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Review article

Blood homocysteine levels in children with autism spectrum disorder: An updated systematic review and meta-analysis

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ABSTRACT

Results of studies on peripheral blood levels of homocysteine (Hcy) in children with autism spectrum disorder (ASD) are inconsistent, and conclusions from two previous meta-analyses on this subject published in 2012 are already outdated. Therefore, we conducted an updated systematic review and meta-analysis to quantitatively summarize the peripheral blood Hcy data in children with ASD compared with healthy controls (HC). We searched PubMed, EMBASE, PsycINFO, PsycARTICLES, Web of Science, and Cochrane Library databases from inception to September 2019 for eligible studies, with no language restriction. Using random-effects model, we computed summary statistics. Thirty-one studies (3304 participants including 1641 cases) were included. The pooled results showed that the peripheral blood Hcy levels were significantly elevated in children with ASD when compared to HC (Hedges's $g = 0.56$, 95% CI = 0.36 to 0.76, $P < 0.001$). By sensitivity analyses, we confirmed that our results were quite robust. Additionally, no publication bias was observed in this meta-analysis. In conclusion, our study support the association of increased circulating Hcy levels with ASD in children, and the involvement of Hcy in the occurrence of ASD. However, in view of the significant between-study heterogeneity, the conclusions should be interpreted cautiously and more investigation is required.

1. Introduction

Autism spectrum disorder (ASD) includes three formerly independent but highly relevant diseases: autistic disorder (AD), Asperger's syndrome (AS), and pervasive developmental disorder-not otherwise specified (PDD-NOS) (American Psychiatric Association, 2013), which is becoming more common. Based on the latest report, there was overall significant increase in the prevalence of ASD among children in the United States between 2009 and 2017 (Zablotsky et al., 2019). However, although growing evidence suggests that ASD is caused by genetic susceptibilities and environmental factors (Schendel et al., 2014), the exact etiologies of ASD have still not been elucidated.

Numerous studies have attempted to identify potential biomarkers in autistic children to advance the diagnosis, treatment and prognosis of ASD (Bjorklund et al., 2018). Thereinto, the peripheral blood levels of homocysteine (Hcy), a sulfur-containing and non-protein amino acid derived from methionine cycle and essential for activated methyl transfer and transsulfuration pathway (Fig. 1), have been widely investigated by researchers (Fuentes-Albero and Cauli, 2018). Nevertheless, the studies examining blood Hcy levels in children with ASD produced conflicting results. For example, some studies revealed that autistic children had elevated levels of blood Hcy compared with healthy controls (HC) (Cai et al., 2016; Wang et al., 2016), whereas others found no correlation between blood Hcy levels and childhood autism (Adams et al., 2007; Main et al., 2015). More than that, several articles reported significantly decreased blood Hcy levels in children with ASD (James et al., 2004; James et al., 2006). In such a context, it is difficult to reach consistent conclusion on the specific blood Hcy levels in children with ASD by a single study.

Assessment of the relationship between blood Hcy levels and children with ASD may help to explain the roles of this non-essential amino acid in ASD and eventually to distinguish possible links between certain biochemical changes and the pathophysiological alterations occurred to ASD (Fuentes-Albero and Cauli, 2018). Furthermore, abnormal deficit or accumulation of Hcy in blood can provide useful information to develop targeted intervention strategies for reconstructing metabolic balance and potentially ameliorate clinical symptoms of ASD (Melnyk et al., 2012). Thus, were the levels of blood Hcy indeed abnormal in children with ASD, it could be very helpful in the diagnosis and treatment of ASD.

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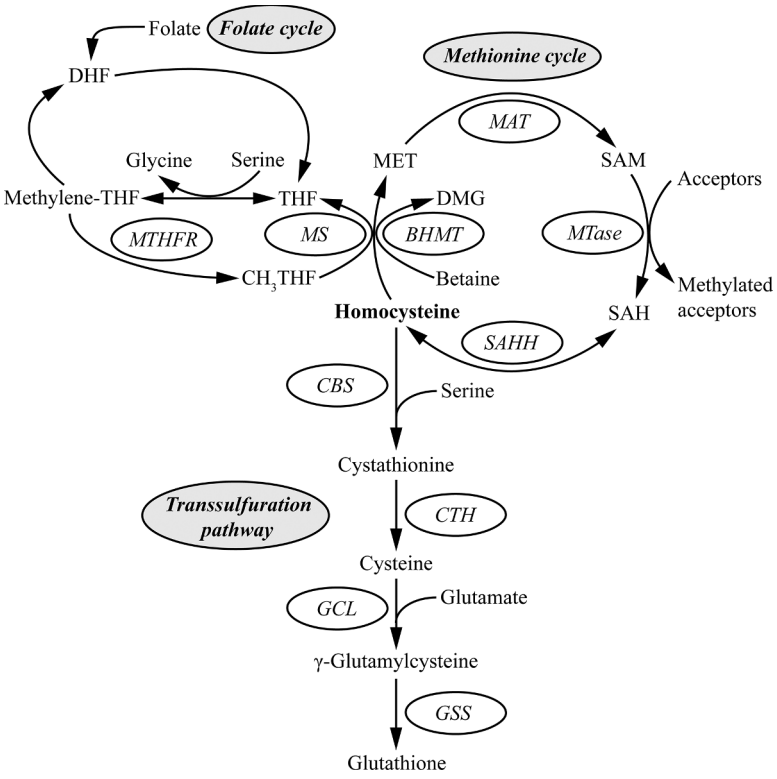

Fig. 1. Metabolic pathways and metabolites related to homocysteine. Abbreviations: BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine beta synthase; CTH, cystathionase; DHF, dihydrofolate; DMG, dimethylglycine; GCL, glutamate-cysteine ligase; GSS, glutathione synthetase; MAT, methionine adenosyltransferase; MET, methionine; MS, methionine synthase; MTase, methyltransferase; MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

Two previous meta-analyses found no statistically significant difference in blood Hcy levels between children with ASD and HC (Frustaci et al., 2012; Main et al., 2012). Nevertheless, all of them were published in 2012 (with literature search until 2011) and each meta-analysis included only 7 studies evaluating blood Hcy levels. Since then, more than 20 articles have additionally become available and added to the evidence base of the association of blood Hcy levels with ASD in children. Therefore, the conclusions of the two previous meta-analyses are already outdated. Accordingly, an updated systematic review and meta-analysis is timely and necessary to summarize the existing evidence from relevant studies (including recently published ones), in order to investigate peripheral blood Hcy alternations in children with ASD and deepen our understanding of the complex biological properties of ASD.

2. Methods

This systematic review and meta-analysis complied with the guideline of Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) (Moher et al., 2009). We did not register or publish our study protocol.

2.1. Search strategy

Two independent researchers (B.-Q.G. and H.-B.L.) searched the PubMed, EMBASE, PsycINFO, PsycARTICLES, Web of Science, and Cochrane Library databases from inception to September 16, 2019, with no language restriction. Literature search was conducted in [All Fields] (PubMed; Web of Science), [All fields] (EMBASE), or [All Text] (PsycINFO; PsycARTICLES; Cochrane Library) using the combination of key terms: ("homocysteine" OR "2-amino-4-mercaptobutyric acid" OR "Hcy" OR "tHcy" OR "hyperhomocysteinemia" OR "HHcy" OR "amino acid*" OR "antioxidant*" OR "oxidant*" OR "oxidati*" OR "redox" OR "transsulfuration" OR "trans-sulfuration") AND ("autism" OR "autistic" OR "pervasive developmental disorder*" OR "Asperger*"). The reference lists of relevant articles were reviewed to find extra studies. In addition, the publications from authors of included articles were also checked.

2.2. Inclusion and exclusion criteria

The eligibility criteria were based on the PECO (Population, Exposure, Comparator, and Outcome) statement. Observational studies performed in children (P) suffering from ASD (E) compared to healthy control individuals (C) to identify the differences in their peripheral blood levels of Hcy (O) were included. Studies were excluded for the following reasons: *a*) no desired data on blood Hcy levels in children with ASD, *b*) no HC, *c*) other biological specimen, *d*) review or meta-analysis, and *e*) partially or completely overlapping samples with other one. In the last case, only the article reporting on the largest study population or the latest published paper was included. Thus, in two studies (Adams et al., 2011a,b) with partially overlapping samples, the one (Adams et al., 2011b) giving the information on the largest sample size was retrieved; in two studies (Al-Farsi et al., 2013; Ali et al., 2011) investigating the same samples, the one (Al-Farsi et al., 2013) published in 2013 was incorporated. In addition, the data used in 4 articles (Howsmon et al., 2017; Li et al., 2018; Melnyk et al., 2012; Vargason et al., 2017) came from the Arkansas Children's Hospital Research Institute's autism IMAGE (Integrated Metabolic And Genomic Endeavor) study, only the article providing the biggest data quantity (Howsmon et al., 2017) was included.

2.3. Data extraction

Two independent researchers (B.-Q.G. and S.-B.D.) extracted the data from all eligible studies using a piloted extraction form. Sample sizes and Hcy levels (means ± SDs) were retrieved as primary outcomes. The standard error (SE) was converted to SD applying the formula: $SD = SE \times \sqrt{(\text{sample size})}$. If data were expressed as medians and the inter-quartile ranges (IQRs), they were converted to means and SDs using the methodology of Wan et al. (2014). When desired data (means

and SDs/SEs, medians and IQRs) were unavailable or statistical measures were not specified, *P*-values were extracted. If data were expressed graphically, the GetData Graph Digitilizer software (version 2.25) was used to acquire the numerical values. Information on author, year of publication, country, age, gender (% of male), matching, ASD subtype, diagnostic method, blood component, fasting status, and analytical technology (unit) was also retrieved. Any disagreements were resolved by group discussion among all the investigators to reach a consensus.

2.4. Quality assessment

Two independent reviewers (B.-Q.G. and H.-B.L.) evaluated the methodological quality of included studies using the Newcastle–Ottawa Scale (NOS) (Wells et al., 2009), which, despite its inherent limitations (Deeks et al., 2003; Stang, 2010), is recommended by the Cochrane Collaboration (Higgins and Green, 2011) and extensively applied by a large amount of research. According to previous standard (Alobaidi et al., 2018), we judged that a study with a score of ≥ 8 is of high quality, 5 to 7 is of medium quality, and ≤ 4 is of low quality.

2.5. Statistical analyses

We used the Comprehensive Meta-Analysis software (version 3.0; Biostat Inc, Englewood, NJ, USA) to analyze the data. Most of the effect sizes were produced by sample sizes and blood Hcy levels (means ± SDs), while the remainders were generated by sample sizes and *P*-values (Qin et al., 2016; Qin et al., 2017). When *P*-value was reported as inequality rather than specific value, the *P*-value was rounded down to the closest decimal value to allow compatibility with the meta-analysis software package (Qin et al., 2016; Qin et al., 2017). $P = 0.000$ means $P < 0.0005$ (Friedman et al., 2014; Wouters and Noyez, 2004). The effect sizes were computed as standardized mean differences in blood Hcy levels between ASD patients and healthy individuals, which converted to Hedges's *g* that not only adjusts the impacts of small sample sizes, but also allows for comparison of effect sizes with diverse measurement scales (Munkholm et al., 2016; Qin et al., 2017). The 95% confidence interval (CI) was simultaneously calculated to evaluate the statistical difference of the pooled effect size. We used the random-effects model to compute summary statistics because of the anticipated heterogeneity from the included studies (Higgins and Green, 2011; Qin et al., 2016). The study weight assigned to each study under this model is the inverse of total variance that is equal to between-study variance (τ^2) plus within-study variance (Higgins and Green, 2011; Qin et al., 2016).

Sensitivity analyses were undertaken, first by leave-one-out analysis (serially removing one study at a time) to confirm whether a single study had influence on the overall effect estimate (Qin et al., 2016; Qin et al., 2017), then by excluding the studies with data conversion and rounded *P*-value, unclear fasting status, or medium quality to assess the impacts of these studies on the result of the meta-analysis. In addition, we performed subgroup analyses based on blood component and ASD subtype. As a rule, there were at least 3 studies available in each subgroup (Song et al., 2019). Univariable meta-regression analyses using the Method of Moments and *Z*-distribution (Munkholm et al., 2016) were performed with moderator variables that were decided *a priori*, including mean age, gender, sample size, differences in mean age/gender/sample size between cases and controls, and total NOS score. The proportion of between-study variance explained by covariates was calculated as R^2 analog (Munkholm et al., 2016). Heterogeneity was quantified by the I^2 statistics (the ratio of between-study variance in total variance), and $P < 0.10$ was considered statistically significant (Higgins and Green, 2011; Higgins et al., 2003). I^2 indices of 25%, 50%, and 75% indicated low, moderate, and high levels of heterogeneity, respectively (Higgins et al., 2003; Qin et al., 2016). Statistical significance was set at $P < 0.05$ unless otherwise noted.

2.6. Publication bias

Publication bias was judged by visual inspection of funnel plot depicting the effect sizes against their precisions (reciprocals of SEs), followed by the Begg's (Begg and Mazumdar, 1994) and Egger's (Egger et al., 1997) tests. The classic Fail-safe N analysis (Soeken and Sripusanapan, 2003) was also employed to evaluate publication bias, which calculates the number of missing studies that could be added to the meta-analysis to render the combined effect size statistically insignificant.

3. Results

3.1. Study identification

A total of 5670 records were obtained after literature search, which was supplemented by 9 records identified from other sources (Fig. 2). After deleting duplicates, 3627 records were screened by going through the titles and abstracts and 3570 records were removed. Subsequently, 26 full-text records were excluded for different reasons (Table S1). Finally, 31 studies (Adams et al., 2007; Adams et al., 2011b; Al-Farsi et al., 2013; Altun et al., 2018; Bala et al., 2016; Cai et al., 2016; Han et al., 2015; Hodgson et al., 2014; Howsmon et al., 2017; James et al., 2004; James et al., 2006; James et al., 2009; Main et al., 2015; Ning et al., 2019; Parellada et al., 2012; Paşca et al., 2006; Paşca et al., 2009; Pastural et al., 2009; Shaik Mohammad et al., 2016; Suh et al., 2008; Sun et al., 2016, 2018, Sun Y et al., 2018; Tu et al., 2012, 2013; Wang et al., 2016; Yan et al., 2015; Yektas et al., 2019; Zhang et al., 2015; Zhou and Li, 2018; Zou et al., 2019) were selected for inclusion in the meta-analysis (Fig. 2).

3.2. Study characteristics

The details of the included 31 studies were displayed in Table 1. Two studies were conducted in Australia (Adams et al., 2007; Main et al., 2015), 13 in China (Cai et al., 2016; Han et al., 2015; Ning et al., 2019; Sun et al., 2016; Sun C et al., 2018; Sun Y et al., 2018; Tu et al., 2012, 2013; Wang et al., 2016; Yan et al., 2015; Zhang et al., 2015; Zhou and Li, 2018; Zou et al., 2019), 1 in India (Shaik Mohammad et al., 2016), 2 in Oman (Al-Farsi et al., 2013; Hodgson et al., 2014), 2 in Romania (Paşca et al., 2006; Paşca et al., 2009), 1 in Spain (Parellada et al., 2012), 3 in Turkey (Altun et al., 2018; Bala et al., 2016; Yektas et al., 2019), and 7 in the United States (Adams et al., 2011b; Howsmon et al., 2017; James et al., 2004; James et al., 2006; James et al., 2009; Pastural et al., 2009; Suh et al., 2008). Twenty-nine articles were published in English language; the remaining 2 articles (Sun C et al., 2018; Sun Y et al., 2018) were published in non-English language (Chinese). Studies were published between 2004 and 2019, and involved a total of 3304 children (1641 cases), ranging from 21 (Paşca et al., 2006) to 276 (Shaik Mohammad et al., 2016) participants per study. The vast majority of studies provided information on mean age; the mean age ranged from 3.46 (Tu et al., 2012) to 10.0 (Adams et al., 2011b) years old for ASD patients, from 3.69 (Cai et al., 2016) to 11.0 (Adams et al., 2011b) years old for HC, and from 3.69 (Cai et al., 2016) to 10.44 (Adams et al., 2011b) years old for all participants. In 1 article (Adams et al., 2011b), Hcy and its oxidized form (homocystine) were measured, both of which belong to total Hcy (Mudd and Levy, 1995). One study reported 2 datasets for distinct blood components (peripheral blood mononuclear cells [PBMC] and plasma) (Suh et al., 2008), 1 study contained 3 datasets for ASD subtypes (AD, AS, and PDD-NOS) (Paşca et al., 2009), and each of 2 studies (Howsmon et al., 2017; Main et al., 2015) presented 2 datasets using diverse control individuals (unrelated children and unaffected siblings).

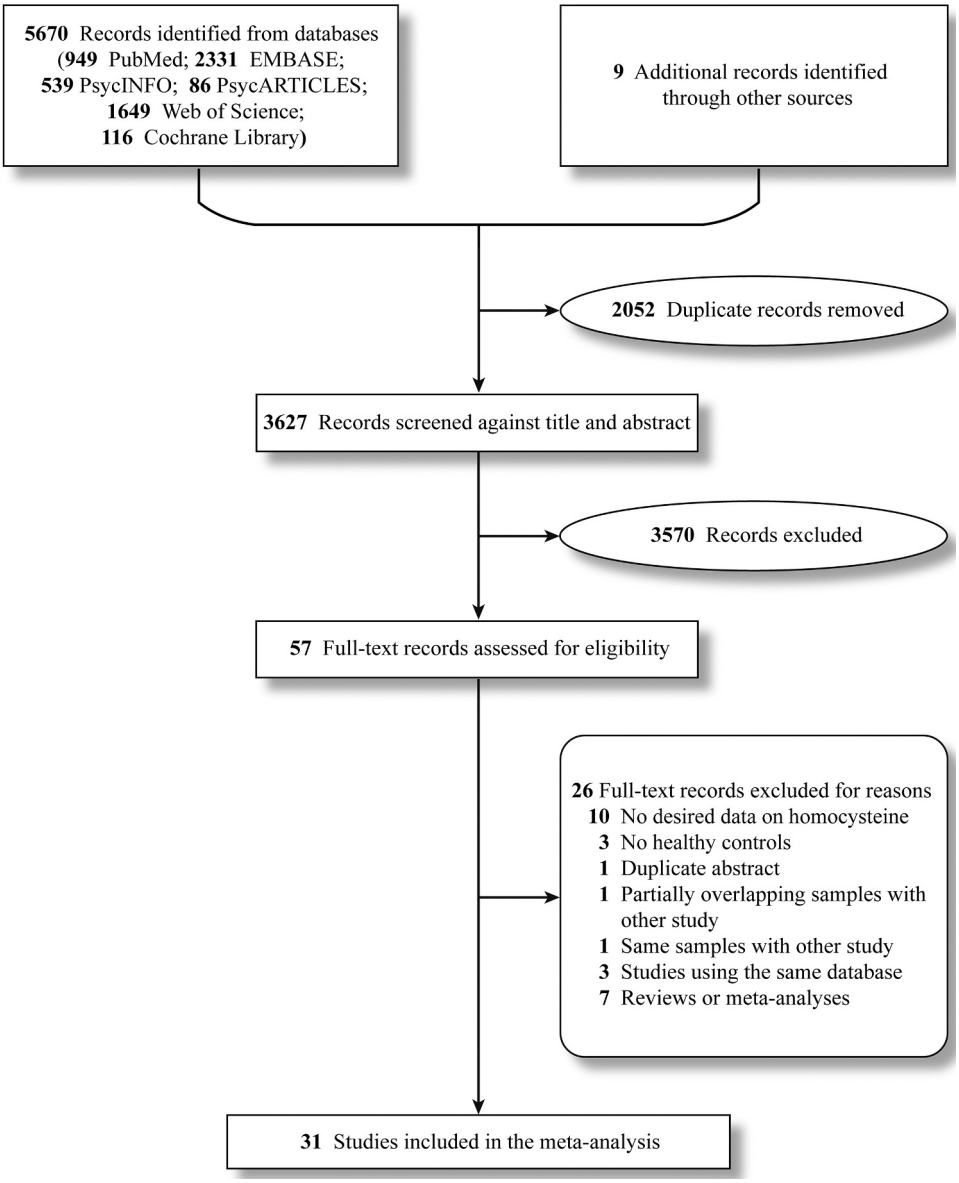

Fig. 2. Flowchart of literature search and selection.

3.3. Quality assessment

The methodological quality of the included studies was adequate overall, with a mean (SD) score of 7.61 (1.05) on the NOS. Twenty studies were of high quality, while 11 studies were of medium quality (Adams et al., 2007; Altun et al., 2018; Hodgson et al., 2014; James et al., 2004; James et al., 2009; Parellada et al., 2012; Paşca et al., 2006; Pastural et al., 2009; Suh et al., 2008; Tu et al., 2012; Yektas et al., 2019) (Table S2).

3.4. Overall analysis (blood Hcy levels in children with ASD)

A random-effects meta-analysis was conducted on the extracted 31 studies (3304 children including 1641 cases). The pooled results showed that peripheral blood levels of Hcy were significantly elevated in children with ASD when compared with the corresponding values in HC (Hedges's g = 0.56, 95% CI = 0.36 to 0.76, $P < 0.001$), with the effect estimates in individual studies ranging from -0.61 (95% CI = -1.22 to -0.01, P = 0.048) (Bala et al., 2016) to 2.62 (95% CI = 2.09 to 3.14, $P < 0.001$) (Cai et al., 2016) (Fig. 3). Nevertheless, high level of heterogeneity was observed (I^2 = 87.00%, τ^2 = 0.30, $P < 0.001$).

3.5. Sensitivity analyses

Leave-one-out analysis indicated that no single study disproportionately impacted the statistically significant difference in blood Hcy levels between autistic children and HC (Fig. S1), confirming that the results of overall analysis were robust. Additionally, no individual study was able to fully explain the between-study heterogeneity, suggesting that the significant heterogeneity could not be attributable solely to any single study. When the studies with data conversion and rounded P-value (Altun et al., 2018; Main et al., 2015; Wang et al., 2016; Yektas et al., 2019; Zhang et al., 2015) were removed, the blood Hcy levels were still higher in children with ASD as compared to HC (Hedges's g = 0.49, 95% CI = 0.27 to 0.71, $P < 0.001$) (Table 2). Further exclusion of the studies with uncertain fasting status (Adams et al., 2007; Al-Farsi et al., 2013; Altun et al., 2018; Bala et al., 2016; Hodgson et al., 2014; Paşca et al., 2006; Suh et al., 2008;

Table 1
Characteristics of the studies included in the systematic review and meta-analysis.

Author, year (country)	No. of participants (cases/controls)	Age (years; cases/controls)	Gender (% of males; cases/controls)	Matching	ASD subtype	Diagnostic method	Blood component	Homocysteine levels (mean ± SD; cases/controls)	Analytical technology (unit)
Adams et al. 2007 (Australia)	17/16	Range: 2–16/NR	NR/NR	NR	AD	Previous diagnosis of AD (DSM-IV) ascertained by a qualified medical specialist. Diagnosis of autism status was verified using a semi-structured interview and the ADOS	Serum	5.6 ± 1.649/5.2 ± 1.6	LBAs (μmol/L)
Adams et al. 2011b (United States)	55/44	Mean ± SD: 10.0 ± 3.1/11.0 ± 3.1	89.0/89.0	Age, gender, and geographical distribution	ASD	Prior diagnosis of autism, PDD-NOS, or Asperger's by a psychiatrist or similar professional, with written verification	Plasma (fasting)	$P = 0.006$	HPLC-MS/MS (μmol/dl)
Al-Farsi et al. 2013 (Oman)	40/40	Mean ± SD: 4.8 ± 0.3/4.8 ± 0.3	50.0/50.0	Age, gender, ethnicity, and sociodemographic status	ASD	According to the CARS, which was developed using gold-standard criteria based on the DSM-IV-TR	Serum	$P = 0.004$	HPLC-ECD (μmol/L)
Altun et al. 2018 (Turkey)	60/45	Mean ± SD: 5.8 ± 2.7/6.7 ± 2.5	86.7/80.0	Age, gender, and/or season of blood collection	ASD	Using the DSM-IV-TR; CARS was used to determine ASD severity	Serum	$P < 0.001$	ELISA (μmol/L)
Bala et al. 2016 (Turkey)	21/21	Mean ± SD: 8.38 ± 5.35/9.80 ± 4.25	61.9/48.0	Age, gender	ASD	According to both DSM-5 and DSM-IV-TR; CARS was used to categorize participants	Plasma	2.40 ± 1.39/3.48 ± 2.01	IEC using Aracus amino acid analyzer (NR)
Cai et al. 2016 (China)	51/51	Mean ± SD: 3.69 ± 1.24/3.69 ± 1.24	82.4/82.4	Age, gender	ASD	According to clinical manifestations and the DSM-IV	Plasma (fasting)	17.9 ± 1.5/14.2 ± 1.3	LC-MS/MS (μmol/L)
Han et al. 2015 (China)	50/50	Mean ± SD: 7.64 ± 4.22/8.38 ± 3.45	78.0/78.0	Age, gender	ASD	By pediatric psychologists using CARS	Serum (fasting)	5.5 ± 2.7/4.5 ± 1.6	HPLC (μmol/L)
Hodgson et al. 2014 (Oman)	27/27	Mean: 5.3/5.5	81.5/74.1	Age, gender	ASD	According to the CARS, which was developed using gold-standard criteria based on the DSM-IV-TR	Serum	6.642 ± 3.352/4.008 ± 2.411	HPLC-ECD (μmol/L)
Howsmon et al. 2017_1 (United States) (cases vs. unrelated neurotypical children)	83/76	Range: 3–10/3–10	NR/NR	Age	ASD	By the DSM-IV, the ADOS, and/or the CARS	Plasma (fasting)	5.066 ± 1.254/4.760 ± 1.042	HPLC-ECD (μmol/L)
Howsmon et al. 2017_2 (United States) (cases vs. unaffected siblings)	83/47	Range: 3–10/3–10	NR/NR	Sibling	ASD	By the DSM-IV, the ADOS, and/or the CARS	Plasma (fasting)	5.066 ± 1.254/4.483 ± 1.025	HPLC-ECD (μmol/L)
James et al. 2004 (United States)	20/33	Mean ± SD: 6.4 ± 1.5/7.4 ± 1.3	70.0/NR	Race	AD	Based on the DSM-IV and by a diagnostic interview conducted by a developmental pediatrician	Plasma (fasting)	5.8 ± 1.0/6.4 ± 1.3	HPLC-ECD (μmol/L)
James et al. 2006 (United States)	80/73	Mean ± SD: 7.3 ± 3.2/10.8 ± 4.1	89.0/NR	Age	ASD	By independent specialists not associated with the study using criteria defined by the DSM-IV, the ADOS, or the CARS	Plasma (fasting)	5.7 ± 1.2/6.0 ± 1.3	HPLC-ECD (μmol/L)
James et al. 2009 (United States)	40/42	Mean ± SD: 4.8 ± 0.8/4.5 ± 0.9	82.0/NR	Age	AD	By the DSM-IV and the CARS	Plasma (fasting)	4.8 ± 1.8/5.0 ± 1.2	HPLC-ECD (μmol/L)
Main et al. 2015_1 (Australia) (cases vs. unrelated neurotypical children)	35/25	Mean ± SD: 7.57 ± 2.92/8.56 ± 2.84	94.3/92.0	Age, gender, and race	AD	Using the DSM-IV-TR; CARS was used to confirm diagnosis and AD severity	Serum (fasting)	7.12 ± 2.36/5.90 ± 1.57	Direct competitive CLIA kit (μmol/L)
Main et al. 2015_2 (Australia) (cases vs. unaffected siblings)	35/27	Mean ± SD: 7.57 ± 2.92/9.31 ± 4.81	94.3/55.6	Sibling, race	AD	Using the DSM-IV-TR; CARS was used to confirm diagnosis and AD severity	Serum (fasting)	7.12 ± 2.36/6.67 ± 1.64	Direct competitive CLIA kit (μmol/L)

(*continued on next page*)

Table 1 (*continued*)

Author, year (country)	No. of participants (cases/controls)	Age (years; cases/controls)	Gender (% of males; cases/controls)	Matching	ASD subtype	Diagnostic method	Blood component	Homocysteine levels (mean ± SD; cases/controls)	Analytical technology (unit)
Ning et al. 2019 (China)	102/102	Mean ± SD: 4.5 ± 1.3/4.5 ± 1.3	78.4/78.4	Age, gender	ASD	By two developmental pediatricians based on the DSM-5; the CARS was used to evaluate ASD severity	Serum (fasting)	13.2 ± 4.5/10.5 ± 3.1	Enzyme cycling method (μmol/L)
Parellada et al. 2012 (Spain)	26/29	Mean: NR/NR	NR/NR	Age, gender, and social status	AS	By the DSM-IV and the ADOS	Plasma (fasting)	7.21 ± 2.19/7.76 ± 5.44	Enzymatic assay (NR)
Pasca et al. 2006 (Romania)	12/9	Mean ± SD: 8.29 ± 2.76/8.33 ± 1.82	75.0/66.7	Age	AD	By a child neuropsychiatrist based on the DSM-IV	Plasma	9.83 ± 2.75/7.51 ± 0.93	RP-HPLC (μmol/L)
Pasca et al. 2009_1 [a] (Romania) (AD vs. controls)	15/13	Mean ± SD: 5.10 ± 1.743/5.89 ± 2.199	86.7/61.5	Age, gender, race, and geographical area	AD	Each patient was assigned a diagnosis based on the DSM-IV-TR	Plasma (fasting)	5.190 ± 1.743/5.860 ± 1.406	GC-MS (μmol/L)
Pasca et al. 2009_2 [a] (Romania) (AS vs. controls)	5/8	Mean ± SD: 9.23 ± 4.07/10.22 ± 2.97	100.0/100.0	Age, gender, race, and geographical area	AS	Each patient was assigned a diagnosis based on the DSM-IV-TR	Plasma (fasting)	5.900 ± 1.901/6.720 ± 1.216	GC-MS (μmol/L)
Pasca et al. 2009_3 [a] (Romania) (PDD-NOS vs. controls)	19/22	Mean ± SD: 8.83 ± 3.661/9.05 ± 4.268	68.4/56.5	Age, gender, race, and geographical area	PDD-NOS	Each patient was assigned a diagnosis based on the DSM-IV-TR	Plasma (fasting)	6.490 ± 1.569/6.880 ± 2.439	GC-MS (μmol/L)
Pastural et al. 2009 (United States)	15/12	Mean ± SD: 7.93 ± 2.91/8.67 ± 3.89	100.0/75.0	Age	AD	By the DSM-IV	Plasma (fasting)	$P = 0.34$	LC-MS/MS (NR)
Shaik Mohammad et al. 2016 (India)	138/138	Mean ± SD: 4.4 ± 1.7/4.4 ± 1.6	87.0/87.0	Age, gender, and ethnicity	AD	Based on the DSM-IV criteria and the ABC scoring	Plasma (fasting)	9.67 ± 4.82/6.99 ± 3.21	RP-HPLC (μmol/L)
Suh et al. 2008_1 [b] (United States) (Plasma)	30/11	Mean ± SD: 4.17 ± 1.3/6.9 ± 1.6	86.7/81.8	NR	ASD	According to the DSM-IV and the ADI-R	Plasma	5.5 ± 2.2/6.7 ± 2.9	LC-MS/MS (μmol/L)
Suh et al. 2008_2 [b] (United States) (PBMC)	30/11	Mean ± SD: 4.17 ± 1.3/6.9 ± 1.6	86.7/81.8	NR	ASD	According to the DSM-IV and the ADI-R	PBMC	0.028 ± 0.025/0.009 ± 0.005	LC-MS/MS (nmol/mg protein)
Sun et al. 2016 (China)	29/29	Mean ± SD: 4.33 ± 1.09/4.78 ± 0.86	82.8/82.8	Age, gender, and socioeconomic status	AD	By a specialist clinician and confirmed by the DSM-IV	Plasma (fasting)	8.80 ± 2.29/7.80 ± 1.13	HPLC (μmol/L)
Sun C et al. 2018 (China)	53/53	Mean ± SD: 4.94 ± 0.62/4.95 ± 0.63	84.9/84.9	Age, gender	ASD	By psychiatrist or pediatrician according to the DSM-IV	Serum (fasting)	10.24 ± 2.31/8.61 ± 2.12	CL (μmol/L)
Sun Y et al. 2018 (China)	110/110	Mean: NR/NR	85.5/NR	Age, gender	ASD	By psychiatrist or pediatrician according to the DSM-5, the ADOS, and the ADI-R	Serum (fasting)	7.54 ± 3.34/6.62 ± 1.79	CLIA (μmol/L)
Tu et al. 2012 (China)	20/20	Mean ± SD: 3.46 ± 0.56/NR	85.0/NR	Age, gender	AD	By the DSM-IV	Plasma (fasting)	9.6 ± 1.2/7.4 ± 1.1	LC-MS/MS (μmol/L)
Tu et al. 2013 (China)	30/30	Range: 2–6/2–6	83.3/83.3	Age, gender	AD	Based on the DSM-IV and by a diagnostic interview conducted by a developmental pediatrician	Serum (fasting)	8.62 ± 1.33/6.94 ± 1.02	Enzyme cycling method (μmol/L)
Wang et al. 2016 (China)	96/96	Mean ± SD: 3.85 ± 1.22/3.85 ± 1.22	79.6/79.6	Age, gender	ASD	Using the DSM-5; CARS was used to evaluate ASD severity	Serum (fasting)	16.967 ± 4.138/11.833 ± 3.085	NR (μmol/L)
Yan et al. 2015 (China)	75/75	Mean ± SD: 3.69 ± 1.22/3.69 ± 1.22	78.7/78.7	Age, gender	ASD	Using the DSM-IV; CARS was used to evaluate ASD severity	Plasma (fasting)	9.42 ± 1.44/7.58 ± 1.09	NR (μmol/L)

(*continued on next page*)

Table 1 (*continued*)

Author, year (country)	No. of participants (cases/controls)	Age (years; cases/ controls)	Gender (% of males; cases/ controls)	Matching	ASD subtype	Diagnostic method	Blood component	Homocysteine levels (mean ± SD; cases/ controls)	Analytical technology (unit)
Yektas et al. 2019 (Turkey) 6.0/9.0	35/35 80.0/100.0	Median: NR	ASD	By the CARS	Serum	P = 0.000 (P < 0.0005)	ELISA (μmol/ L)		
Zhang et al. 2015 (China)	80/100	Mean ± SD: 3.82 ± 1.34/ 3.79 ± 1.25	78.75/79.00	Age, gender	ASD	Based on the DSM-IV; CARS was used to evaluate ASD severity	Serum (fasting)	17.667 ± 5.889/ 11.467 ± 3.911	NR (μmol/L)
Zhou and Li 2018 (China)	81/81	Mean ± SD: 3.80 ± 1.22/ 3.80 ± 1.22	79.0/79.0	Age, gender	ASD	A series of structured interviews were conducted by two developmental pediatricians according to the DSM-5	Serum (fasting)	10.62 ± 4.10/7.13 ± 2.04	Enzyme cycling method (μmol/L)
Zou et al. 2019 (China)	89/89	Mean ± SD: 6.68 ± 2.92/ 6.71 ± 2.95	87.6/87.6	Age, gender, and ethnicity	ASD	By two independent specialist clinicians according to DSM-5 criteria; the ADOS and ADI-R were used as an aid to diagnose	Serum (fasting)	6.96 ± 2.16/6.20 ± 1.36	Commercial immunoassay (μmol/L)

Abbreviations: ABC, Autism Behavior Checklist; AD, autistic disorder; ADI-R, Autism Diagnostic Interview-Revised; ADOS, Autism Diagnostic Observation Schedule; AS, Asperger's syndrome; ASD, autism spectrum disorder; CARS, Childhood Autism Rating Scale; CL, chemiluminescence; CLIA, chemiluminescent immunoassay; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, fourth edition; DSM-IV-TR, Diagnostic and Statistical Manual of Mental Disorders, fourth edition, Text Revision; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, fifth edition; ELISA, enzyme-linked immunosorbent assay; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; HPLC-ECD, high performance liquid chromatography with electrochemical detection; HPLC-MS/MS, high performance liquid chromatography-tandem mass spectrometry; IEC, ion-exchange chromatography; LBAs, ligand-binding assays; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NR, not reported; No., number; PBMC, peripheral blood mononuclear cells; PDD-NOS, pervasive developmental disorder-not otherwise specified; RP-HPLC, reversed-phase high performance liquid chromatography; SD, the standard deviation.

[a] In this study, the total number of healthy controls for all the 3 autism subgroups was 25. [b] The sample size of the control group in this study was 30 rather than 31.

Yektas et al., 2019) or medium quality (Adams et al., 2007; Altun et al., 2018; Hodgson et al., 2014; James et al., 2004; James et al., 2009; Parellada et al., 2012; Paşca et al., 2006; Pastural et al., 2009; Suh et al., 2008; Tu et al., 2012; Yektas et al., 2019), the results of the main analysis also did not change substantially (Hedges's g = 0.59, 95% CI = 0.35 to 0.82, P < 0.001; Hedges's g = 0.61, 95% CI = 0.37 to 0.85, P < 0.001; respectively) (Table 2). These findings further corroborated the reliability of our results. Besides, the heterogeneity was not evidently influenced by each exclusion (I^2 = 86.80%, τ^2 = 0.31, P < 0.001; I^2 = 89.12%, τ^2 = 0.33, P < 0.001; and I^2 = 88.91%, τ^2 = 0.30, P < 0.001; respectively) (Table 2).

3.6. Subgroup analyses

When grouping by blood component, increased Hcy levels were found in ASD children as compared to HC in both serum (Hedges's g = 0.75, 95% CI = 0.46 to 1.03, P < 0.001) and plasma (Hedges's g = 0.37, 95% CI = 0.09 to 0.64, P = 0.01) subgroups, with moderate (I^2 = 74.89%, τ^2 = 0.11, P < 0.001) and high (I^2 = 90.39%, τ^2 = 0.51, P < 0.001) levels of heterogeneity, respectively (Table 2). There was no statistically significant difference in the pooled effect estimates between subgroups (Q = 3.54, P = 0.06), suggesting that the blood component was not a possible source of the heterogeneity in this study.

When stratified by ASD subtype, elevated Hcy levels were observed in children diagnosed with AD (Hedges's g = 0.47, 95% CI = 0.12 to 0.83, P = 0.01) and children diagnosed with ASD (Hedges's g = 0.69, 95% CI = 0.44 to 0.94, P < 0.001) when compared with HC, with marked heterogeneity in both subgroups (I^2 = 79.94%, τ^2 = 0.28, P < 0.001 and I^2 = 89.42%, τ^2 = 0.31, P < 0.001, respectively) (Table 2). No significant difference in the summary effect sizes was found between subgroups (Q = 0.97, P = 0.32), manifesting that the ASD subtype was not a contributor to the heterogeneity in the present meta-analysis.

3.7. Meta-regression analyses

Subsequently, we performed meta-regression analyses to evaluate whether certain continuous variables could be responsible for the heterogeneity among studies. Univariate meta-regression analyses indicated that the mean age (cases, controls, and all participants), and the differences in mean age/gender (% male) between cases and controls had moderating effects on the outcome of the meta-analysis (P < 0.05 in all of these analyses) (Table 3), suggesting that these 5 variables were confounding factors for studies analyzing blood Hcy levels in children with ASD and potential reasons of the heterogeneity in this study.

3.8. Publication bias

Visual inspection of the funnel plot suggested no publication bias (Fig. 4), which was verified by the Begg's (P = 0.38) and Egger's (P = 0.45) tests. The classic Fail-safe N analysis revealed that 2211 missing studies would be needed to bring about P > 0.05 for this meta-analysis, further confirming that the positive findings observed in our meta-analysis were unlikely to be owing to publication bias.

4. Discussion

4.1. Overview

Circulating levels of Hcy in children with ASD have been the subject of controversy over the years, and therefore its significance with regard to ASD etiology is not fully clarified. Here, the overall analysis of this study found that in comparison to the corresponding values in HC, the peripheral blood levels of Hcy from autistic children were elevated significantly. By sensitivity analyses, it can be confirmed that our results were quite robust. Subgroup analyses revealed that blood Hcy

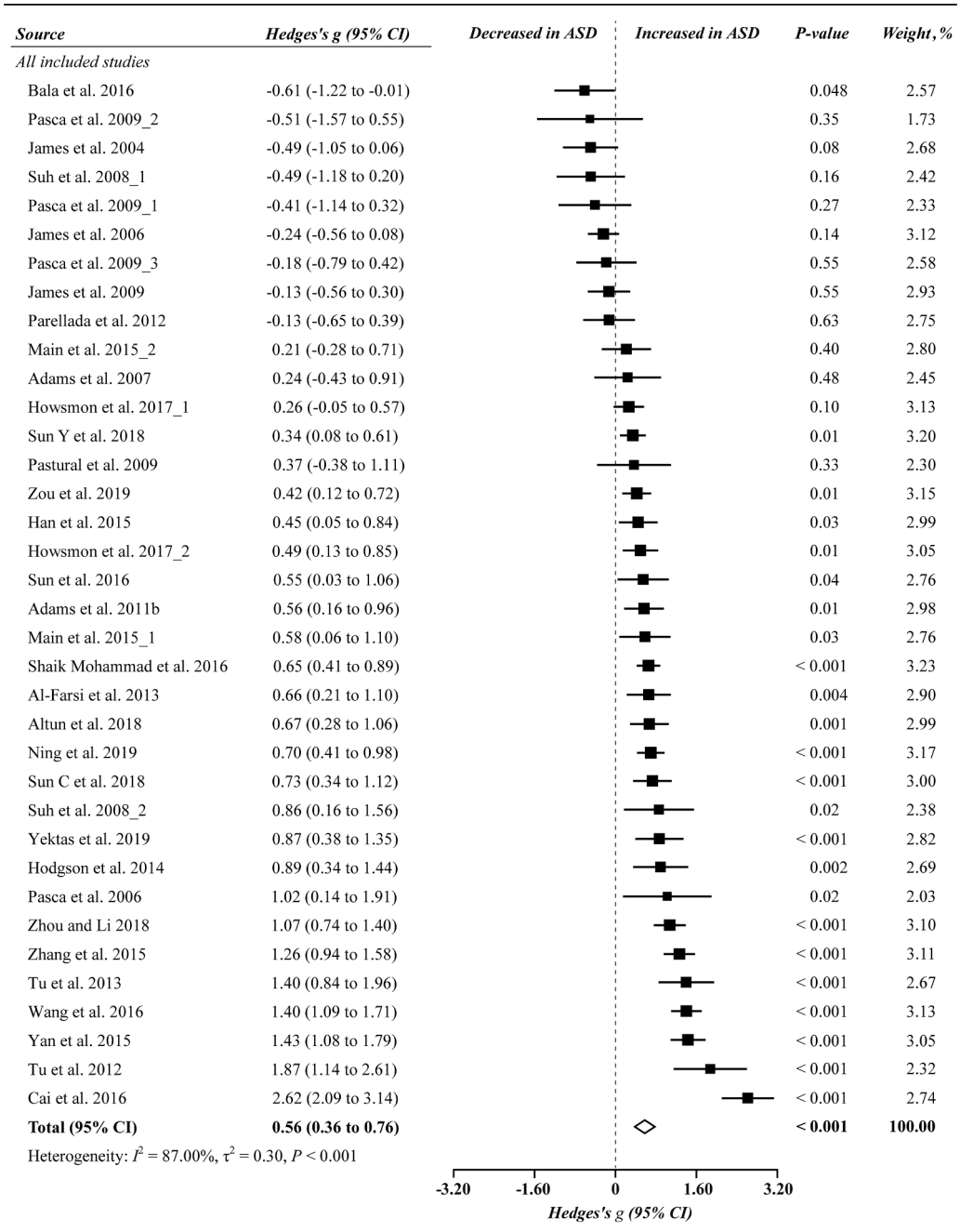

Fig. 3. Forest plot for overall analysis using random-effects model. The size of each square is proportional to the study weight. Diamond symbol indicates the pooled effect size. Abbreviations: ASD, autism spectrum disorder; CI, confidence interval.

levels in children with ASD were still increased regardless of blood component and ASD subtype. Additionally, no publication bias was observed in this meta-analysis. Therefore, in contrast to the negative findings from two previous meta-analyses (Frustaci et al., 2012; Main et al., 2012), the present study indicates that ASD is associated with increased circulating Hcy levels and that Hcy may contribute to the development of ASD.

4.2. Investigation of heterogeneity

Significant heterogeneity among studies was detected in this meta-analysis. Thus, we used sensitivity, subgroup and meta-regression analyses to identify potential sources that are also the feature and strength of this work. Sensitivity analyses showed that the marked heterogeneity could not be attributable exclusively to any single study, the studies with data conversion and rounded *P*-value, the studies with unclear fasting status, or the studies with medium quality. By subgroup analyses, we found that the levels of between-study heterogeneity could also not be explained by blood component and ASD subtype. Through meta-regression analyses, however, we identified significant correlations between the summary effect size and the mean age of cases, controls, and all participants, showing that the heterogeneity between studies could be partially explained by these moderators. According to our viewpoint, it seems reasonable, because the mean age of cases, controls, and all participants in the included studies of this meta-analysis varied broadly. In fact, of additional concern are the slopes in these regression equations, because they are negative values. It means that the higher age of autistic children, controls, or all participants will

Table 2
Statistics on sensitivity and subgroup analyses.

Sensitivity/subgroup analyses	No. of studies for analysis	No. of children		Pooled effect size		Heterogeneity		
		Cases	Total	Hedges's *g* (95% CI)	*P*-value	I^2 statistic (%)	τ^2	*P*-value
Sensitivity analyses								
Excluding studies with data conversion/rounded *P*-value	26	1333	2666	0.49 (0.27 to 0.71)	< 0.001	86.80	0.31	< 0.001
Excluding studies with uncertain fasting status	23	1399	2858	0.59 (0.35 to 0.82)	< 0.001	89.12	0.33	< 0.001
Excluding studies with medium quality	20	1339	2723	0.61 (0.37 to 0.85)	< 0.001	88.91	0.30	< 0.001
Subgroup analyses								
Blood component								
Serum	15	907	1835	0.75 (0.46 to 1.03)	< 0.001	74.89	0.11	< 0.001
Plasma	16	734	1469	0.37 (0.09 to 0.64)	0.01	90.39	0.51	< 0.001
ASD subtype								
AD	11	371	765	0.47 (0.12 to 0.83)	0.01	79.94	0.28	< 0.001
ASD	19	1220	2448	0.69 (0.44 to 0.94)	< 0.001	89.42	0.31	< 0.001

Abbreviations: AD, autistic disorder; ASD, autism spectrum disorder; CI, confidence interval; No., number.

produce the smaller difference in blood Hcy levels between children with ASD and HC. These indicate that the levels of Hcy in blood samples from autistic children may vary by age, or more accurately, the blood Hcy concentrations are likely to be higher in younger children than in older children who were diagnosed with ASD. However, it's just a hypothesis; so far there is no more data available to suggest and/or verify this hypothesis. Therefore, whether it is tenable remains to be verified by additional studies. Besides, our results also showed a significant positive interaction of the difference in mean age between cases and controls on the summary effect size. This implied that blood Hcy levels in children with ASD would elevate with increasing difference in the mean age between cases and controls. Notably, although there is a sex difference in ASD prevalence, with a male-to-female ratio of closer to 3:1 (Loomes et al., 2017), in this study we did not reveal a relationship between the gender (% male; patients, HC, and all participants) and the pooled effect estimate. Nevertheless, we found a significant negative correlation of the difference in gender (% male) between cases and controls on the main outcome. This represented that the greater the percentage of males in cases than that in controls is, the smaller the difference in blood Hcy levels between autistic children and control individuals will be. Accordingly, this confounding factor should be taken into account in future work analyzing Hcy in children with ASD.

4.3. Possible roles of Hcy in the pathogenesis of ASD

Hyperhomocysteinemia is not specific for ASD, and has been reported as a risk factor for neural tube defects, cardiovascular diseases, and other neuropsychiatric disorders such as schizophrenia, Alzheimer's disease, Parkinson's disease, and depression, emphasizing the pivotal role of the Hcy and/or its metabolism in the pathogenesis of various diseases (Muntjewerff et al., 2006; Numata et al., 2015). Hcy is located at the intersection of two metabolic pathways: methionine cycle (in which S-adenosylmethionine [SAM], the universal methyl donor for various methyl-transfer reactions, is synthesized) and transsulfuration pathway (in which glutathione [GSH], a key antioxidant present in cells and tissues, is synthesized) (Fig. 1). Theoretically, the changes in Hcy levels could influence both of the two metabolic pathways. Notably, it has been indicated that impaired methionine cycle and abnormal transsulfuration metabolism may be implicated in the development of autism (James et al., 2004, 2006, 2009). Thus, we hypothesize that the two metabolic pathways might play indirect roles in the association between Hcy and the pathogenesis of ASD. Hcy is also closely correlated to folate cycle (Fig. 1). Genetic alteration in key metabolic enzymes, including methionine synthase (MS) and methylenetetrahydrofolate reductase (MTHFR), or deficiency in cofactors (folate, vitamin B6, and B12) may result in metabolic disturbance of Hcy (Bhatia and Singh, 2015). Moreover, accumulating evidence from animal and in vitro studies suggests that the nervous system is particularly sensitive to folate deprivation and elevated Hcy levels (Lipton et al., 1997; Mattson and Shea, 2003). On the other hand, available data strongly suggest that Hcy has neurotoxic properties. For example, extracellular Hcy has been found to show toxic effects on cultured neurons, eventually bringing about apoptosis and deleterious impacts on synaptic and glial function (Ho et al., 2002; Kruman et al., 2000; Lipton et al., 1997). Additionally, Hcy may activate N-methyl-D-aspartate (NMDA) receptors, leading to neuronal death via Ca^{2+} influx and phosphorylation of the extracellular signal-regulated kinase-mitogen-activated protein (ERK-MAP) kinase (Poddar and Paul, 2009). Furthermore, Hcy can accumulated in the brains of animals exposed to this compound (Algaidi et al., 2006), resulting in restricted growth, abnormal brain

Table 3
Statistics on meta-regression analyses.

Moderator	No. of comparisons	No. of children		Meta-regression				Proportion of variance explained
		Cases	Total	Slope	95% CI (Lower)	95% CI (Upper)	*P*-value	R^2 analog
Mean age of cases	29	1340	2660	**-0.20**	-0.31	-0.10	< **0.001**	0.36
Mean age of controls	28	1320	2620	**-0.18**	-0.26	-0.10	< **0.001**	0.44
Mean age of all participants	28	1320	2620	**-0.19**	-0.28	-0.10	< **0.001**	0.41
Gender (% male) of cases	32	1515	3010	0.00001	-0.02	0.02	1.00	0.00
Gender (% male) of controls	27	1245	2462	0.02	-0.001	0.03	0.06	0.03
Gender (% male) of all participants	27	1245	2462	0.01	-0.01	0.03	0.36	0.00
Sample size of cases	36	1641	3304	0.01	-0.001	0.01	0.11	0.00
Sample size of controls	36	1641	3304	0.01	-0.001	0.01	0.08	0.00
Sample size of all participants	36	1641	3304	0.003	-0.0004	0.01	0.09	0.00
Difference in mean age [a]	28	1320	2620	**0.35**	0.13	0.57	**0.002**	0.29
Difference in gender (% male) [a]	27	1245	2462	**-0.03**	-0.05	-0.01	**0.01**	0.14
Difference in sample size [a]	36	1641	3304	-0.01	-0.03	0.02	0.54	0.00
Total NOS score	36	1641	3304	0.04	-0.17	0.24	0.73	0.00

Abbreviations: CI, confidence interval; No., number; NOS, Newcastle–Ottawa Scale.
[a] Between cases and controls.

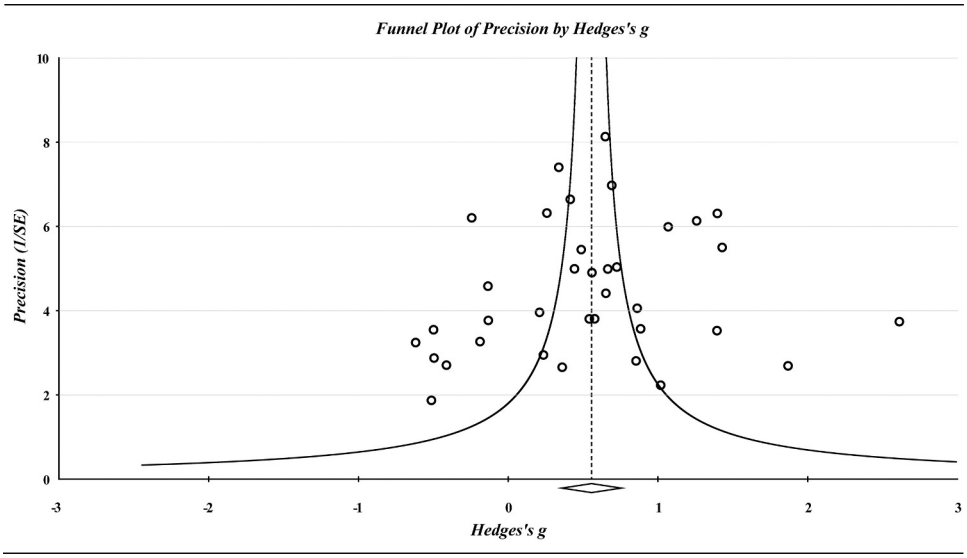

Fig. 4. Funnel plot evaluating publication bias in observed studies comparing blood homocysteine levels between autistic children and healthy controls. The figure depicts the effect sizes (Hedges's *g*) of studies against their precisions (inverse of SE). Circles represent observed studies. Diamond symbol indicates the pooled effect size based on observed studies. Abbreviation: SE, the standard error.

energy metabolism (Streck et al., 2003), and neural or cognitive dysfunction (Algaidi et al., 2006; Streck et al., 2004). Accordingly, we speculate that the neurotoxicity of Hcy might also be an important contributor to the occurrence of ASD and could aggravate the clinical manifestations of ASD. Despite our understanding about possible roles of Hcy in the pathogenesis of ASD, the specific mechanisms are very complicated and have not been fully elucidated. Therefore, further investigation is warranted.

4.4. Implications

The current study support the notion that increased Hcy levels may be a risk factor for ASD. Meanwhile, our findings have important clinical significance for diagnosis, prevention and treatment of ASD, because elevation in blood Hcy levels in autistic children suggests that blood Hcy could be used as a peripheral biomarker of childhood autism and, more importantly, that pharmacological interventions for lowering Hcy levels may have beneficial effects on ASD. A randomized control trial showed that oral vitamin/mineral supplementation may be considered as a reasonable adjunct therapy for autism by improving the nutritional and metabolic status (including a decrease of blood levels of Hcy) of autistic children and reducing their symptoms (Adams et al., 2011a). Another open-label trial indicated that folic acid intervention could improve autism symptoms as measured by the Autism Treatment Evaluation Checklist (ATEC), which was accompanied by a significant decline in blood Hcy concentrations (Sun et al., 2016). In summary, our findings support the hypothesis that efforts toward early measurement of blood Hcy in combination with enforcement of nutritional and lifestyle changes to improve Hcy metabolism may represent a feasible strategy to reduce the risk of childhood autism.

4.5. Strengths and limitations

The current study has major strengths. First, this systematic review and meta-analysis was performed in strict adherence to the PRISMA guidelines. Second, by thorough literature search through multiple databases, we captured all the relevant articles to date, especially including recently published studies. Third, we used strict inclusion and exclusion criteria and conducted extensive data collection to avoid the omission of important information. Fourth, we made a detailed exploration on the latent sources of heterogeneity between studies by means of sensitivity, subgroup and meta-regression analyses. Consequently, the present study is rigorous systematic review and meta-analysis, and can offer high precision results for objectively evaluating the blood Hcy levels in children with ASD.

Despite its strengths, this study also has several limitations. First, the current meta-analysis pooled data originating from case-control studies. Thus, whether an increase in blood Hcy levels is a pathogeny for the genesis of ASD or just an epiphenomenon accompanying other etiologic factors, such as a counterbalance effect of ASD development, is not yet clear. That is, the increase of Hcy could be secondary to ASD rather than a cause of ASD. Second, high levels of heterogeneity were observed in this study. It was not surprising owing to obvious variations in study characteristics. Third, we used the NOS for the quality assessment of the studies included, while this scale suffers from inherent weaknesses. Fourth, some factors may impact the intake, absorption, and metabolism of Hcy, including genetic and nutritional factors, lifestyle behaviors, disease states, certain drugs, and hormone levels (Guo et al., 2015). However, only a few included studies asked about and controlled for some of these factors. Consequently, direct evaluation in these aspects was not possible. Fifth, there were significant differences in the diagnostic methods of ASD and the analytical technologies of blood Hcy among the included studies, therefore these categorical variables have not been investigated in this meta-analysis. Sixth, we did not register or publish our study protocol.

5. Conclusions

Our systematic review and meta-analysis showed significantly increased peripheral blood levels of Hcy as a manifestation of children with ASD. These findings strengthen the clinical evidences of an aberrant Hcy profile in autistic children. However, in view of the significant between-study heterogeneity, the conclusions of this study should be interpreted with caution. Thus, more investigation is required to confirm the results of this meta-analysis.

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Declaration of Competing Interest

The authors declare that they have no actual or potential conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2020.113283.

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