spreadsheet. When authors find multiple publications from the same dataset, the article reported the prevalence and factors associated of anemia in extractable form will be used.

Moreover, for prospective cohort and repeated cross-sectional studies that reported the outcomes of interest, the baseline data will be used for our systematic review and meta-analysis to facilitate comparability of results across studies and to reduce loss to follow-up bias.

Data analysis

The data extracted from primary eligible studies will be entered into Microsoft excel, and then will be exported to STATA version 14 (StataCorp LLC, Texas, USA) for analysis.

The magnitude of heterogeneity between included studies will be quantitatively measured by index of heterogeneity (\(I^2\) statistics) \[14\]. \(I^2\) values of 25%, 50% and 75% are assumed to represent low, medium and high heterogeneity, respectively.

The significance of heterogeneity will be determined by p-value of \(I^2\) statistics; and a p-value of <0.05 will be an evidence of heterogeneity. If \(I^2\) value is greater than 50%, we will use Dersimonian and Liard random effect model to determine the pooled estimates \[15\].

Sensitivity analysis will be carried out if there is heterogeneity between studies. Subgroup analysis will be done considering age group, region and study design as grouping variables.

Small-study effects and publication bias will be evaluated using the visual funnel plot test, and Egger’s and Begg’s statistics in the random effect model. In the evidence of small-study effect, trim and fill methods (Duval and Tweedie’s) will be used to estimate the pooled prevalence anemia and to estimate effect size of factors associated with childhood anemia, as the method is robustness for estimation due to publication bias \[16\]. Odds ratio with its 95% confidence will be used to estimate the measure of association between anemia and associated factors. The results will be presented both in text and Forest plot.

Ethical statement and dissemination

As this systematic review will based on published data, ethical approval is not required. The final report will be disseminated through publication in a peer-reviewed scientific journal and will also be presented at Local, regional, national and international conferences.

DISCUSSION

Anemia has been shown to be a public health problem affecting low, middle and high-income countries. It has also been significantly associated with negative consequences on health, social and economic development \[17, 18\]. Globally, it is a moderate to severe public health problem in children.

As to the WHO 2015 estimate, the highest prevalence of anemia is found in children, 42.6% (95% CI: 37- 47%) of children were suffering from anemia \[19\]. The consequence of anemia is worse, as it limits the physical growth \[20\], mental \[21\], social \[22\] as well as the behavioral development of children \[23\].

Anemia prevalence data remains an important indicator of public health as anemia is related to morbidity and mortality, particularly in vulnerable segments of the population such as in children \[10\].

In Ethiopia, the magnitude of anemia is expected to be higher among children who are malnourished and economically disadvantaged; and among children who are living in areas where infectious diseases are highly prevalent and drought is common.
Previous studies highlighted that the prevalence of anemia among children in Ethiopia ranged from 5.83% [24] to 83% [10].

At a global level, anemia prevalence data is a useful indicator to assess the impact of widespread or highly effective interventions and to track the progress made towards the goal of reducing anemia in children [25].

In order to design more adaptable intervention and control strategies to the local context, national estimate of anemia and its associated factors needs to be generated. Given the importance of such data, there is no published meta-analysis estimate on the prevalence of anemia and its associated factors among children in Ethiopia.

Therefore, the authors believe that, this systematic review and meta-analysis will generate concrete evidence on the national burden of anemia and its associated factors among children. The review will provide substantial evidence for the government and other stakeholders working on child health; and it will help them in designing intervention and control strategies to reduce anemia in a better adaptable and more effective manner.

STRENGTH AND LIMITATION OF THIS SYSTEMATIC REVIEW

Strengths

- This will be the first published systematic review and meta-analysis that comprehensively estimate the burden of anemia, and its associated factors among children in Ethiopia.
- The involvement of experts from public health, biomedical and clinical fields during searching, screening, appraisal, and data extraction processes will help to make the evidence generated more comprehensive.
- The data reporting will adhere as to the guideline outlined in the Preferred Reporting Items for Systematic reviews and Meta-Analyses protocol (PRISMA-P 2015 Statement).

Limitations

- A potential limitation of this systematic will be the heterogeneity between published studies that can be raised from differences in study design, study period and geographic areas. Besides, studies reported in other than English language may not be included.

Amendments of the protocol

- In case there is a need to amend this protocol, the reasons for the amendment and the date when it has been amended will be explicitly described.

Conflicts of Interest

The authors declared that they have no competing interests.

Availability of data and materials

Not applicable.

Authors’ contributions

MM, ZA, ZG and WWT: have been developing the search strategy;
MM, ZA, ZG, DTE, WWT, DZA and MA: will search, screen and appraise the studies, and will extract the data;
MM, ZA, DZA, MA and WWT: will analyze the data;
ZA, MM, ZG, WWT and MA: involved during the drafting of the protocol.

All authors read and approved the final protocol for publication.

Funding Statement

No funding was secured for this review.
REFERENCES


Acute myeloid leukemia with severe coagulation disorder and concomitant central nervous system bleeding – a clinical diagnostic case report

S. Hatzl¹, B. Uhl¹, M. Hinterramskogler², S. Leber³, F. Eisner⁴, M. Haring¹, P. Jud⁵

¹ Division of Hematology, Department of Internal Medicine, Medical University of Graz, Austria
² Department of Anesthesiology and Intensive Care Medicine, Medical University of Graz, Austria
³ Department of Radiology, Medical University of Graz, Austria
⁴ Intensive Care Unit, Department of Internal Medicine, Medical University of Graz, Austria
⁵ Division of Angiology, Department of Internal Medicine, Medical University of Graz, Austria

INFO

Corresponding author:
Stefan Hatzl
Division of Hematology
Department of Internal Medicine
Medical University of Graz
Austria
E-mail: stefan.hatzl@medunigraz.at

Key words:
acute myeloid leukemia, disseminated intravascular coagulopathy, bleeding

Conflict of interest:
All authors declare that no conflict of interest exists.

Funding:
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

ABSTRACT

We report a case of severe central nervous system bleeding in a patient with acute monocytic leukemia. The patient was admitted to our emergency department because of massive back pain and positive meningeal signs. MR imaging yielded a spontaneous epidural hematoma of the thoracic vertebral column. Coagulation studies revealed fibrinogen levels below the linear measuring range and blood smears showed myeloid blast cells in the peripheral blood. The diagnosis of acute monocytic leukemia was confirmed by flow cytometric analysis. Despite substitution with more than 12 g fibrinogen per day over 3 days plasma fibrinogen levels couldn’t be stabilized. After starting the induction chemotherapy with cytarabine, laboratory coagulation test results were improved. Despite all intensive medical efforts, the patient died due to cerebral epidural hematoma.
INTRODUCTION

Acute myeloid leukemia (AML) is a result of malignant transformation of hematopoietic stem or progenitor cells and is the most frequent acute leukemia in adults. (1, 2) DIC is one of the most feared and fatal complication in acute promyelocytic leukemia (APL), however it is rare in all other subtypes of acute myelogenous leukemia (AML). (3) The incidence of disseminated intravascular coagulation (DIC) induced by hematologic malignancies was 12.7% in a large cohort of patients, studied by Okajima et al., with an overall mortality of more than 50% (4) A typical feature of DIC in acute leukemia is hyperfibrinolysis and reduction of natural anticoagulants like protein C and antithrombin III. (5, 6)

In our case report we show that early eradication of the leukemia by using high dose chemotherapy is the foremost option to control DIC and improve the coagulation test results as well as overall survival.

CLINICAL-DIAGNOSTIC CASE

A 36-year-old woman presented to the emergency department with acute back pain, fatigue and fever. Physical examination revealed positive meningeal signs (Lhermitte, Laseque). For further evaluation of the back pain, a magnetic resonance imaging was performed, which yielded massive epidural bleeding from the first thoracic vertebral body to the fourth lumbar vertebral body. (Figure 1 A)

Laboratory testing revealed leukocytosis [18,520 /µL] and mild thrombocytopenia [100,000 G/l], additionally a severely decreased fibrinogen level [< 80 mg/dL] using the Clauss method as well as a massively elevated D-Dimer value of >33 mg FEU/L could be detected. A hematological cause of hyperfibrinolysis was suspected and blood smear as well as flow-cytometric analysis (FACS) was performed. Because of the severe clinical and laboratory findings pointing towards disseminated intravascular coagulation, the most probable diagnosis was APL and a therapy with all-trans-retinoic acid 45mg/m²/day was administered. The patient was transferred to the intensive care unit and substitution of fibrinogen was initiated.
During the first day of hospitalization, a total amount of 16 g fibrinogen was given, despite substitution fibrinogen levels in plasma remained undetectable, no signs of bleeding could be detected. The patients’ condition improved and was hemodynamically stable with pain controlled by continuous morphine infusion.

During the second day, results of the blood smears including cytochemistry revealed 10% myeloid blasts with Auer rods, 15% atypical monocytes, 40% lymphocytes as well as 35% band stage and mature neutrophil granulocytes. (Figure 1B) FACS confirmed these results resulting in the diagnosis of an acute monocytic leukemia (FAB M4 according to French American British classification of acute leukemia). On the second day, again 12 g fibrinogen and 1 g tranexamic acid were administered without any effect on plasma fibrinogen level (Figures 1C; 2A).

After confirmation of the diagnosis FAB M4, a bone marrow biopsy was performed and chemotherapy with cytarabine 200mg/m²/day was administered. Due to DIC and possible tumor lysis syndrome, daunorubicine 60mg/m² was postponed to day 5.

Directly after administration of cytarabine and fibrinogen, plasma fibrinogen levels increased into normal ranges for the first time after initial diagnosis (Figure 1C). Due to chemotherapy, platelet count started to decrease and one irradiated

![Figure 1B](image-url) Peripheral blood smear showing myeloid differentiated blast cells
thrombocyte concentrate was administered to keep thrombocyte count above 50,000 G/L.

On the third day of hospitalization, the patient developed pronounced macrohematuria and multiple disseminated skin hematomas. Four irradiated erythrocyte concentrates were needed to maintain a hemoglobin level of 7.0 g/L. Subsequently, the patients’ neurological deficits were relieved, reaching a Glasgow Coma Scale score of 15 points.

However, on the morning of the fourth day the patient was comatose and did not respond to any stimulation reaching a Glasgow coma scale of 3 points. Due to neurological impairment, immediate endotracheal intubation was performed and total intravenous anesthesia with propofol and remifentanil as continuous infusion was initiated.

Cranial computer tomography (CCT) revealed a temporally right sided newly developed epidural bleeding causing a midline-shift of the brain towards left. Emergency craniotomy was successfully performed for intracranial decompression. A Thrombelastography (TEG) was performed to re-evaluate the coagulation status again revealing low fibrinogen levels and additionally a newly developed thrombocytopenia was detected. This triggered a subsequent continuous infusion of fibrinogen (3 g/h) together with administration of tranexamic acid 3g daily plus Factor XIII concentrate with the aim
S. Hatzl, B. Uhl, M. Hinterramskogler, S. Leber, F. Eisner, M. Haring, P. Jud
Acute myeloid leukemia with severe coagulation disorder and central nervous system bleeding

Figure 2A Initial TEG (citrated kaolin and citrated functional fibrinogen)*

![Figure 2A]

**Figure 2B Follow Up TEG day 4 (citrated kaolin and citrated functional fibrinogen)*

![Figure 2B]

*Before administration of fibrinogen and factor XIII concentrate

*After administration of fibrinogen and factor XIII concentrate

to enhance the clot stability which was achieved as demonstrated in following TEGs. (Figure 2B)

On the fifth day after the diagnosis of AML, intravenous anesthesia was stopped and the patient was extubated. On this day, the patient reached again a Glasgow Coma Scale score of 15 points, the coagulation status remained stable and fibrinogen substitution was stopped. Seven hours later, however, the patient’s neurological status deteriorated and mechanical ventilation was necessary again. CCT scan showed a massive haemorrhagic infarction of the occipital lobe with massive brain edema.

In the evening of the sixth day after the diagnosis, electroencephalography revealed zero-line consistent with the brain death of the patient.

DISCUSSION

The standard of care therapy for acute myeloid leukemia is a 7 + 3 days chemotherapy regimen including continuous infusion of cytarabine (200 mg/m²) for seven days combined with bolus infusion of an anthracycline for three days. (1)

All other forms of acute leukemia like acute lymphoblastic leukemia and acute promyelocytic leukemia also respond to administration of cytarabine. (7, 8)

The paradigm for treating acute promyelocytic leukemia with impaired coagulation test results is that even if acute promyelocytic leukemia is just suspected, treatment with all-trans retinoic acid should be immediately started.
However, the main issue in treatment of patients with acute leukemia associated DIC and without correct subtype diagnosis is that all-trans retinoic acid is only effective in acute promyelocytic leukemia.

In our case, the patient did not receive any effective treatment for more than one day with active central nervous bleeding. Additionally, plasma fibrinogen level did not respond to massive replacement. We could observe that coagulation parameters ameliorated and leukemic blast cells decreased after starting chemotherapy. In summary, the main goal to stop DIC and bleeding complications must be the early eradication of the leukemia. Coagulation management in these patients is very difficult to handle, thus by using frequent TEG testing a better control of bleeding complications could be achieved.

We conclude that immediate continuous infusion with cytarabine would have been adequate to start with because of expectable response in any form of acute leukemia. This might have allowed an early control of DIC even before receiving results of acute leukemia subtype diagnostics. An early detection of DIC and early improvement of the coagulation status can potentially improve the outcome of such patients.

**TAKE HOME MESSAGES/LEARNING POINTS**

- In case of unknown differentiation of acute leukaemia, Cytarabine can be used as the cytoreductive agent of choice because of the high effectiveness in all forms of acute leukaemia whether lymphoblastic or myelogenous.
- TEG result-based consequent substitution of pro-coagulative agents like fibrinogen, factor XIII and tranexamic acid independently may help to improve coagulation in such patients with DIC and underlying haematological malignancy.

**REFERENCES**

Vitamin B12 immunoassay interference in a patient with multiple myeloma – troubleshooting in a two step reagent kit based on enhanced chemiluminescence testing

V. Pant, A. Tumbapo, B. Kumar Yadav

Department of Biochemistry, Institute of Medicine (IOM), Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal

INFO

Corresponding author:
Dr. Vivek Pant
Department of Biochemistry
Institute of Medicine (IOM)
Tribhuvan University Teaching Hospital (TUTH)
Kathmandu, Nepal
E-mail: drvpant@gmail.com

Key words:
endogenous antibody, monoclonal gammopathy, immunoassay

Informed consent:
Informed consent was given by the patient for the publication of this case report.

ABSTRACT

Immunoassays are widely used for quantification of serum analytes however they are subjected to interference by endogenous antibodies. The laboratory procedures used to identify these endogenous antibodies is the demonstration of response to dilution or use of nonimmunoglobulin protein to block the interfering antibodies or the use of an alternate immunoassay. We report a clinical-diagnostic situation where serum vitamin B12 determination was interfered in an immunoassay due to excess of endogenous antibodies from monoclonal gammopathy that resulted in excess of analyte concentration. Reporting of such cases may be beneficial when assaying sera of multiple myeloma to avoid false results and in addition to avoid costs due to unnecessary repeat testing and further delay reporting of results.
**CLINICAL-DIAGNOSTIC CASE**

Quantification of Serum B$_{12}$ was ordered by the on-duty doctor from a forty-five years old female inpatient admitted at medical ward with provisional diagnosis of multiple myeloma. The patient serum sample was processed for testing B$_{12}$ using Vitros “ECiQ Immunodiagnostic system” Enhanced Chemiluminescence Immunoassay (ECI) after performing daily maintenance and running internal quality control samples from Bio-Rad, which were found to be within the acceptable range [Level 1 range = 268-608 pg/mL]. The analyser failed to give a result for the sample and the error message displayed “No Result.” Repeated testing of the sample failed again. A new sample from the patient was requested and processed, yielding again no result. The test was repeated with a dilution series of this sample, after 25 folds dilution of the sample a result of 3512.0 pg/mL was obtained (Normal range: 239-931 pg/mL) [1].

After reporting results to the clinician, the patient’s medical records were reviewed, and it was observed that the patient was diagnosed with grade III Multiple Myeloma. Serum protein electrophoresis on cellulose acetate paper revealed a significant M-gradient. On immunofixation, the immunological nature of M-gradient was found to be a monoclonal gammopathy in gamma globulin region with corresponding light-chain correlates in kappa light chains [Figure 1]. Beta 2 microglobulin in serum was increased up to 11494 ng/mL (Reference: 609.0-2366.0 ng/mL) [1], based on which the grading of multiple myeloma was done. Beta 2 microglobulin was quantified using Siemens Nephelometer. There was absence of myeloproliferative disorder, eosinophilia, and iatrogenic intake of cyanocobalamin in this patient. Absence of heterophile antibody interference was confirmed using heterophile antibody blocking tube.

To eliminate the interference generated by endogenous antibodies from multiple myeloma, the serum of patient was then subjected to precipitation with Polyethylene Glycol (PEG) 6000 to remove interfering antibodies after performing control studies with another patient’s serum.

---

**Figure 1** Immunofixation showing monoclonal gammopathy in gamma globulin region and kappa light chains (arrows)

---

**Table 1** Testing procedure and obtained values of serum vitamin B12

<table>
<thead>
<tr>
<th>Testing of Serum B12</th>
<th>Value (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted serum sample (repeat testing)</td>
<td>Not determinable</td>
</tr>
<tr>
<td>Diluted serum sample (1:25)</td>
<td>3521</td>
</tr>
<tr>
<td>PEG precipitation (1:2)</td>
<td>887</td>
</tr>
</tbody>
</table>

$(1:25) = 25$ times dilution; $(1:2) = 2$ times dilution
sample. This resulted in the value of 887.0 pg/mL which was within normal range. (Normal range: 239-931 pg/mL) [1]. Values of serum vitamin B\textsubscript{12} obtained after various methods applied in ECI is shown in Table 1.

DISCUSSION

Immunoassays are based on antigen-antibody reaction. Despite advances in immunoassay, they may be subjected to interference, depending on the assay used. According to International Federation of Clinical Chemistry (IFCC), interference in immunoassay is described as a systematic error of analytic measurement caused by a sample tested, which does not allow for a signal in the measuring system. [2] Interferences can rise from the presence of endogenous antibodies like heterophilic antibodies, anti-animal antibodies or autoantibodies. [3] In most cases, these interfering antibodies tend to produce false high values of the analyte tested. [4] Vitros Enhanced Chemiluminescence Immunoassay (ECI) is based on competitive binding immunoassay technique.

First, there is a competition between vitamin B\textsubscript{12} present in the patient’s serum sample with a Horseradish peroxidase (HRP)-labeled vitamin B\textsubscript{12} conjugate for binding sites on a biotinylated Intrinsic Factor. When this competitive binding occurs, the vitamin B\textsubscript{12} Intrinsic Factor complexes are formed which are captured by streptavidin on the wells. The complex bound with HRP thus formed is then measured by a luminescent reaction. This amount of complex bound with HRP is indirectly proportional to the concentration of vitamin B\textsubscript{12} present. [5] We report a clinical-diagnostic situation where serum vitamin B\textsubscript{12} testing was interfered in this immunoassay due to excess endogenous antibodies produced from monoclonal gammopathy which resulted in excess analyte concentration.

On average, 15-20 samples are analysed daily for Vitamin B\textsubscript{12} in our Central Biochemistry Laboratory of the Institute of Medicine by Vitros Diagnostic immunoassay which is based on the principle of ECI. High serum vitamin B\textsubscript{12} concentrations may be seen in myeloproliferative disorders, hyper eosinophilic syndromes, hepatic diseases, renal disease, and presence of heterophile antibodies or iatrogenic intake of cyanocobalamin for long duration. [6]

In this patient, myeloproliferative disorder was absent as bone marrow biopsy was normal. There was no raised eosinophil count in blood and patient had normal liver and renal function test. The patient was not taking cyanocobalamin during and six months before the sampling. Absence of heterophilic antibodies interference for the cause of the increased vitamin B\textsubscript{12} in our patient was confirmed using heterophilic antibody blocking tubes. Possible cause for the excess vitamin B\textsubscript{12} in this patient serum was due to high endogenous antibodies saturating all available sites on the IF in the first incubation step.

Subsequently, in the second incubation step there were no vacant sites on the IF for Vitamin B\textsubscript{12} to bind. Thus, no signal was generated. In this case the patient has high endogenous antibodies from monoclonal gammopathy. Presence of excess IgG immunoglobulin also forms cobalamin complex and results in high value of vitamin B\textsubscript{12} in serum and interferes with its assay. [7]

High vitamin B\textsubscript{12} concentration in this patient was due to immune complexes composed of IgG as seen in immunofixation and to prove this we treated the serum with PEG and measured B\textsubscript{12} in the supernatant. PEG precipitation test can be used as an alternative to size exclusion chromatography and is an easy method to confirm presence of immunocomplex. [8] An equal quantity of 25% PEG was added to 200 µl of the B\textsubscript{12}-elevated serum sample.
After full mixing, the mixture was centrifuged at 1500×g for 30 minutes, and then the supernatant was isolated for $B_{12}$ analysis. This gave a value of 887.0 pg/mL which was within the normal range. This decrease in serum $B_{12}$ level after PEG treatment can be due to effect of PEG on assay system and not precipitation of interfering antibody.

So, control studies with other samples were done to investigate this observation and no effect of PEG was seen in the assay system. Thus, we present a case of positive interference in the Ortho $B_{12}$ assay because of paraprotein interference in a patient with monoclonal gammopathy. To our knowledge, this particular paraprotein interference has not been previously reported.

Thus, for troubleshooting for assay interference when other samples can be quantitatively measured by two step reagent kit based on ECI, various steps can be taken.

Obtaining history of patient regarding the clinical status and dilution of sample should be done if there is a prior history of intravenous administration of Vitamin $B_{12}$. Likewise, the single step incubation method may be used. Others have also used heterophilic antibody blocking tubes, protein G–sepharose, size-exclusion High Performance Liquid Chromatography (HPLC), sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). [7]

But these methods are expensive and time consuming. The proper algorithmic knowledge is essential. Communication and collaborative approach between clinicians and laboratory staff is crucial in such cases yielding benefit for the patient. Furthermore, reporting these types of cases help clinicians and laboratory staffs to be aware of unnecessary repeated investigations or even false interpretation of results.

**TAKE HOME MESSAGES/LEARNING POINTS**

- This case presents a positive interference in the Ortho $B_{12}$ assay due to paraprotein interference in a patient with monoclonal gammopathy. Laboratory staff should be aware of such a phenomenon to avoid false results, unnecessary delay in reporting and unnecessary reagent wastage due to repeat tests.

- In such situations, the laboratory staff should consider dilution of sample and PEG precipitation before analysis.

**REFERENCES**


The eJIFCC is a member of the Committee on Publication Ethics (COPE).

The eJIFCC (Journal of the International Federation of Clinical Chemistry) is an electronic journal with frequent updates on its home page. Our articles, debates, reviews and editorials are addressed to clinical laboratorians. Besides offering original scientific thought in our featured columns, we provide pointers to quality resources on the World Wide Web.

This is a Platinum Open Access Journal distributed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.