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ORIGINAL ARTICLE

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ABSTRACT

Background: Stillbirth remains a major problem in both developing and developed countries. Omics evaluation of stillbirth has been highlighted as a top research priority.

Objective: To identify new putative first-trimester biomarkers in maternal serum for stillbirth prediction using metabolomics-based approach.

Methods: Targeted, nuclear magnetic resonance (NMR) and mass spectrometry (MS), and untargeted liquid chromatography-MS (LC-MS) metabolomic analyses were performed on first-trimester maternal serum obtained from 60 cases that subsequently had a stillbirth and 120 matched controls. Metabolites by themselves or in combination with clinical factors were used to develop logistic regression models for stillbirth prediction. Prediction of stillbirths overall, early (<28 weeks and <32 weeks), those related to growth restriction/placental disorder, and unexplained stillbirths were evaluated.

Results: Targeted metabolites including glycine, acetic acid, L-carnitine, creatine, lysoPCaC18:1, PCaeC34:3, and PCaeC44:4 predicted stillbirth overall with an area under the curve [AUC, 95% confidence interval (CI)] = 0.707 (0.628–0.785). When combined with clinical predictors the AUC value increased to 0.740 (0.667–0.812). First-trimester targeted metabolites also significantly predicted early, unexplained, and placental-related stillbirths. Untargeted LC-MS features combined with other clinical predictors achieved an AUC (95%CI) = 0.860 (0.793–0.927) for the prediction of stillbirths overall. We found novel preliminary evidence that, verruculotoxin, a toxin produced by common household molds, might be linked to stillbirth.

Conclusions: We have identified novel biomarkers for stillbirth using metabolomics and demonstrated the feasibility of first-trimester prediction.

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Stillbirth prediction; metabolomics; nuclear magnetic resonance; mass spectrometry; metabolites

Introduction

Stillbirth remains a problem in both developing and developed countries. In the USA it complicates 6.25 per 1000 deliveries [1]. Overall frequencies for developed and developing countries are 5.3 and 25.5 per thousand deliveries, respectively [2]. In the US, a marked disparity in stillbirth frequency along racial lines raises additional challenges beyond those reflected by the overall frequency [1]. The need for further research in both the prediction and prevention of stillbirth has been previously emphasized by experts in the field [3].

A significant body of literature on the association between maternal first and second trimester blood biomarker concentrations and stillbirth risk exists. Two serum analytes, pregnancy-associated plasma protein-A (PAPP-A), and free beta-human chorionic gonadotropin (β-hCG) [4], have been extensively studied for first trimester stillbirth prediction. Similarly, elevated midtrimester serum markers such as alpha-fetoprotein (AFP) [5] and intact hCG [6], are known to be associated with increased risk of subsequent stillbirth. Additionally, in a study with small number of stillbirth cases ($n = 5$), the imbalance in the ratio of angiogenic/antiangiogenic factors, including placental growth factor (PGF)/soluble vascular endothelial growth factor receptor-1 (sVEGFR-1) ratio at 30–34 weeks of gestation was shown to have a sensitivity of 80%, a specificity of 94%, and a likelihood ratio of a positive test of 14.2 for the identification of a

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Supplemental data for this article can be accessed here.

subsequent stillbirth [7]. In addition to the plasma biomarkers, first-trimester sonographic markers, such as reversed Doppler a-wave in the ductus venosus and nuchal translucency measurement, have also been evaluated for stillbirth prediction [8,9]. A systematic review and meta-analysis concluded, however, that overall, first and second trimester algorithms were not clinically useful for the prediction at this time [10]. Despite these negative findings, early prediction of stillbirth appears scientifically plausible. First trimester placentation abnormality is thought to contribute to some categories of stillbirth [11]. Further, there are potential clinical benefits to very early stillbirth prediction. There is evidence that prophylactic aspirin given before 16 weeks reduces preeclampsia and fetal growth restriction, which are two important causes of stillbirth. The benefits of aspirin prophylaxis were confirmed by the US Preventative Task Force [12]. Indeed aspirin prophylaxis was noted to reduce fetal deaths linked to these disorders [13]. Recently, a multivariable prediction model using a combination of first trimester serum analytes and sonographic markers were evaluated in the general obstetric population. These achieved a sensitivity of 45% and specificity of 90% for stillbirth <34 weeks [8]. Comparable accuracy was achieved for the prediction of stillbirth related to small for gestational age (SGA) status.

The etiology of stillbirth is heterogeneous, which makes it difficult to develop high-performance biomarkers. Hypertension, preeclampsia, fetal growth restriction, and placental dysfunction are significant risk factors for stillbirth [14]. Metabolomics has been effectively used by us and others for both first and second trimester prediction, detection and for evaluating the pathogenesis of these disorders [15,16]. For the above reasons, we performed a metabolomic study to evaluate the feasibility of developing first trimester maternal serum metabolite biomarkers for the prediction of stillbirth and secondly to evaluate the diagnostic accuracy of these markers.

Materials and methods

Details regarding how patients were recruited and how the specimens were handled and stored have been previously published [15]. Briefly, pregnant women from the general obstetric population were recruited as part of an on-going first-trimester study for the prediction of obstetric and fetal complications. Recruitment occurred between 11^{+0} and 13^{+6} weeks gestation at the time of first-trimester aneuploidy screening. The study was approved on 14 March 2003 by the Institutional Review Board of the King's College Hospital, London, England (Project #02-03-033). Patients provided written consent to screening studies for pregnancy disorders. Women with singleton pregnancies were prospectively screened between 2003 and 2012. Patients with known or suspected major structural, chromosomal, or genetic abnormalities that were identified pre- or postnatally were excluded from the study. Stillbirths were defined as losses after 24-week gestation. Overall 60 stillbirth cases and 120 age-matched controls were included in the study. Stillbirth was further subclassified based on gestational age at death i.e. early stillbirth (<32 weeks and <28 weeks), fetal growth restriction/placental-related stillbirth, defined as birthweight <10th percentile or unexplained stillbirth in which there was no identified cause for the loss. Controls consisted of first trimester pregnant women who subsequently delivered at term with no major obstetrical complications.

Maternal history and demographic information were obtained. Ultrasound measurements including crown rump length (CRL) and uterine artery Doppler pulsatility index (UtPI) measurements were performed as previously described [15]. Serum was obtained through venipuncture and taken to the lab within 5 minutes of collection. The sample was left at room temperature for 10–15 minutes. After clotting, the specimens were centrifuged at 3000 rpm for 10 minutes to separate the serum. Serum was aliquoted in 0.5-mL quantities and samples were temporarily stored in a −20 °C freezer and then transferred to a −80 °C freezer within 24 hours.

Targeted and untargeted metabolomic analysis

Both direct injection liquid chromatography coupled with mass spectrometry (DI-LC-MS/MS) and nuclear magnetic resonance (NMR) platforms were used for targeted metabolomic analysis. In addition to targeted metabolomics we also performed untargeted metabolomics analysis using dansylation labeling technique. The targeted and untargeted metabolomics laboratory methods are described in detail in the Supplemental Methods section.

Overall statistical analysis

Our statistical approaches have been previously described in detail [17]. Log-transformation and Pareto-scaling were performed to normalize the metabolite concentration data for following multivariate analysis. Principal component analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were performed to identify distinct metabolite

patterns. To assess the significance of the separations achieved by PLS-DA, permutation testing was performed (using 2000 data resampling steps) to determine the corresponding *p* values. Permutation testing allows one to determine the likelihood that the observed separation between cases and controls on a PLS-DA plot is due to chance. In addition, variable importance in projection (VIP) plots was generated from the PLS-DA data. VIP plot scores estimate the importance of each variable in the projection used in a PLS-DA model which is often used for variable selection. MetaboAnalyst was used to perform univariate analysis as well as PCA and PLS-DA analyses [17]. Custom programs written using the R statistical software package (http://www.r-project.org) and Stata 12.0 (http://www.stata.com) were used to perform all other statistical analyses.

Mean (SD) metabolite concentrations in cases and controls were compared using a two-tailed *t* test. The Mann–Whitney *U* test was performed for non-normal distribution of each metabolite. Logistic regression analysis was used to generate the optimal predictive models for stillbirth prediction. Independent variables and potential confounders were considered in each of the prediction models. These included clinical or demographic factors such as maternal age, ethnicity, parity (multi- versus nulliparous), tobacco use, prior history of chronic hypertension, other medical disorders, conception method (spontaneous versus others), BMI, fetal CRL (crown-rump length) measurement, delta NT (observed – expected based on CRL), UtPI, maternal serum PAPP-A (MoM), and β-hCG (MoM) concentrations. We evaluated the diagnostic accuracy of these prediction models for overall stillbirth as well as several stillbirth subgroups: (i) early occurring <28 weeks, (ii) <32 weeks, (iii) stillbirth related to fetal growth restriction/placental abnormality, and (iv) unexplained stillbirth. Areas under the receiver operating characteristic curve (AUROC or AUC) along with sensitivity and specificity values were calculated. Details of logistic regression modeling and cross-validation (CV) are provided in supplemental methods section.

Results

In our analysis cohort there were a total of 60 stillbirths and 120 controls (Table 1). There were significantly higher frequencies of chronic hypertension and African ethnicity, higher BMI, and UtPI values in the stillbirth group. It should be noted that for the subsequent regression analyses used to develop the predictive algorithms, these and other potential confounders were taken into consideration. Supplemental Figure 1 ranks the important targeted metabolites for overall stillbirth prediction displayed as a VIP plot. Table 2 shows the predictive equations for overall stillbirth, which are based on metabolites that in turn based on targeted methods (NMR and DI-LC-MS/MS) by themselves and also combined with clinical predictors. Untargeted metabolomic analysis generated 2169 "features" or potential metabolites. The performance of untargeted metabolites alone and combined with other potential predictors is also shown in Table 2. Each of the metabolite-based algorithms was found to be statistically significant predictors of stillbirth based on the AUC (95%CI).

Table 1. Demographic and other characteristics, stillbirths versus controls.

	Mean (SD)		
Variables	Stillbirth (overall)	Control	*p* value
Number of cases (%)	60 (33.3)	120 (66.7)	–
PAPP-A MoM	1.180 (0.737)	1.239 (0.755)	.616[a]
β-hCG MoM	1.091 (0.693)	1.306 (0.892)	.077[a]
CRL	63.80 (7.98)	62.62 (7.54)	.330[a]
Maternal age	32.43 (5.65)	30.94 (6.01)	.112[a]
BMI	28.03 (5.51)	25.21 (4.70)	**<.001**[a]
Ethnicity, *N* (%)			
White	21 (35.0)	80 (66.7)	**<.001**[b]
Black	33 (55.0)	24 (20.0)	
Asian	3 (5.0)	13 (10.8)	
Mixed	3 (5.0)	3 (2.5)	
Conception, *N* (%)			
Spontaneous	57 (95.0)	115 (95.8)	1.000[c]
Others	3 (5.0)	5 (4.2)	
Smoking	3 (5.0)	13 (10.8)	.270[c]
Nullipara, *n* (%)	27 (45.0)	58 (48.3)	.673[b]
Chronic hypertension	6 (10.0)	0 (0.0)	**.001**[c]
Delta NT	0.102 (0.320)	0.116 (0.379)	.804[a]
Ut PI	1.962 (0.607)	1.647 (0.524)	**.005**[a]

[a]t-test.
[b]Chi-square test.
[c]Fisher's exact test.
p value < .05.

Similar analyses were performed for early stillbirth (defined as occurring at <32-week gestation). The 22 cases of stillbirths in this category were compared to the 120 controls. Targeted metabolites alone and combined with other clinical predictors significantly predicted early stillbirth defined, as occurring <32 weeks (Table 3). In addition, we explored the performance of the prediction algorithm by redefining early stillbirth as occurring <28 weeks. Targeted metabolites, plus UtPI and BMI similarly achieved good predictive accuracy for early stillbirth thus defined (Table 3). It appears that with the exception of UtPI, conventional markers of stillbirth risk-status (namely clinical, historical, and demographic factors) did not achieve significance in early stillbirth prediction against metabolites. This is in contrast with overall stillbirth prediction where clinical characteristics including UtPI were significant contributors. This could be due to the small number of cases

Table 2. Comparison of diagnostic performance of metabolite algorithms: overall stillbirth prediction.[a]

Model	Metabolites/clinical factors	AUC (95%CI)	Sensitivity	Specificity
NMR + DI-LC-MS/MS	LysoPC a C18:1, PC ae C34:3, PC ae C44:4, Glycine, Acetic acid, L-Carnitine, Creatine	0.707 (0.628–0.785)	66.7%	62.5%
NMR + DI-LC-MS/MS + other factors	MA, BMI, Ut PI, lysoPC a C20:3, Glycine, Acetic acid, L-Carnitine	0.740 (0.667–0.812)	68.3%	68.3%
Untargeted LC-MS metabolites	DAN_1076, DAN_1183, DAN_1808, DAN_2488, DAN_-2626, DAN_2730	0.697 (0.619–0.775)	75.0%	60.8%
Untargeted LC-MS metabolites + other factors	MA, Ethnicity, BMI, Ut PI, DAN_1076, DAN_1808, DAN_2626, DAN_2730	0.722 (0.697–0.846)	70.0%	78.3%

NMR: nuclear magnetic resonance; DI-LC-MS/MS: direct injection liquid chromatography coupled with mass spectrometry; LC-MS: liquid chromatography – mass spectrometry.
("DAN_" for dansylation): refers to the metabolite "features" in the untargeted analyses whose exact chemical identities remain to be determined (see Materials and methods).
[a]60 stillbirth cases and 120 controls.

Table 3. Comparison of diagnostic performance: metabolite–based algorithms for prediction of stillbirth subgroups.

Subgroup	Model	Metabolites/clinical factors[a]	AUC (95%CI)	Sensitivity	Specificity
Early stillbirth (<32 weeks)[b]	NMR + DI-LC-MS/MS	PC ae C44:3, Acetylornithine, t4-OH-Pro	0.608 (0.477–0.740)	68.2%	50.0%
	NMR + DI-LC-MS/MS + other factors	Ut PI, Acetylornithine, t4-OH-Pro	0.730 (0.616–0.884)	72.7%	73.3%
	Untargeted LC-MS metabolites + other factors	Ut PI, DAN_1033, DAN_1039, DAN_1654, DAN_1808, DAN_1833	0.860 (0.793–0.927)	81.8%	75.8%
Early stillbirth (<28 weeks)[c]	NMR metabolites	Tyrosine, t4-OH-Pro, Spermidine	0.721 (0.604–0.837)	66.7%	68.3%
	DI-LC-MS/MS metabolites	Tyrosine, Creatine, Glycerol	0.656 (0.460–0.852)	60.0%	75.0%
	NMR + DI-LC-MS/MS + other factors	BMI, Ut PI, Creatine, Glycerol	0.773 (0.636–0.910)	80.0%	73.3%
FGR/placenta-related Stillbirth[d]	NMR + DI-LC-MS/MS	PC ae C44:3, SM C26:1, t4-OH-Pro, 2-Hydroxybutyrate	0.727 (0.622–0.831)	73.3%	59.2%
	NMR + DI-LC-MS/MS + other factors	BMI, Ut PI, t4-OH-Pro, Serotonin, 2-Hydroxybutyrate	0.784 (0.702–0.865)	86.7%	60.0%
	Untargeted LC-MS metabolites + other factors	BMI, Ut PI, DAN_1076, DAN_1126, DAN_1519, DAN_1682, DAN_1954, DAN_2119, DAN_3682	0.855 (0.789–0.922)	86.7%	73.3%
Unexplained stillbirth[e]	NMR + DI-LC-MS/MS	lysoPC a C20:3, Glycine, Acetic acid	0.694 (0.579–0.809)	70.0%	70.8%
	NMR + DI-LC-MS/MS + other factors	MA + Ethnicity (Black =1), lysoPC a C20:3	0.748 (0.652–0.843)	73.3%	63.3%
	Untargeted LC-MS metabolites + other factors	BMI, DAN_23, DAN_179, DAN_2138, DAN_2626, DAN_2951, DAN_3257, DAN_3385	0.785 (0.676–0.893)	73.35	85.8%

NMR: nuclear magnetic resonance; DI-LC-MS/MS: direct injection liquid chromatography coupled with mass spectrometry; LC-MS: liquid chromatography – mass spectrometry.
DAN_ refers to the metabolites in the untargeted analyses whose exact chemical identities remain to be determined.
[a]Clinical factors: These potential confounders were considered (a) PAPP-A MoM + β − hCG MoM + CRL + MA + BMI, (b) Ethnicity (black: 1 versus others: 0), (c) Conception (spontaneous: 1 versus others: 0), (d) Smoking, (e) Parity (Multi: 1 versus Nullipara: 0); (f) Chronic hypertension (CH: 1 versus normal: 0), (g) Delta NT, (h) Uterine artery PI.
[b]Analysis limited to the Discovery Group (early stillbirth <32 weeks)–22 cases and 120 controls.
[c]Analysis limited to the Discovery Group (early stillbirth <28 weeks)–15 cases and 120 controls.
[d]Analysis limited to the Discovery Group (FGR/placenta-related)–30 cases and 120 controls.
[e]Analysis limited to the Discovery Group (unexplained)–15 cases and 120 controls.

in the early stillbirth subgroup, however, this still suggests that metabolite markers were more robust stillbirth predictors.

Placental impairment is known to be an important factor in the development of stillbirths including unexplained stillbirths [18]. We therefore evaluated the efficacy of metabolite biomarkers in stillbirth prediction for the subgroup associated with placental disorder (Table 3). Targeted metabolite markers by themselves achieved a 73.3% sensitivity for placental-related stillbirth with a statistically significant AUC = 0.727 (0.626 − 0.831). Similarly, untargeted metabolites combined with clinical markers also achieved an AUC = 0.855 (0.789 − 0.922) with a sensitivity of 86.7% for placental-related stillbirth prediction. Additionally, metabolites alone or in combination with clinical risk factors significantly predicted unexplained stillbirth which is the most common and the most troubling form of stillbirth given the late gestational age at which they occur (Table 3).

Finally, we have been able to putatively identify the chemical identity of three untargeted metabolites in this analysis. These are verruculotoxin, 2-aminomuconic acid, and 24-nor-5 beta-cholane-3 alpha, 22, 23-tetrol (Supplemental Table S1). These metabolites by themselves or combined with clinical markers significantly

predicted stillbirth overall (Supplemental Table S2), early stillbirth <32 weeks (Supplemental Table S3), and unexplained stillbirth (Supplemental Table S4).

Discussion

The main findings of our study were that first-trimester metabolites from untargeted and targeted metabolomic analysis (NMR and mass spectrometry (MS) platforms) either by themselves or in combination with commonly used clinical risk factors were statistically significant predictors of stillbirth overall. Moreover, metabolite biomarkers also predicted important subcategories of stillbirth including early, unexplained, and those associated with fetal growth restriction/placental dysfunction. NMR-based metabolomics was previously reported to predict fetal growth restriction at first trimester by using maternal urine metabolites [19]. This supports our results of first trimester-targeted metabolomics predicting stillbirth that is associated with growth restriction/placental dysfunction. Further, our novel approach of combining NMR and MS platforms also allowed us to predict clinically challenging subcategories of stillbirth including the ones that are unexplained.

While targeted metabolomics is very accurate, highly reproducible and quantitative, it is not always ideal for characterizing a broad "swath" of the metabolome. Indeed, a typical targeted metabolomics experiment can only generate data for, a very small fraction (1–5%) of the total metabolome [20]. On the other hand, untargeted (otherwise called "unbiased" or "undirected") metabolomics is capable of generating data for a far larger fraction of the metabolome, with up to 8000 "features" being detected, depending on the experimental approach used. In untargeted analysis, thousands of MS peaks or "features" such as *m/z* (mass-to-charge ratios) and retention times are initially generated. The challenge in untargeted metabolomics is to ultimately identify these features by finding/verifying the metabolite matches in published databases or using available libraries of internal standards. The ultimate objective in untargeted metabolomic analysis is to maximize the breadth of metabolite or feature "space" to better characterize disease phenotype. This allows one to generate novel biomarker leads that could not otherwise be found via targeted metabolomics. Therefore, we utilized untargeted analysis and putatively identified three novel potential biomarkers for stillbirth prediction, namely verruculotoxin, 2-aminomuconic acid, and 24-nor-5 beta-cholane-3 alpha, 22, 23-tetrol. Together, they effectively predicted stillbirth overall, stillbirth <32 weeks and unexplained stillbirth. To the authors' knowledge this has not been previously reported in the stillbirth literature. Two of the above markers have reported immunoregulatory function. The 24-nor-5-beta-cholane-3-alpha,22,23-tetrol has been included in a list of bile acids and bile alcohols that are agonists for TGR5 (G protein-coupled plasma membrane receptor) and postulated to mediate immunomodulation as well as having possible roles in glucose homeostasis and energy expenditure in response to thyroid hormone [21]. Aminomuconic acid is generated by the oxidative degradation of tryptophan. Tryptophan metabolites including kynurenine play critical roles in feto-maternal immune tolerance and induce arterial relaxation. Kyurenine in cancer cells promote immune tolerance, angiogenesis, and cancer cell invasion. Tryptophan depletion can reduce fetal growth and potentially trigger immune rejection [22].

Verruculotoxin is one of the so-called "tremorigenic" (inducing tremors) mycotoxins that affect the CNS of vertebrates including humans. Mycotoxins are a large, diverse group of small molecular weight substances produced by molds. Most are produced by common species of mold such as Aspergillus and Penicillium and are toxic in low concentrations [23]. The main sources of risk to exposure are in the developing countries as a result of mold overgrowth on food/grains due to high humidity. Human mycotoxin-related diseases are often not recognized my medical personnel. In the USA, risk groups include inner city residents who are exposed to higher levels of household mold while women in agrarian communities in India are exposed to contaminated grains [24]. Both groups have the increased rates of stillbirths. The authors could not locate any existing literature linking mold and stillbirth. Ergot alkaloids are mycotoxins and their relationship to miscarriage has long been recognized. Extensive medical problems were previously reported among family members occupying a house with mold overgrowth post flooding [25]. The mother was pregnant during this period and the newborn had total body flare (of the skin). Mycotoxins were identified in the placenta, umbilical cord and in breast milk after birth while amniotic fluid was not tested.

High priority research themes for stillbirth for high-income countries include improving antenatal screening for stillbirth risk including detecting fetal growth restriction. The need to use systems biology-based approaches such as metabolomics has been specifically recommended [26]. Investigation of the early pregnancy environment has been prioritized given the role of early placental development in late complications [27]. Particularly for stillbirth, analysis before demise is

important for elucidating disease pathogenesis. Together, these justify the use of first trimester metabolomics.

Maternal age, black ethnicity, smoking, and BMI are known to correlate with stillbirth [8,28,29]. Generally, these did not appear to perform as well as metabolites as for stillbirth prediction. However, depending on the stillbirth category, demographic/clinical factors did contribute to prediction. Recently it has been proposed that menstrual factors remote from pregnancy itself might govern the adequacy of placentation and the future development of placental-related pregnancy disorders [30]. This gives further scientific plausibility in the early (i.e. first trimester) evaluation for the prediction of placental complications including stillbirths. The potential clinical advantages of early prediction could include the opportunity for timely counseling, antenatal testing, and early intervention and even prophylaxis [13]. There is evidence that increased awareness and vigilance among caregivers and parents about decreased fetal movement could reduce stillbirth risk [31]. The potential benefits of early screening should be tempered by the realization that development of effective screening does not automatically translate into effective management strategies. On the other hand, effective first-trimester prophylaxis is particularly attractive as it could potentially reduce stillbirth without increasing iatrogenic morbidities.

The strengths of this study include the fact that the data and specimens were collected prospectively in an average-risk population for the purposes of first-trimester prediction of pregnancy complications, including stillbirth, making the results more relevant to the general population. Given the absolute frequency of stillbirths in the general British pregnant population, approximately 5/1000, a relatively large number of cases were screened ~12,000 to perform our study. We also looked at early-prediction of important subcategories of stillbirth, including cases occurring remote from screening, and were able to generate novel pathogenic insights. A potential limitation of this study, however, is that analyses performed later in gestation or even at near term could improve the predictive accuracy and provides better pathogenic information into the late subgroup.

In conclusion, we report novel metabolomic markers for the first-trimester prediction of subsequent stillbirths. Overall, metabolites appeared to remain significant even when we took commonly recognized clinical risk factors into consideration. Validation studies in larger and different populations appear warranted.

Disclosure statement

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