Biomarkers in Medicine

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Short Communication

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Assessment of biomarker stability and assay performance parameters for medical diagnosis: a case study of diagnosis of major depressive disorder

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Aim: Assessing the stability profiles and assay performance of 24 biomarker assays in 32 biomarker/body fluid combinations identified as relevant for prediction of major depressive disorder. **Materials & methods:** Combinations were tested for stability and assay performance with ELISA at different storage and freeze–thaw conditions in pooled samples of 40 patients. **Results:** Stability and assay performance issues were found in almost all cases except three biomarkers in urine and three in serum. **Conclusion:** This study shows that, to produce reliable measurement data, assessments of stability and assay performance are essential. In development, other quality assurance parameters might be implemented to increase the level of measurement reliability by increasing assay performance control.

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Keywords: biomarkers • ELISA • major depressive disorder • method development • quality assurance • stability

Biomarkers are defined as biological indicators of normal and pathological biological processes and can be used for both diagnostic and monitoring purposes across medicines to, for example, identify subsets of tumors based on a genetic biomarker profile [1]. Types of biomarkers range from imaging biomarkers to genetic biomarkers to more molecular biomarkers, which can be measured in different matrix types [2]. In the field of psychiatry, the development of biomarker-based diagnostic assays is relatively recent. Current diagnostic methods in psychiatry rely heavily on the clinical assessment of psychiatric disorders using classification systems such as the Diagnostic and Statistical Manual of Mental Disorders (DSM) or the International Classification of Diseases (ICD-10) [3,4]. However, the diagnosis of psychiatric disorders is particularly complex due to both heterogeneity within disorder categories and high comorbidity rates with other psychiatric and nonpsychiatric disorders. This overlap in symptomology may lead to the initiation of suboptimal treatment procedures, a high psychological burden for patients and increased healthcare costs [5–7]. In addition, multiple biological pathways are believed to correlate with psychiatric disorders [2,8,9]. Recently, focus has shifted to finding disease-associated biomarker patterns since it is unlikely that a single biomarker will be identified with sufficient discriminative power for clinical usefulness. Such an approach has been shown to be fruitful in several studies, revealing promising biomarker panels for major depressive disorder (MDD) [10–14]. As multiple biomarkers are needed to identify a disorder, and the margin of error in terms of reliability is quite small, the resulting high application stringency currently hampers the advancement of these panels from research settings to clinical stages [15,16].

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One issue impeding the development of these clinical panels is the lack of understanding of the importance of biomarker reliability and assay performance in terms of correctly interpreting results, leading to variation in reported results in historically published papers on the subject of biomarker development [17]. The vast majority of these biomarker studies utilize ligand-binding assays (LBAs) such as ELISA. Due to the biological nature of these methods, they are prone to variations in assay performance and protein stability, negatively impacting the reliability and reproducibility of measured analytes [18–22]. Therefore, during early method development, it is important to obtain data on assay performance and protein stability with respect to factors involving sample-specific variations, such as hemolytic and lipidemic content, storage condition, storage time and freeze–thaw cycles [23,24].

The present study was designed to show the importance of assessing the stability of various biomarkers in serum and urine under different conditions and how this may affect the reliability and performance of the measured analyte. Findings will not only be relevant to the field of psychiatry but also extend to biomarker research in other fields where LBAs are used as the method of choice. Performing this evaluation at an early stage of biomarker method development will improve the selection of appropriate biomarkers for use in clinical panels. In addition, this study also shows which biomarkers are more or less suitable for further development of a biomarker panel for diagnostic use in MDD.

Materials & methods

Sample material & biomarker selection

To determine biomarker stability, sample materials (i.e., urine and serum) were obtained from an existing cohort of 40 patients with MDD included based on the Mini-International Neuropsychiatric Interview (MINI) and the Hamilton Depression 17 scale (HAM-D) for which a score ≥11 was used as an inclusion criterion. For a comprehensive list of inclusion/exclusion criteria and demographic characteristics, please see van Buel *et al.* [13]. The investigation was carried out in accordance with the latest version of the Declaration of Helsinki and the initial study design was reviewed by an appropriate ethical committee. Informed consent of the participants was obtained after the nature of the procedures had been fully explained. Biomarker selection was performed on the basis of the current authors' previous work [13,14] and supplemented with the biomarker acetyl-L-carnitine, as recent literature suggests this metabolite is implicated in the pathophysiology of MDD [25]. All selected biomarkers play a role in one or more of the major and minor hypotheses associated with MDD [2].

Sample pooling

All tests were performed on pooled patient samples. For biomarkers for which data about biomarker levels were available, eight samples per biomarker that were known to have sufficiently high levels were chosen and pooled. For serum, this included calprotectin. For urine this included aldosterone, cGMP, isoprostane 2, leptin, midkine, substance P and thromboxane B2. For those biomarkers for which information about urine or serum levels was absent, a general pool was made of eight randomly selected patients, separately for both urine and serum. The general serum and urine pool contained material from the same eight individuals. Notably, this study was not designed to assess individual biomarker levels in patients with MDD, but to investigate biomarker stability and assay performance. Pooled samples were prepared by quickly thawing at 37 °C in a water bath, homogenizing via vortex and storing on ice until refreezing. All samples for each pool were thoroughly combined and mixed. Samples were aliquoted such that one aliquot contained a sufficient volume for use in a single biomarker assay.

Study design

Stability parameters investigated include storage temperature (-80 °C, -20 °C, 4 °C, 22 °C and 37 °C), duration (Table 1) and number of freeze–thaw cycles (0, 1, 5 and 10). Depending on its applicability, the study was performed on samples from urine, serum or both. For temperature treatments, samples were stored at each temperature for the indicated time periods and subsequently placed back at -80 °C until testing. For short incubations (up to 4 h), samples were stored at -80 °C and thawed immediately prior to the start of temperature treatment. Freeze–thaw samples were placed in an open box within a 37 °C incubator for 20 min, adequate time to thaw without warming. Samples were homogenized by vortexing and refrozen in an open box at -80 °C.

Biomarker measurements

For each time point as shown in Table 1, biomarker stability was measured. Biomarker levels in urine and serum were determined using various available research and Conformité Européenne (CE)-marked ELISA kits (Table 2).

Table 1. Storage temperature conditions and time duration when biomarker stability was measured.

Storage condition	Hours					Days			
	0	1	2	4	8	1	2	7	14
-80 °C	x								X
-20 °C	x					x	x	x	X
4 °C	x				x	x	x	x	X
22 °C	x	x	x	x	x	x	x	x	X
37 °C	x	x	x	x	x	x			

Each cross represents conditions tested. Empty fields were not tested.

Table 2. Biomarkers tested, sample origin and manufacturer of commercially available assays.

Number	Biomarker	Sample	Manufacturer
1	Aldosterone	Urine	R&D Systems (MN, USA)
2	Alpha1 antitrypsin	Serum	Immundiagnostik (Bensheim, Germany)
3	Alpha1 antitrypsin	Urine	Immundiagnostik (Bensheim, Germany)
4	Apolipoprotein A1	Serum	R&D Systems (MN, USA)
5	cAMP	Serum	R&D Systems (MN, USA)
6	Total BDNF†	Serum	R&D Systems (MN, USA)
7	Calprotectin	Urine	Hycult Biotech (Uden, Netherlands)
8	Calprotectin	Serum	Hycult Biotech (Uden, Netherlands)
9	cGMP	Urine	R&D Systems (MN, USA)
10	Cortisol	Urine	Diagnostics Biochem Canada Inc. (London, Canada)
11	Cortisol	Serum	Diagnostics Biochem Canada Inc. (London, Canada)
12	EGF	Serum	R&D Systems (MN, USA)
13	Endothelin-1	Serum	R&D Systems (MN, USA)
14	HVEM	Urine	R&D Systems (MN, USA)
15	Isoprostane-2	Urine	Northwest LLC (WA, USA)
16	Leptin	Urine	R&D Systems (MN, USA)
17	Leptin	Serum	R&D Systems (MN, USA)
18	LTB4	Urine	R&D Systems (MN, USA)
19	Midkine	Urine	R&D Systems (MN, USA)
20	Myeloperoxidase	Serum	R&D Systems (MN, USA)
21	Myeloperoxidase	Urine	R&D Systems (MN, USA)
22	Prolactin	Serum	Diagnostics Biochem Canada Inc. (London, Canada)
23	Prolactin	Urine	Diagnostics Biochem Canada Inc. (London, Canada)
24	Resistin	Serum	R&D Systems (MN, USA)
25	Resistin	Urine	R&D Systems (MN, USA)
26	Substance P	Urine	R&D Systems (MN, USA)
27	Thromboxane	Serum	R&D Systems (MN, USA)
28	Thromboxane	Urine	R&D Systems (MN, USA)
29	TNF-α receptor 2	Serum	R&D Systems (MN, USA)
30	Zonulin	Serum	Immundiagnostik (Bensheim, Germany)
31	Acetyl-L-carnitine	Urine	Abbexa (Cambridge, UK)
32	Acetyl-L-carnitine	Serum	Abbexa (Cambridge, UK)
33	Lipocalin	Urine	R&D Systems (MN, USA)

† Total BDNF is both free and bound BDNF in serum.
BDNF: Brain-derived neurotropic factor; cGMP: Cyclic guanine monophosphate; HVEM: Herpes virus entry mediator; LTB4: Leukotriene B4.

Each biomarker was measured according to the procedures provided by the vendor. An ELISA plate washer (Bio-Rad PW40, CA, USA) was used for all washing steps. Tetramethylbenzidine (TMB) absorption measurements were performed on a microtiter plate reader (Thermo Multiskan Spectrum, MA, USA) at 450 nm, using 620 nm

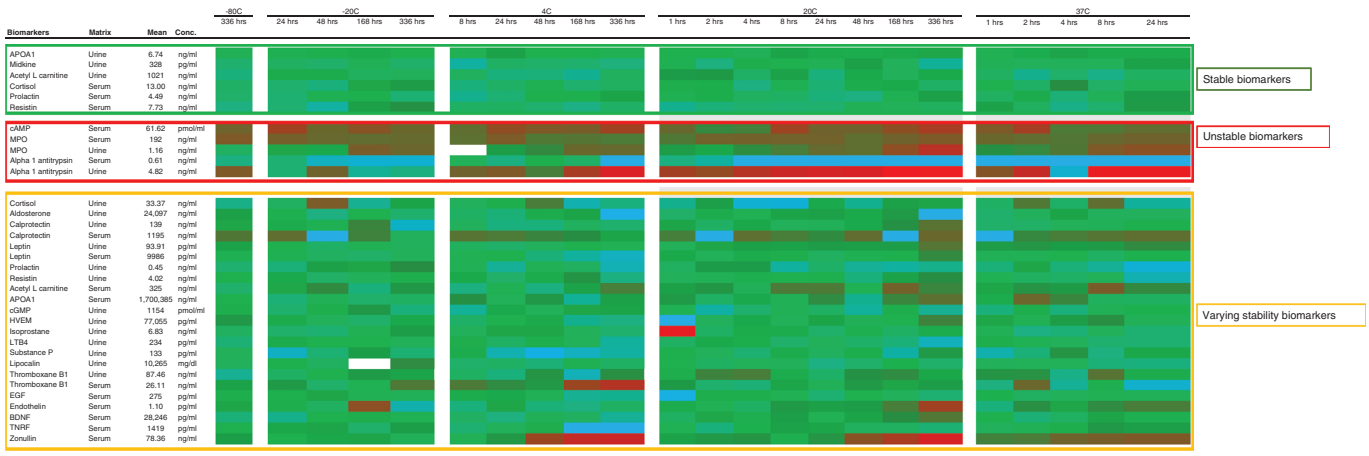

Figure 1. Heatmap representing biomarker stability under various temperature storage conditions.
Stability determined based on bias of mean concentration with an acceptance criteria of ±25% bias of mean biomarker concentration. Biomarkers clustered based on overall stability under various storage conditions. Red represents high values with a plus bias percentage; blue represents a minus bias percentage and green represents a low bias from mean concentration for each biomarker. White indicates N/A values for which no results were obtained. Absolute bias values are presented in Supplementary Table 1. Heatmap created with the following criteria: blue: minimum value = -50; green: percentile value set at 50; red: highest value.

as a reference wavelength. Biomarker concentrations were determined using an Excel-based 4-Parameter Logistic (4-PL) curve-fitting algorithm, in which the optical density (OD) differences between 450 nm and 620 nm (OD_{450}–OD_{620}) of the unknown samples were plotted against the measured ODs of the known calibrators.

Stability & acceptance criteria

Differences in measured sample concentrations were compared with the mean of frozen T = 0, -80 °C samples. Samples stored at -80 °C remain stable for long periods and can therefore be used as both a measure of assay performance and a reference to assess stability under various storage conditions. Bias (as a measure of stability) was determined by the percent difference from the reference sample and acceptance was set at 25% following Organisation for Economic Co-operation and Development guidelines for bioanalysis [23,26] for initial assessment of the biomarker data. All samples were measured in singlicate whereas calibrators were measured in duplicate. For each biomarker, all conditions were measured in one run on a single plate to exclude interrun and interplate variances. A 15% coefficent of variation for the calibrators was used as a run acceptance criterion.

Results

Storage temperature biomarker stability

The results of the biomarker stability studies are shown in Figure 1. Six biomarkers were identified as stable biomarkers meeting all acceptance criteria under all storage conditions. These include APOA1, midkine and acetyl-L-carnitine in urine and cortisol, prolactin and resistin in serum. Five biomarkers showed low stability with a high percentage in variation of the bias under various storage conditions, not meeting most acceptance criteria. These include MPO and alpha 1 antitrypsin in urine and cAMP, MPO and alpha 1 antitrypsin in serum. The remaining biomarkers showed various patterns of stability as indicated by the heatmap indicating either decreased stability and/or assay performance variability. Absolute bias values are shown is Supplementary Table 1.

Freeze–thaw biomarker stability

Figure 2 shows the results of the freeze–thaw experiments. The urine biomarkers MPO, aldosterone, APOA1, calprotectin, cGMP, HVEM, prolactin, LTB4, resistin, acetyl-L-carnitine, Substance P and lipocalin were stable under all freeze–thaw conditions with maximum bias under 25%. In serum, this was also true for the biomarkers alpha 1 antitrypsin, calprotectin, cortisol, EGF, leptin, MPO, BDNF, resistin, TNRF and zonulin. In urine, alpha 1 antitrypsin and thromboxane B1 show the least freeze–thaw stability, with measured concentrations above 25% after five and ten cycles. With respect to the remaining biomarkers in both serum and urine, freeze–thaw stability varied. Some biases varied above 25% after one cycle, within 25% after five cycles and again above 25% after ten

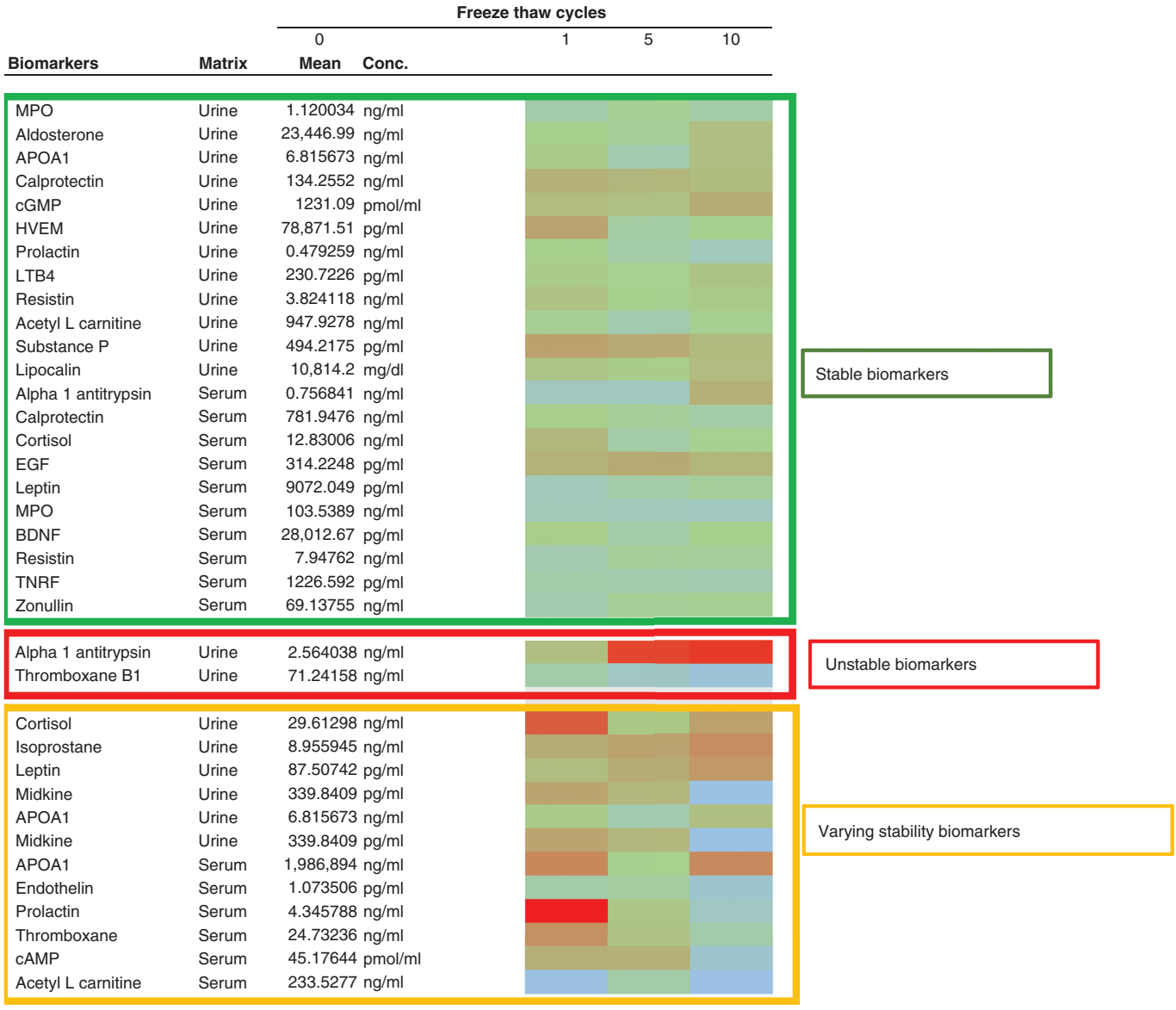

Figure 2. Heatmap representing biomarker stability after various freeze–thaw cycles. Freeze–thaw stability determined based on bias of mean concentration with an acceptance criteria of ±25% bias of mean biomarker concentration. Red represents high values with a plus bias percentage; blue represents a minus bias percentage and green represents a low bias from mean concentration for each biomarker.

cycles, indicating assay performance variability rather than stability issues. This was the case for cortisol in urine and thromboxane, APOA1, prolactin and acetyl-L-carnitine in serum.

Discussion

To our knowledge, this paper is the first to present stability data on various urine and serum biomarkers measured with ELISA from a nonbioanalytical perspective while also demonstrating that control of assay performance variability is essential during scientific biomarker research and development. The diagnosis of MDD and associated biomarkers were used for illustration.

In both urine and serum, three biomarkers passed the stability and acceptance criteria under all conditions whereas the other biomarkers varied in bias compared with the reference -80 °C concentration. With respect to the freeze–thaw stability of the various serum and urine biomarkers, some remained stable after ten freeze–thaw cycles whereas others lost stability after only five cycles. In light of further biomarker panel development for MDD, the failing stability and freeze–thaw properties of some biomarker/body fluid combinations indicate the need for

appropriate considerations to be made in determining both the sampling procedure and the test design. Failing assay performances are much more difficult to control and, therefore, these biomarker/body fluid combinations should be rejected or additional assay performance measures should be implemented.

This study was performed as part of the development of a biomarker panel for MDD diagnostics. Although the results of this study provide some points for further development, their relevance is not limited to MDD but extends to all biomarker studies involving LBAs as the method of choice. Due to the nature of LBAs like ELISA, a starting point for every matrix-based biomarker study should be the assurance of accurate and reproducible results. In this biomarker study, off-the-shelf commercial ELISA kits were used, which are ideal for early studies focused on biomarker discovery. Vendors of commercial ELISA kits provide data on cross-reactivity, limits of quantification, calibration range, assay accuracy and precision, assay linearity, inter/intra-assay precision and level of analyte recovery by performing spike experiments. Accuracy and precision, inter/intra assay precision and spike recovery are often reassessed prior to widespread use to increase the level of confidence of the assay used. To determine suitability for the study in question, these assay parameters can be supplemented with data on frozen storage stability and freeze–thaw stability as was done in this biomarker stability experiment. During the process of biomarker assay development, additional assay parameters may be utilized to increase confidence in assay performance and reproducibility of results [27–29]. Biomarker assay development, however, requires a more unconventional approach due to the biological nature of the analytes of interest. The development and validation of a biomarker assay should follow the context-of-use principle and be developed as fit for purpose. Structural pillars for this development include assay parameters such as parallelism, selectivity, sensitivity and stability [30,31]. Based on data obtained for these parameters, assay acceptance criteria can be set that can differ among various biomarker assays [30]. These factors do not need to be determined in an early phase but are considered an imperative part of biomarker assay development to ensure that the method is fit for purpose.

Following the context-of-use and fit-for-purpose principles, early-phase biomarker assay development could benefit from implementation of assay performance parameters such as acceptance criteria for calibrators (bias and coefficient of variation) and the utilization of quality control (QC) samples. Acceptance criteria for calibrators confirm that the calibration curve is of sufficient quality and also allow for tracking assay performance over time. QC samples can be used in every analytical run as an acceptance control while also providing a way to track plate shifts and assay drifts over time [32,33]. QC samples should be representative of the study samples used (preferably the same matrix) and the analyte concentrations. Preparation of QC samples for biomarker assays are complicated due to the endogenous nature of the analytes, but various options are available. The QC sample concentration should be determined over several runs after which a nominal value can be set. Acceptance criteria can be determined based on the precision of the measurements over several runs [33].

In the field of psychiatry, several studies have identified potential biomarkers to be utilized in a diagnostic setting but results have varied and the road to practical clinical application remains long [2,10,13,14,34–36]. The complex underlying biological background of psychiatric disorders may, however, not be the only explanation for the huge variations in biomarker studies. The vast majority of these biomarker studies utilize LBAs, which are often off-the-shelf research kits and no resources are spent to implement even basic assay performance criteria, such as analyte/body fluid stability. For example, cytokines are often an interesting target for psychiatric biomarker research [37–39]. In these studies, blood samples are regularly used from cohorts in which the samples have already been stored for up to 5 years prior to analysis. A study from 2009 [40] showed that, after long-term storage, cytokines are prone to degradation. Combined with increased variations in ELISA antibody-binding capacity, this leads to increased variations in measured concentrations and reduced reliability as a result of assay performance issues. This not only indicates that stability data on biomarkers is essential for assessing the suitability of samples but also shows that implementation of basic assay performance criteria may be of high value in improving the reliability of study results. The suitability of a biomarker assay may be further improved by incorporating acceptance criteria related to the clinical concentration range of a certain biomarker. Preliminary data (not shown) indicates that, with the application of clinical acceptance criteria based on variability in QC samples (assay performance parameter) relative to overall variability of the clinical samples, a valuable tool to discriminate suitability of a specific biomarker body/fluid combination is gained.

With respect to the current study, three biomarkers in urine (i.e., APOA1, midkine and acetyl-L-carnitine) and three biomarker in serum (i.e., cortisol, prolactin and resistin) are clinically relevant and analytically stable MDD biomarkers. These biomarkers are interesting analytical targets since they remain stable under different temperature and freeze/thaw conditions In addition to being analytically stable, these biomarkers can also be considered clinically

relevant. Lower protein levels of APOA1 are associated with MDD [41]. Midkine contributed significantly in two studies aimed at creating a biodepression score model suitable for discriminating between patients with MDD and healthy individuals [13,14]. Changes in acetyl-L-carnitine levels are associated with mitochondrial dysfunction in MDD [42]. The markers cortisol, prolactin and resistin are also known to play roles in MDD pathophysiology [43–45]. Furthermore, these three biomarkers also contributed significantly to the biodepression score model as described by van Buel *et al.* and Jentsch *et al.* [13,14]. Due to the analytical stability of these biomarkers in combination with their potential clinical relevance, these biomarkers are interesting targets for further assay development toward a clinically relevant diagnostic tool for MDD. Additionally, these biomarkers can be supplemented with other biomarker fluid combinations such as, for example, LTB4 and resistin in urine. These biomarkers were not stable under all time point conditions but still showed good stability at -80, -20, 4, 20 and 37 °C and are therefore interesting targets for further development. On the other hand, cAMP in serum and MPO in serum and urine can be considered unfit biomarker combinations for further development. These biomarkers have been shown to be analytically unstable under different conditions. Overall, the combination of analytically (relatively) stable biomarkers in combination with implementing additional quality assurance parameters will increase the likelihood that a clinically relevant biomarker panel method will be developed in the near future.

In this study, assay performance acceptance criteria were set but additional assay performance parameters could have increased both the reliability of the results and led to a decrease in inconclusive results. Incorporation of duplicate or triplicate analysis in combination with a low coefficient of variation acceptance criteria, for example, could further increase the validity of the measured biomarker concentration by allowing better control of overall analytical variability. Still, singlicate measurements are analytically acceptable, valid data when early method development data concerning accuracy and inter/intra assay precision is available. This view is also supported by recent shifts in the bioanalytical field [46,47]. The addition of low and high QC samples would have provided a level of assay performance measurement to ensure that the assay performed as intended and could have been used as an assay acceptance criteria. The large number of inconclusive results in the literature may also be the result of pipetting errors, which could have been missed due to measuring singlicate samples rather than duplicate. Pipetting errors can occur when using relatively small amounts of sample volume ($\leq$10.0 µl) in some biomarker assays. Dilution errors may also have contributed to the inconclusive results. In the current assay, alpha 1 antitrypsin was diluted at least 1000× through the course of several dilution steps before adding samples to the ELISA plate. Due to the limited amount of information available with respect to the used assay, possible dilution effects that impact assay performance cannot be ruled out. To assess assay performance with extremely dilute samples, a dilution linearity experiment could have been performed [48].

Future analyses of stability of biomarkers and assay performance could also benefit from a change in study design. The current study was performed as a descriptive study with a single measurement of biomarkers in pooled sample material sufficient for testing stability under various conditions. The design of the study did not, however, allow for more advanced statistical measurements such as a regression analysis, which could have been used to detect possible trends among storage conditions and biomarker concentrations/bias. Notably, with an improved design, assay performance indicators are key in generating reliable data for correct interpretation.

Conclusion

Although this study was designed as a descriptive study, it showed that assessing biomarker stability is an essential aspect of biomarkers studies using LBAs as the method of choice. Assessing biomarker stability and assay performance in an early phase not only allows for better method development and optimization of the assay but also enables the researcher to select suitable biomarkers with potential for further development, and to assure that the assay generates valid and reliable measurement data. In this study in which the diagnosis of MDD with a biomarker panel was used as an example, three biomarkers in serum and urine were identified as interesting targets for further development that are stable under different conditions and increase the level of reliability of the measurement data. Basic assay performance and analyte stability parameters should constitute the foundation of matrix-based biomarker assay studies using either commercial or in-house-developed assays. Depending on the status of an assay, different requirements may be necessary. In an early phase, a short investigation of reproducibility may be sufficient. The more weight ascribed to an assay, the more time should be invested to verify that the assay performs adequately and is fit for purpose. By doing so, the level of confidence of reported findings of future biomarker studies might improve compared with previous studies in several medical fields including psychiatry.

Summary points

Determining biomarker stability & assay performance improves reliability of ELISA assay measurements

- Most biomarker studies are measured with off-the-shelf research and development kits with limited information available in terms of biomarker stability and assay performance, which could negatively impact assay measurement outcomes.

Biomarker combinations associated with major depressive disorder show variability in stability profiles & possibly assay performance

- In the current study, 24 biomarkers assays were measured in 32 biomarker/body fluid combinations, showing only six with good stability profiles and assay performance. Others showed various stability profiles potentially related to issues in assay performance.

Developing ELISA-based biomarker assays for psychiatry should follow fit-for-purpose principle

- The development of ELISA-based biomarker assays should focus on the fit-for-purpose principle, implementing in a early phase basic biomarker stability and assay performance indicators followed by expanding stability and assay performance parameters along the way depending on the weight ascribed to an assay.

Limitations & future perspectives

- The current study showed stable biomarker profiles for six biomarkers but no firm conclusions could be determined for the other biomarkers due to the relationship between biomarker stability and assay performance. Basic assay performance parameters were implemented but additional assay-performance parameters could have increased the reliability of results. Future biomarker studies for psychiatry but also other biomarker studies utilizing ELISA assays might benefit from determining biomarker stability and implementing basic assay performance parameters, thereby improving the reliability of measurements allowing for a better interpretation of results and ultimately less variability in results reported in future papers.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/bmm-2023-0416

Author contributions

Conceptualization: MB Meddens and MJ Meddens; methodology: MB Meddens and MJ Meddens; formal analysis: M Jentsch, MB Meddens and MJ Meddens; investigation: M Jentsch and MB Meddens; data curation: M Jentsch, MB Meddens and MJ Meddens; Writing original draft: M Jentsch; review and editing: B van der Strate, R Schoevers and MB Meddens; visualization: M Jentsch and B van der Strate; Supervision: MJ Meddens and R Schoevers; Funding acquisition: MJ Meddens. All authors read and agreed to the published version of the manuscript.

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Competing interests disclosure

MJ Meddens holds stock in Brainscan. Brainscan has filed separate patents covering the diagnostic use of the biomarkers as described in this manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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