Research Article

Unraveling the need for standardization of Homocysteine assay: Insights from the results of External Quality Assurance

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Article Info

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Keywords

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Abstract

Homocysteine is an intermediate product of the biosynthesis of methionine and cysteine and a sulfhydryl-containing amino acid. The study aims to assess the difference between the estimated levels of homocysteine by different automated systems on Biorad External Quality Assurance Services samples and the imprecision of examination of homocysteine levels below the limit of detection in External Quality Assurance Services samples by proficiency testing providers. This study involves the analysis of Biorad External Quality Assurance Services sample reports of two cycles (cycles 10 and 11) for homocysteine level evaluation performance. Our study demonstrates the wide difference in the bias percentage of homocysteine levels when bias was calculated with the peer mean and the method mean. There was a substantial increase in bias percentage for the Siemens Advia Centaur XP machine for samples with levels of homocysteine below the limit of detection. Our study also demonstrated that Siemens Advia and Siemens Atellica were evidently underestimating homocysteine levels when compared with other platforms. The use of Standard Reference Material for traceability of calibrators will ensure the standardization of assays between different manufacturers. The variation observed in the External Quality Assurance Services results of homocysteine highlights the impending requirement of standardization of assays using Standard Reference Materials.

Introduction

Homocysteine is an endogenously derived intermediate product of methionine metabolism. The normal range of homocysteine is from 5 to 15 µmol/L [1]. Human plasma contains homocysteine in three forms: free or unbound, protein-bound, and homocysteine-cysteine or homocystine dimers. Homocysteine level in humans is regulated by three enzymatic pathways based on the metabolic status. The various genetic factors that determine homocysteine levels in an individual include homozygosity or heterozygosity for Cystathionine-beta Synthase defects or Methylene Tetrahydrofolate Reductase defects, cobalamin mutations, and Down's syndrome [2,3]. Apart from genetic factors, physiological determinants such as gender, aging, decreased renal function, increased muscle mass, and lifestyle determinants such as habit of smoking, coffee, or ethanol intake also affect the homocysteine levels [4-6]. Clinical conditions such as folate deficiency, vitamin B12 deficiency, vitamin B6 deficiency, hypothyroidism, hyperproliferative disorders, and drugs such as folate antagonists, antiepileptics, contraceptives, vitamin B12 and B6 antagonists, aminothiols, etc also play a role in determining homocysteine levels [7]. Among the various determinants, only a few are responsible for lowering the level of homocysteine, such as the use of contraceptive pills or hormone therapy, aminothiols (penicillamine, acetylcysteine), or clinical conditions such as Down's syndrome [8,9]. The majority of the determinants increase the level of homocysteine. Hyperhomocysteinemia can be defined as plasma homocysteine levels of more than 15 µmol/L. Mild hyperhomocysteinemia is defined as the level of homocysteine between 15 to 30 µmol/L, moderate as the level of homocysteine between 30 to 100 µmol/L, and severe as the level of homocysteine greater than 100 µmol/L [10]. Hyperhomocysteinemia can clinically indicate cardiovascular, neurological, and psychiatric pathology. The mechanism underlying hyperhomocysteinemia contributing to cardiovascular, neurological, or psychiatric pathology points towards oxidative stress, deoxyribonucleic acid damage, protein homocysteinylation, or protein thiolation [11-13]. Homocysteine is auto-oxidized in plasma, leading to the formation of homocysteine disulfides, homocysteine, and homocysteine thiolactones. The initiation of lipid peroxidation is triggered by superoxide anion radical, hydrogen peroxide, and hydroxyl radical generated during the oxidation of homocysteine, which is ultimately responsible for the endothelial cytotoxicity of homocysteine. It has also been shown that a higher concentration of homocysteine restricts the activity of the antioxidant enzyme glutathione peroxidase [10,14,15]. Studies have shown that hyperhomocysteinemia induces inflammation in the mouse retina, brain, cultured retinal and microglial cells, and hence, targeting the reduction of inflammation is essential for mitigating damage associated with hyperhomocysteinemia age-related disorders such as diabetic retinopathy, age-related macular degeneration, and Alzheimer's disease [16]. It has also been shown that prenatal hyperhomocysteinemia in mother rats can induce behavioural impairments and oxidative stress in offspring rats. Some studies correlate the level of homocysteine with disease susceptibility [14]. A meta-analysis by Boushley et al had shown that the odds ratio of coronary artery disease in men with a 5 µmol/L increase in total homocysteine levels is 1.6, and that in women it is 1.8, whereas the odds ratio of cerebrovascular disease with an increase of 5 µmol/L in total homocysteine levels is 1.5. Hence, a change of 5 µmol/L is a clinically significant change [17]. The evaluation of homocysteine level is also important in neonates in diagnosing hemolytic uremic syndrome subtype and infantile tremor syndrome [18,19]. In the pediatric population, the elevated serum homocysteine levels correlate with autistic spectrum disorder and attention deficit hyperactivity disorder [20]. Our lab estimates homocysteine using the chemiluminescence method on the Siemens Advia Centaur XP platform. The assay range for the kits used in Siemens Advia Centaur XP is from less than 0.50 to 65 µmol/L. The quality control of the homocysteine parameter is performed by three levels of control samples. The range of level 1 is 4.03 to 8.16 µmol/L, whereas the range of level 2 is from 7.18 to 13.4 µmol/L, and that of level 3 is from 16.50 to 28.80 µmol/L. The Advia homocysteine assay is traceable to an internal standard manufactured using highly purified materials. The lab is enrolled in the Biorad External Quality Assurance Services cardiac markers programme for proficiency testing. Interestingly, on analysis of Biorad External Quality Assurance Services reports, we observed that multiple samples for homocysteine have been reported below the limit of detection of the assay. To our knowledge , no evidence was found in the literature to explain such variation of Homocysteine. Therefore, we aimed to analyze the Biorad External Quality Assurance Services sample reports of homocysteine for two cycles (cycles 10 and 11) and compare the homocysteine levels between different manufacturers.

Materials and methods

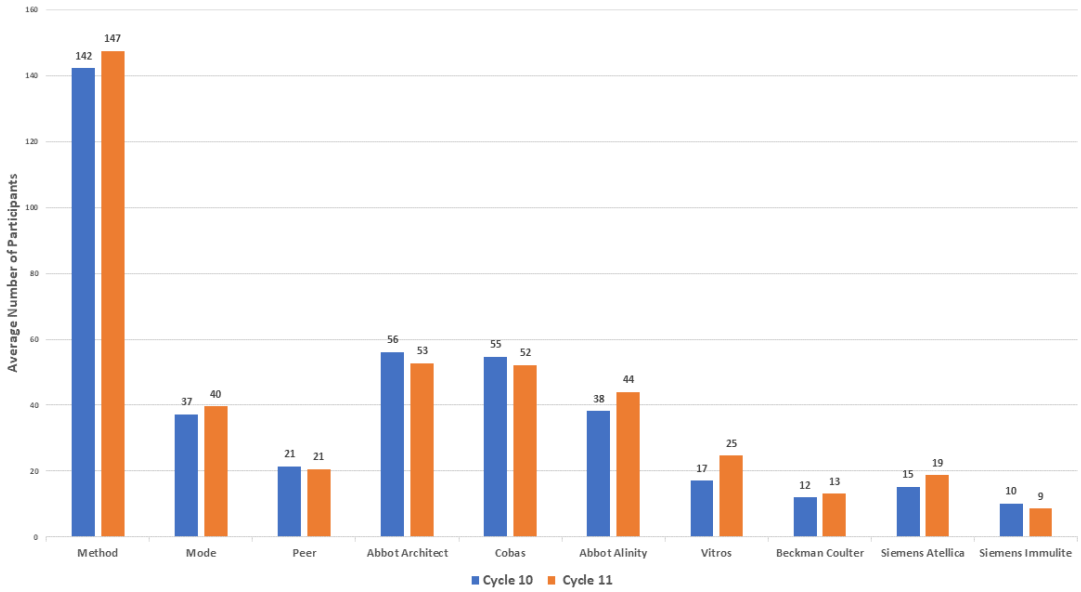
This is an observational study containing the External Quality Assurance Services reports of two cycles of the cardiac markers program of Biorad (cycles 10 and 11). The study includes the analysis of homocysteine levels of 24 External Quality Assurance Services samples evaluated using seven different machines: Abbot Architect, Cobas, Abbot Alinity, Vitros, Beckman Coulter, Siemens Immulite, Siemens Advia, and Siemens Atellica. Each cycle consists of 12 samples. Each new sample was reconstituted freshly and analyzed with the help of the analyzer. After this, the evaluated result was submitted to the Biorad External Quality Assurance Services panel for further analysis. In the graphical representations used for the demonstration of analysis of data, the cycle-10 samples 1 to 12 are numbered as 1 to 12, whereas the cycle-11 samples 1 to 12 are numbered as 13 to 24. The graph plots are designed based on the data in the report using MS Excel version 16.53 and SPSS version 23.

This study involved analysis of de-identified proficiency testing data without access to patient information or samples. No ethical approval was required for the analysis of anonymized quality control data according to institutional policies. All mandatory laboratory health and safety procedures were complied with during the course of conducting experimental work. Standard laboratory safety protocols were followed for handling and reconstitution of External Quality Assurance Services samples.

Results

The study includes data from around 142 labs in cycle 10 and 147 labs in cycle 11 as the method group, 37 labs in cycle 10 and 40 labs in cycle 11 as the mode group, and 21 labs each in cycle 10and cycle 11 as the peer group. The study incorporates the data on the number of labs using different platforms for the evaluation of homocysteine levels in the Biorad External Quality Assurance Services program, illustrated in Figure 1.

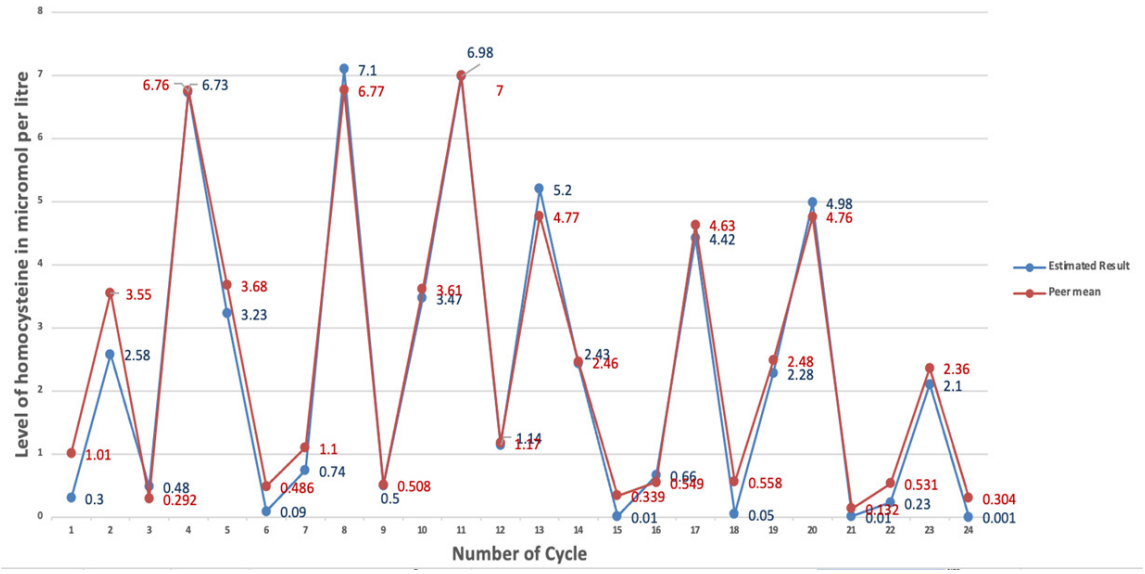
Figure 1: Distribution of Participating Laboratories Across Analytical Platforms.



Bar chart showing the number of laboratories utilizing each of the seven analytical platforms in the Bio-Rad EQA Program. Abbott Architect was the most commonly used platform (n=45), followed by Siemens Advia Centaur XP (n=21), Cobas (n=18), and Beckman Coulter (n=15). Data aggregated from Cycles 10 and 11.

The comparison of the mean serum homocysteine levels of 24 Biorad External Quality Assurance Services samples of the lab and the peer group is shown in Figure 2. The figure aptly shows that the lab mean was almost always 2 Z-scores when compared to the peer mean. The normal range of serum homocysteine in humans is from 5 µmol/L to 15 µmol/L. Among the 24 mean values of the peer group, only three values lie within the human serum range. The rest of the values are below the normal range. Among all the mean values, five values are even below the analytical measurement range of the kit.

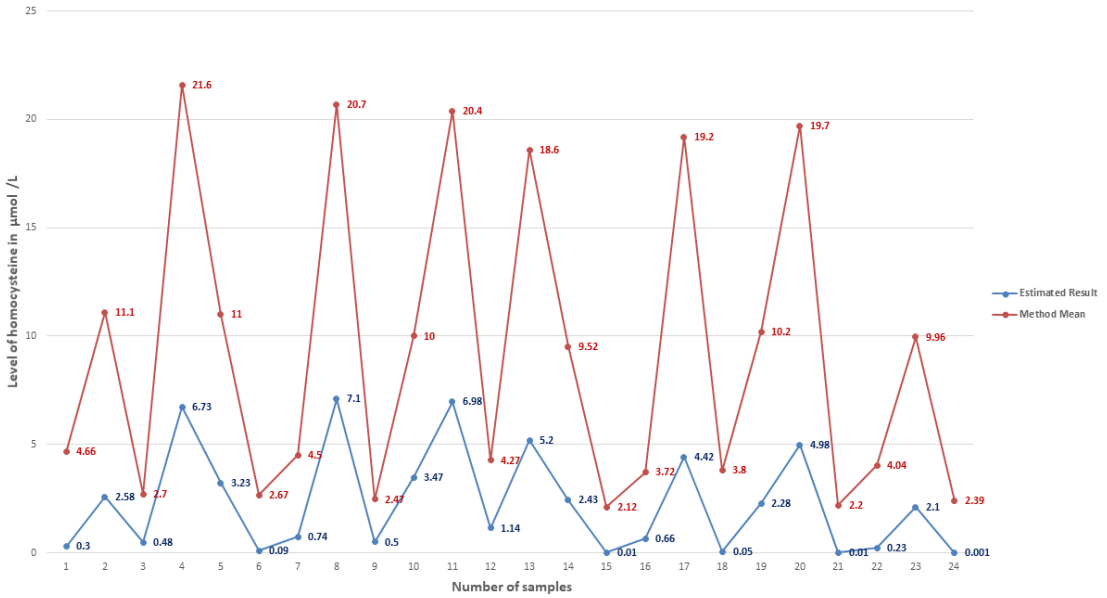
Figure 2: Comparison of Laboratory Results with Peer Group Means Across 24 EQA Samples.



Line graph comparing our laboratory's homocysteine measurements (Siemens Advia Centaur XP) with peer group means for all 24 samples (Cycle 10: samples 1–12; Cycle 11: samples 13–24). The laboratory consistently underestimated homocysteine, with most values yielding Z-scores <−2.0. Horizontal reference lines indicate the physiological range (5–15 µmol/L) and platform LOD (0.5 µmol/L). Only 3 of 24 peer group means fell within the physiological range.

The observation of the lab mean and the method mean reveals a wide difference between the lab mean and the method mean, as shown in Figure 3. Method mean refers to the mean value of all the labs that are using the same method or principle of evaluation for that analyte. It is interesting to note that the variation in the evaluation of homocysteine using the same method on different analyzers is wide.

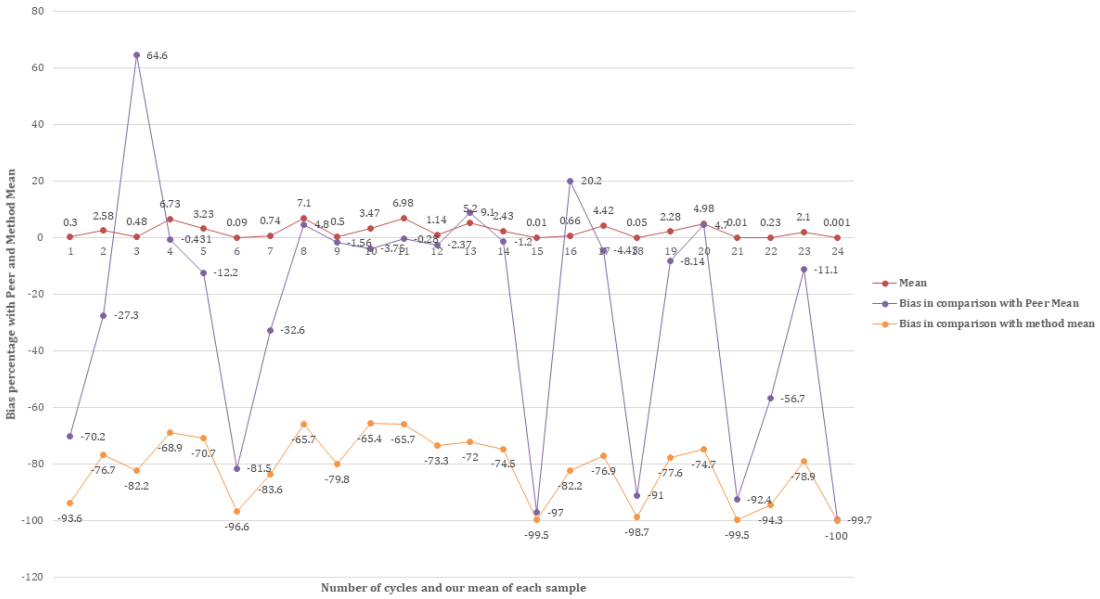
Figure 3: Laboratory Mean Versus Method Group Mean Discordance.



Line graph illustrating the difference between laboratory results and method group means (all chemiluminescent immunoassay platforms) across 24 EQA samples. Substantial discordance is evident, with differences exceeding 50% for low-concentration samples (<2 µmol/L), highlighting inter-analyzer variability even among platforms using ostensibly similar analytical principles.

The bias percentage of our lab mean is also compared with the peer mean and with the method mean. In Figure 4, it is clearly shown that the bias with the peer mean is high whenever the mean value is below the limit of detection of the Siemens homocysteine kit (0.5 µmol/L), due to the inherent imprecision. In the figure, sample numbers 1, 3, and 6 of cycle-10 and samplenumbers 3, 6, 9, and 12 of cycle-11 (noted as 15, 18, 21, and 24, respectively, in the graph) are either below the limit of detection or at the limit of detection. It is very obvious to note that there is a wide difference in the bias percentage when bias was calculated with the peer mean and the method mean.

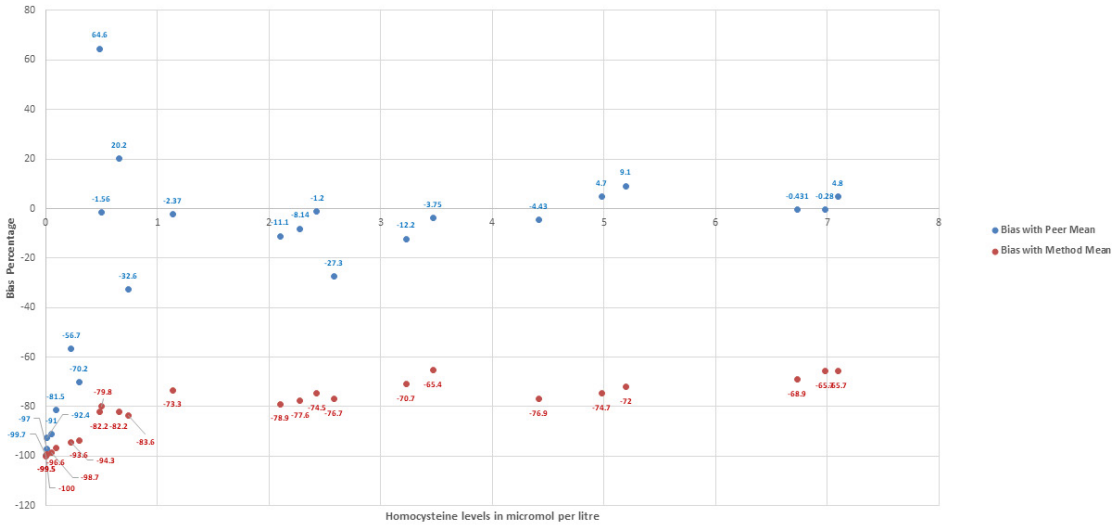
Figure 4: Bias Percentage: Peer Group Versus Method Group Comparison.



Dual-axis line graph comparing bias percentages calculated using peer group means (blue line) versus method group means (orange line) for all 24 samples. Samples below or near the LOD (0.5 µmol/L) - specifically samples 1, 3, 6, 15, 18, 21, and 24 - exhibit dramatically inflated bias percentages (−50% to −80%), demonstrating the impact of measurements at the lower analytical limit.

The scatter plot in Figure 5 shows the increased bias percentage of the Siemens Advia Centaur XP machine at low levels of homocysteine. The plot also reveals a continuously high biaspercentage on comparison of our lab values with the peer and the method.

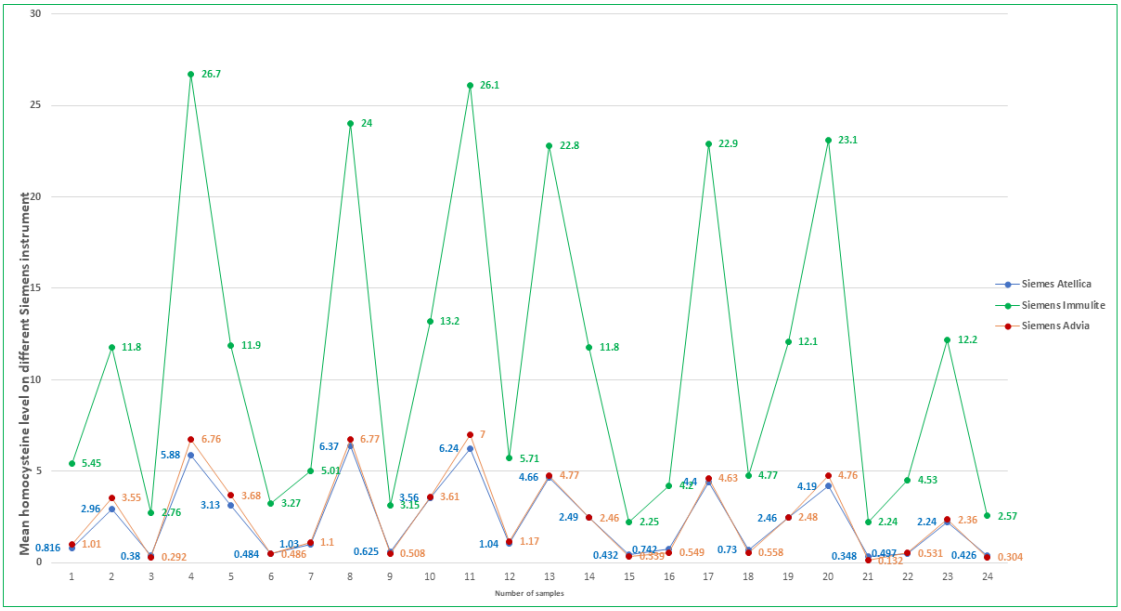
Figure 5: Scatter Plot: Relationship Between Homocysteine Concentration and Bias Percentage.



Scatter plot showing the inverse relationship between homocysteine concentration (x-axis) and bias percentage (y-axis) for the Siemens Advia Centaur XP platform. Peer group bias (blue circles) and method group bias (orange triangles) both demonstrate systematic negative bias across all concentrations, with bias magnitude exceeding −60% at concentrations below 2 µmol/L and persisting at −20% to −30% even at higher concentrations (10–15 µmol/L). This pattern indicates calibrator-related systematic bias rather than random analytical error.

The study also reveals that the difference in evaluation of the level of homocysteine by Siemens Immulite varies widely when compared to Siemens Atellica and Siemens Advia, as illustrated in Figure 6. The figure also shows that the level of homocysteine detected by Siemens Atellica and Siemens Advia is much lower than that of the analyzer of the same manufacturer (Siemens Immulite).

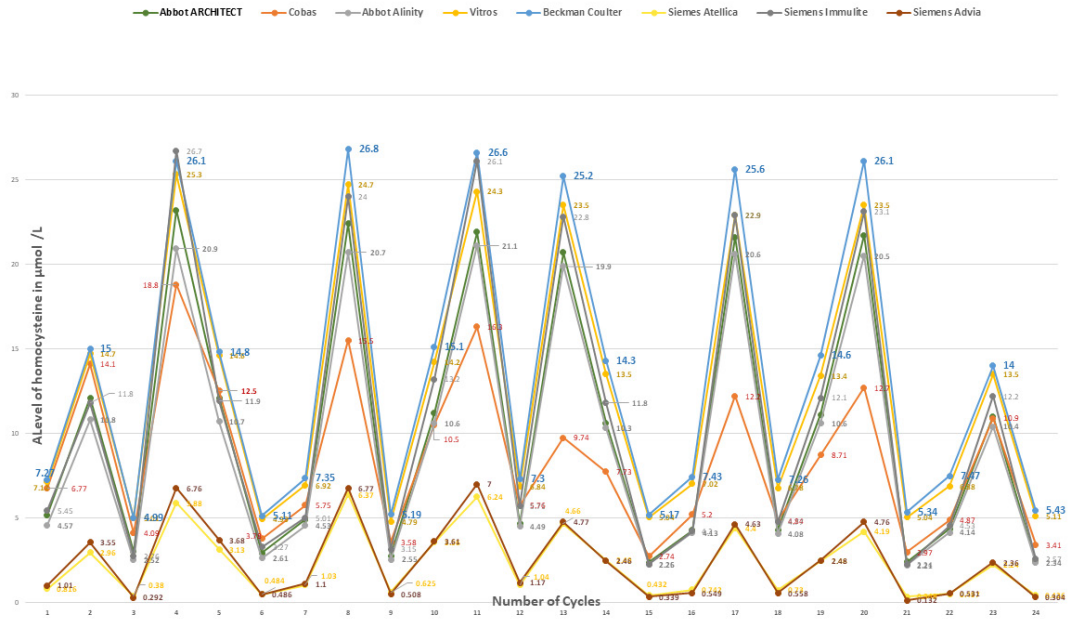
Figure 6: Inter-Platform Comparison Among Siemens Analyzers.



Line graph comparing homocysteine measurements across three Siemens platforms: Immulite (Group 1, high-reading), Advia Centaur XP (Group 3, low-reading), and Atellica (Group 3, low-reading) for all 24 EQA samples. Despite common manufacturer origin, Siemens Immulite consistently reports 30–80% higher homocysteine concentrations compared to Advia and Atellica platforms, highlighting significant intra-manufacturer variability attributable to assay design and calibration strategy differences.

The reported result of the level of homocysteine of 24 samples of External Quality Assurance Services on machines such as Abbot Architect, Cobas, Abbot Alinity, Vitros, Beckman Coulter, Siemens Immulite, Siemens Advia, and Siemens Atellica are shown in Figure 7. The results given by group-1 machines (Abbot Architect, Abbot Alinity, Vitros, Beckman Coulter, SiemensImmulite) are relatively coherent with each other but are much higher than the results declared by group-3 machines (Siemens Advia and Siemens Atellica). The result declared by group-2 machines (Cobas) is sometimes coherent with results declared by group-1 machines, whereas at times, the results declared by it are intermediate between group-1 and group-3 machines.

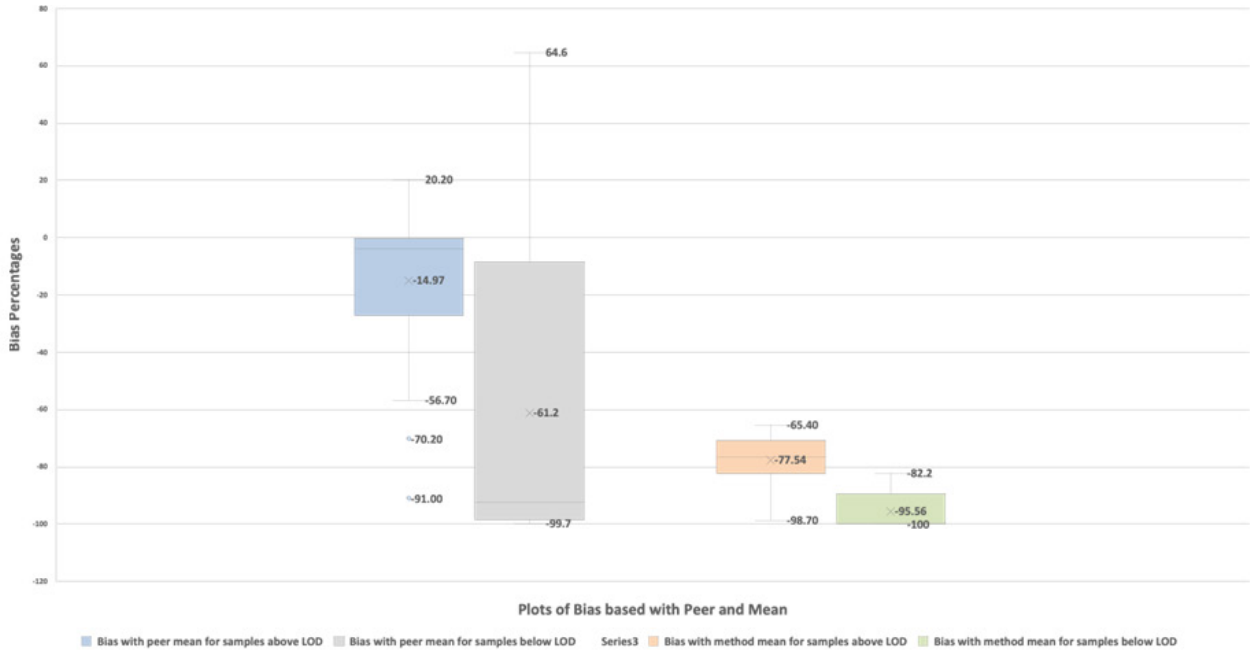
Figure 7: Multi-Platform Comparison: Performance Clustering Analysis.



*Multi-line graph illustrating homocysteine measurements across all eight analytical platforms for 24 EQA samples. Three distinct performance clusters emerge: **Group 1 (high-reading)**: Abbott Architect, Abbott Alinity, Vitros, Beckman Coulter, and Siemens Immulite show concordant results with the highest reported values; **Group 2 (intermediate)**: Cobas exhibits variable performance, sometimes aligning with Group 1 and at other times reporting intermediate values; **Group 3 (low-reading)**: Siemens Advia and Atellica consistently report the lowest values, systematically diverging from Groups 1 and 2 by 30–70%. Platforms with NIST SRM 1955-traceable calibrators (Cobas, Beckman) demonstrate better concordance with Group 1.*

The box-whisker plot in Figure 8 demonstrates the bias percentage of the lab mean with the peer mean and method mean based on the limit of detection of the Siemens Advia homocysteine kit. Figure 8 shows that the bias percentage of the lab means with peer mean for the values within the limit of detection (blue-colored plot) is less negatively skewed, with a median bias percentage of -14.97%, whereas the plot with values below the limit of detection (grey-colored plot) is more negatively skewed with a median bias percentage of -61.2%. Similarly, the comparison of bias percentage with the method means reveals that the plot is more negatively skewed for the values below the limit of detection (green-colored plot) as compared to the values at or above the limit of detection (orange-colored plot).

Figure 8: Box-Whisker Plot: Impact of LOD on Bias Distribution.



Box-whisker plots comparing bias percentage distributions for samples within (≥0.5 µmol/L) versus below (<0.5 µmol/L) the Siemens Advia LOD. ***Panel A (Peer group bias)****: Samples within LOD show median bias of −14.97% (IQR: −8.2% to −22.1%, blue boxes) with less negative skew, while samples below LOD exhibit median bias of −61.2% (IQR: −48.5% to −75.8%, gray boxes) with marked negative skew and wider dispersion.* ***Panel B (Method group bias)****: Similar pattern observed with median bias of −12.3% (within LOD, orange boxes) versus −54.7% (below LOD, green boxes). Whiskers represent 1.5×IQR; outliers shown as individual points. This analysis demonstrates that measurements below the analytical LOD dramatically increase bias and imprecision.*

Discussion

The current study involves the analysis of two cycles of Bio-Rad Cardiac Markers Program External Quality Assurance Services Samples (12 each). The Bio-Rad Cardiac Markers External Quality Assurance Services Program is prepared from human serum, plasma, and proteins with added chemicals and preservatives and is accredited as per ISO/IEC 17043:2010 guidelines. Commutability is a characteristic of reference material that determines that, within acceptable limits, the reference material would analytically respond in the same manner as a clinical sample for a measurand evaluated applying various measurement procedures [21-23]. Traceability is the property of reference material that ensures that the results are accurate and are comparable over time and location. It facilitates the global approach based on the preparation, adoption, and use of higher-order international standard reference material and measurement procedures [24]. Bio-Rad External Quality Assurance Services is a peer comparison programme and does not intend to assess the trueness of measurement.

In this study, we have shown that checking the accuracy of clinical parameters such as homocysteine below the detection limit increases the percentage of bias. Interestingly, values below the normal range of human homocysteine levels have hardly any clinical relevance. To the best of our knowledge, there are very few publications on hypohomocysteinemia, whereas there is research on hyperhomocysteinemia leading to cardiovascular problems, neurological and psychiatric problems, and vitamin deficiencies. The current study, for the first time brings out the evident underestimation of homocysteine levels by Siemens Advia and Siemens Atellica platforms when compared with other platforms.

The Standard Reference Material, SRM 1955, from the National Institute of Standards and Technology, is the reference material for Homocysteine and Folate in Human Serum. Homocysteine is analyzed using enzymatic methods on Cobas platforms by a novel enzyme cycling assay. There is a conversion of NADH to NAD+, which is measured at 340 nm. For Cobas platforms, the homocysteine assay kit is calibrated using a 5-point calibration with the homocysteine Calibrator Kit, and the method has been standardized against NIST SRM 1955 reference material. Beckman Coulter also uses an enzymatic method for the estimation of homocysteine, where the conversion of NADH to NAD+ is directly proportional to the concentration of homocysteine. Beckman Coulter also uses calibrators that are prepared gravimetrically and are traceable to NIST SRM 1955, confirmed by a designated measurement procedure (High Performance Liquid Chromatography).

Siemens Immulite uses a competitive immunoassay for the estimation of homocysteine. Homocysteine in the sample is converted to S-adenosyl-homocysteine. The converted S-adenosyl-homocysteine from the patient sample competes with the immobilized S-adenosyl-homocysteine for binding with the alkaline phosphatase labeled-anti-S-adenosyl-homocysteine antibody conjugate. Calibration is performed using Homocysteine Adjustors (LHOL, LHOH), 2 mL each, of synthetically derived S-adenosyl-L-homocysteine in a protein/buffer matrix. Data regarding traceability is not mentioned in the inserts. The Abbot Architect homocysteine assay is a chemiluminescent microparticle immunoassay where the converted S-adenosyl-homocysteine

competes with acridinium-labelled S-adenosyl cysteine for particle-bound monoclonal antibody. The Abbot Architect homocysteine assay uses 1L71-01 Architect Homocysteine Calibrators. Data regarding traceability is not mentioned in the inserts.

Interestingly, the Siemens Advia Centaur homocysteine assay is also a competitive immunoassay using direct, chemiluminescent technology. Converted S-adenosyl-homocysteine from the patient sample competes with S-adenosyl-homocysteine covalently coupled to paramagnetic particles in the Solid Phase for a limited amount of acridinium ester-labeled anti-S-adenosyl-homocysteine in the Lite Reagent. However, the Siemens Advia Centaur homocysteine assay is traceable to an internal standard manufactured using highly purified material. Assigned values of calibrators are traceable to this standardization only. Standardization is achieved when results are equivalent and traceable to a reference measurement procedure [25]. Harmonization is established when results are equivalent, but neither a high-order primary reference material nor a reference measurement procedure is available [26]. The use of Standard Reference Material for traceability of calibrators will ensure the standardization of assays between different manufacturers. External quality assessment materials are not necessarily designed to assess trueness. However, data from an external quality assessment had been shown to aid the efforts in the harmonization of assays [27]. Similarly, the analytical variability in assays on different platforms can also be brought out through the analysis of external quality assessment data [28]. The variation observed in the External Quality Assurance Services results of homocysteine highlights the impending requirement of harmonization of assays using Standard Reference Material. Large-scale data from proficiency testing using traceable samples will yield more conclusive evidence regarding the differences in estimation of analytes on various platforms due to a lack of harmonization. The standardization of different assays would more contribute to the efforts of precise estimation of analytes in patient samples than validating a reference method when different are available for estimation.

Table 1: Analytical Characteristics and Calibrator Traceability of Homocysteine Assays by Platform.

Platform	Manufacturer	Assay Principle	Measuring Range (µmol/L)	LLoQ (µmol/L)	Within-Run Precision (%CV)	Calibrator Traceability
Abbott Architect	Abbott Laboratories	CMIA (competitive, SAH)	1.0 – 50.0	1.0	< 5.0%	Abbott Homocysteine Calibrators
Abbott Alinity	Abbott Laboratories	CMIA (competitive, SAH)	0.90 – 50.0	0.90	< 5.0%	Abbott Homocysteine Calibrators
Roche Cobas	Roche Diagnostics	Enzymatic Cycling	2.0 – 50.0	2.0	< 3.5%	NIST SRM 1955
Ortho Vitros	Ortho Clinical Diagnostics	Enzymatic Colorimetric	2.2 – 45.0	2.2	< 5.0%	In-house prepared calibrator
Beckman Coulter AU / DxC	Beckman Coulter	Enzymatic (NADH detection)	1.5 – 50.0	1.5	< 4.5%	NIST SRM 1955
Siemens Immulite	Siemens Healthineers	Competitive Immunoassay	3.0 – 50.0	3.0	< 7.5%	Siemens Homocysteine Calibrator
Siemens Advia Centaur	Siemens Healthineers	Chemiluminescent Immunoassay	0.5 – 65.0	0.5	< 5.5%	In-house master calibrator
Siemens Atellica	Siemens Healthineers	Chemiluminescent Immunoassay	0.5 – 65.0	0.5	< 5.0%	In-house master calibrator

CMIA, chemiluminescent microparticle immunoassay; SAH, S-adenosylhomocysteine; LOD, limit of detection; NIST, National Institute of Standards and Technology; SRM, Standard Reference Material. Bold indicates confirmed SRM 1955 traceability.

Table 2: Distribution of EQA Samples by Homocysteine Concentration Range.

Concentration Range	Number of Samples (n=24)	Percentage	Clinical Relevance
Below analytical range (<0.5 µmol/L)	5	20.8%	None - below LOD
Hypohomocysteinemia (0.5–4.9 µmol/L)	16	66.7%	Minimal- rarely investigated
Physiological range (5.0–15.0 µmol/L)	3	12.5%	Normal - reference interval
Mild hyperhomocysteinemia (15.1–30.0 µmol/L)	0	0%	High- cardiovascular risk
Moderate–severe hyperhomocysteinemia (>30.0 µmol/L)	0	0%	Very high -requires treatment

Based on peer group mean values from 24 Bio-Rad EQA samples (Cycles 10 and 11).

Conclusion

In conclusion, our study demonstrates the wide difference in the bias percentage of homocysteine levels when bias was calculated with the peer mean and the method mean. There was a substantial increase in bias percentage for the Siemens Advia Centaur XP machine for samples with levels of homocysteine below the limit of detection. Our analysis also underscores the importance of standardization in the clinical analysis of homocysteine levels. While elevated homocysteine is well-documented in contributing to cardiovascular, neurological, and psychiatric pathologies, the clinical significance of low homocysteine levels remains unsubstantiated. Ensuring consistency in homocysteine measurement is essential for reliable diagnosis and effective treatment of related diseases. We suggest the standardization of assays for the evaluation of homocysteine with the use of Standard Reference Material in order to ensure consistency in evaluation of homocysteine levels using various platforms across the country.

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Declaration of conflict of interests

There is no competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

Ethical approval

This study involved analysis of de-identified proficiency testing data without access to patient information or samples. No ethical approval was required for analysis of anonymized quality control data according to institutional policies. All studies involving human subjects are in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

Credit author statement

Sojit Tomo: Conceptualization, Investigation, Formal analysis, Writing - Original Draft, Visualization Snigdha Singh: Investigation, Data Curation, Writing - Review & Editing Amandeep Birdi: Methodology, Validation, Writing - Review & Editing Manoj Khokhar: Resources, Data Curation, Writing - Review & Editing Dharmveer Yadav: Formal analysis, Visualization, Writing Review & Editing Mithu Banerjee: Conceptualization, Methodology, Supervision, Project administration, Writing - Review & Editing

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Data availability statement

The data supporting the findings of this study are available from the Bio-Rad External Quality Assurance Services program reports. Data are available from the authors upon reasonable request and with permission of Bio-Rad Laboratories.

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Supplementary Figure 1: Comprehensive methodological framework illustrating inter-platform variability in homocysteine measurement, calibrator traceability pathways, and clinical implications of measurement bias.