Biomarkers and Attention-Deficit/Hyperactivity Disorder: A Systematic Review and Meta-Analyses

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Objective: To determine whether peripheral biochemical markers (biomarkers) might differentiate patients with attention-deficit/hyperactivity disorder (ADHD) from non-ADHD individuals. **Method:** We conducted a systematic search and a series of meta-analyses of case-control studies comprising studies from 1969 to 2011. **Results:** We identified 210 studies in the following categories: 71 studies of the main metabolites and metabolism enzymes of monoaminergic neurotransmission pathway; 87 studies of environmental risk factors divided into heavy metals (18 studies), substance/chemical exposures (16 studies), and nutritional factors (trace elements: 29 studies; essential fatty acids: 24 studies); 22 studies of the hypothalamic–pituitary–adrenal axis (HPA) pathway; 31 studies indicated with "other." After screening for the availability for meta-analyses of drug naïve/free case-control studies and Bonferroni correction, five comparisons were statistically significant (Norepinephrine [NE], 3-Methoxy-4-hydroxyphenylethylene glycol [MHPG], monoamine oxidase [MAO], Zinc [Zn], cortisol), five of the significant findings found support in studies of response to ADHD medications (NE, MHPG, MAO, b-phenylethylamine [PEA], cortisol), six in studies of symptoms severity (NE, MHPG, MAO, ferritin, Zn, cortisol) and three in studies of neurophysiological or cognitive functioning (lead–ferritin–Zn). No evidence of publication bias was found, whereas significant heterogeneity of effect sizes across studies was found for three of the five biomarkers that differentiated ADHD from control subjects. Suggestive associations were evidenced for neuropeptide Y (NPY), manganese, and dehydroepiandrosterone (DHEA). **Conclusions:** This study provides evidence for several peripheral biomarkers as being associated with ADHD both in diagnosis and in treatment efficacy. Further studies are warranted to replicate these findings, to assess their specificity for ADHD, and to quantify the degree to which they are sufficiently precise to be useful in clinical settings. J. Am. Acad. Child Adolesc. Psychiatry, 2012;51(10):1003–1019. **Key words:** attention-deficit/hyperactivity disorder, biochemical markers, diagnosis, drug treatment efficacy, meta-analyses

The identification of peripheral biochemical markers (biomarkers), measurable in vivo with noninvasive methods, might facilitate the differential diagnosis of attention-deficit/hyperactivity disorder (ADHD) and the development of individualized therapies. Although the discovery of an accurate biomarker test for ADHD would be valuable for these reasons, potential negative consequences should be considered. Most notably, if it became possible to diagnose psychiatric disorders from a simple blood test, such a test could be used by employers or insurance companies to discriminate against persons with mental illness.

Following Schmidt *et al.*,[1] we define a biomarker as a characteristic that can be objectively measured and evaluated as an indicator of a normal biological process, a pathogenic process, or a response to a therapeutic intervention. Most biomarker studies compare case patients and control subjects to determine the sensitivity and specificity of the biomarker for detecting the disorder. In interpreting such results, one must be aware that the presence of a case–control


Supplemental material cited in this article is available online.

difference does not clarify the mechanistic status of the biomarkers. A biomarker detected by such a study could be a measure of vulnerability to the disorder, processes that occur with the onset of the disorder, or processes that lead to chronicity or to epiphenomena of the disorder. They could also reflect effects of treatment or physiological responses to the stress of living with a chronic disorder. Our review will not differentiate these types of biomarkers because the studies that we reviewed could not address that distinction.

An important step toward identifying a biomarker is the validation of the results reported by single independent studies using a meta-analytic approach. In this review, we meta-analyzed studies assessing the association between ADHD, or response to treatment, and biomarkers in the following categories: dopaminergic, noradrenergic, serotoninergic, biogenic trace amines systems, and their principal metabolites; environmental risk factors, including heavy metals and substance/chemical exposures and nutritional factors; hypothalamic–pituitary–adrenal axis (HPA) alterations; and markers involved in other aspects of brain functioning (growth hormone and thyroid function, oxidative stress cascade, cytokine unbalance, other neurotransmission systems, neurotrophic factors, complement C4-B, pineal hormone melatonin).

METHOD

Literature Search

To identify eligible studies for the review and meta-analysis, we searched two online electronic databases (PubMed and Human Genome Epidemiology Network [HuGeNet]), from inception until September 2011, for all available studies for the association between biomarkers and ADHD in childhood. The diagnostic search terms used to query the databases were "ADHD" and "Attention Deficit Hyperactivity Disorder." These two terms were used to conduct searches with all relevant names of the biomarkers of interest along with different combinations of the following key words: levels, peripheral, serum, plasma, urine, saliva, blood, platelets, cerebrospinal fluid, red blood cells, hair, treatment, clinical trial. Once articles had been collected, bibliographies were manually searched for additional eligible studies. The literature search was performed by two individuals independently.

Inclusion and Exclusion Criteria

We reviewed three types of studies: first, comparisons of biomarker assays between treatment-naive/free patients with ADHD and controls; second, studies of the effects of ADHD medications on biomarker assays; and third, studies of the association between biomarkers and clinical features of ADHD (severity, neurophysiological and cognitive functioning). We included studies that met the following inclusion criteria: investigated one or more of the peripheral biomarkers described in the introductory section of this article; compared cases with ADHD defined by *DSM* or ICD criteria with controls; and provided statistics required for meta-analysis. We excluded studies that met the following exclusion criteria: used patients having current or prior use of psychotropic medications or where drug status was not described; were case reports; were commentaries or reviews; were not in English; used adults or animal models; or selected samples based on a disorder other than ADHD. We excluded Shekim *et al.*[2] and Eppright *et al.*[3] because no response was obtained after contacting the authors for these data. Meta-analyses were performed for all biomarkers levels for which usable data were reported in at least four published studies. Figure 1 presents a flow diagram depicting our selection procedure for review and meta-analysis.

Data Extraction for Meta-analyses

For all studies suitable for meta-analysis, we extracted the following data from the original publications: first author and year of publication, population, biological fluid, number of participants, percentage of males, mean age in years, biomarkers analyzed, and diagnostic system used to diagnose ADHD (Table S1, available online).

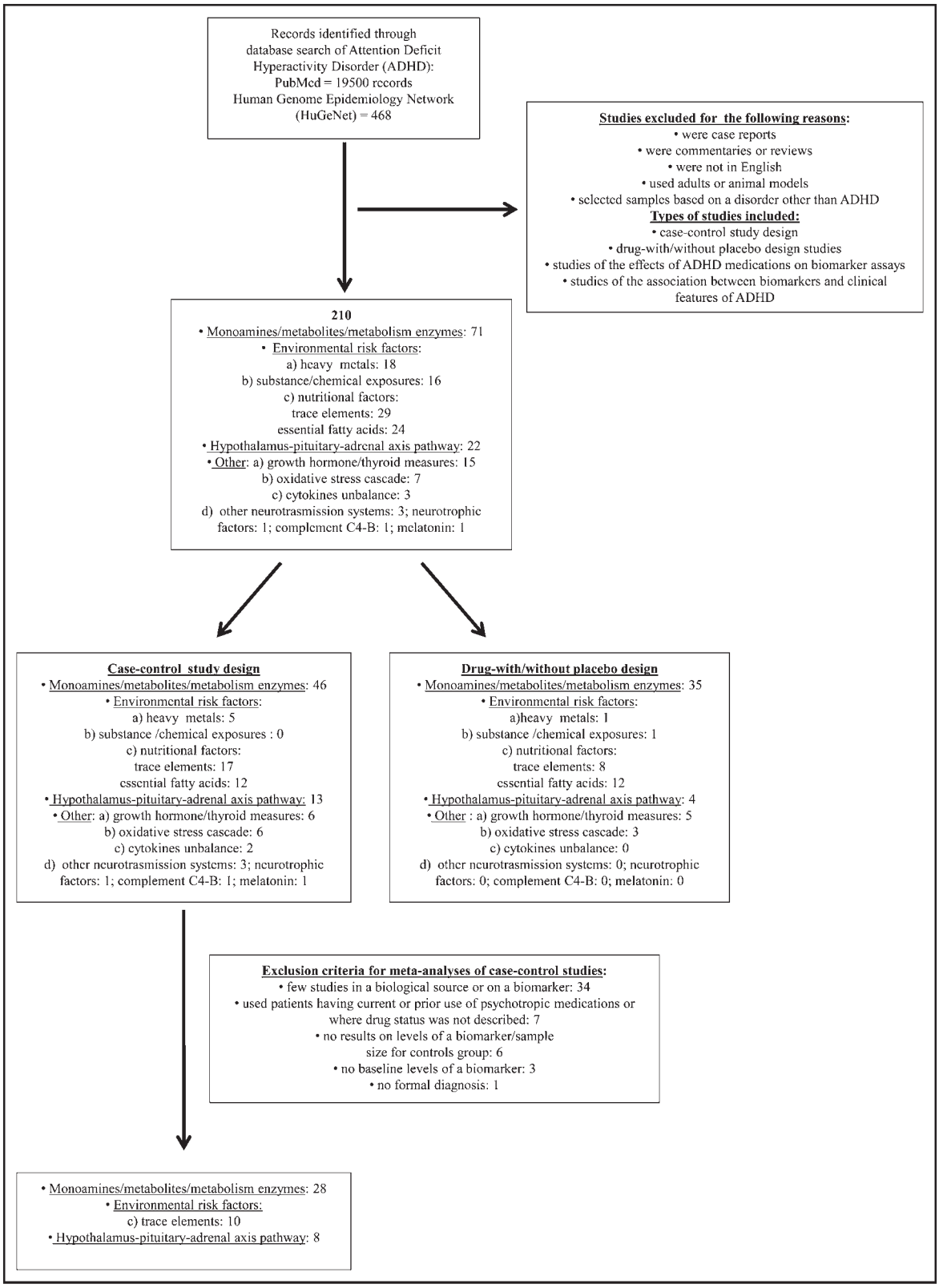
Statistical Analyses

Review Manager was used to analyze the data (RevMan Version 5.1.6; Copenhagen, The Nordic Cochrane Centre, The Cochrane Collaboration, 2008).

We used the fixed-effects model to generate a pooled effect size and 95% confidence interval (CI) from individual study effect sizes (Cohen's d or the standardized mean difference [SMD]) using the inverse variance method. The significance of the pooled effect sizes was determined by the z-test. Between-study heterogeneity was assessed using a χ^2 test of goodness of fit test and the I^2 statistic. We used a p value of .05 to assert statistical significance. In a fixed-effects model, the fundamental assumption is that a single true effect size underlies all study results and that observed estimates vary only as a function of chance. The error term in a fixed-effects model represents only within-study variation, and between-study variation is ignored.

Where the results showed a significant effect in the presence of significant between-study heterogeneity, a random effects model was used, with effect sizes pooled using the DerSimonian and Laird method. The

FIGURE 1 Flow chart depicting the selection procedure for review and meta-analyses.



random effects model assumes that each study estimates different, yet related, true effects and that the distribution of the various effects is normally distributed around a mean effect size value. This model takes both within-study and between-study variation into account. When there is little heterogeneity, both models yield essentially identical results. When heterogeneity is extensive, however, the analyses will yield different estimates of the mean effect size, and the confidence intervals around the estimates will differ. When there is heterogeneity across studies, the random effects model yields wider confidence intervals than the fixed effects model and is thus usually more conservative.

Publication bias was estimated by the method of Egger *et al.*,[4] which uses a linear regression approach to measure funnel plot asymmetry on the natural logarithm scale of the OR. The significance of the intercept (a) was determined by the t test.[4] We repeated all analyses using the weighted mean difference (WMD) rather than the SMD (Cohen's d) to assess the sensitivity of our results to using SMD methodology. The rank correlation method and regression method tests were conducted by MIX version 1.7. (http://www.mix-for-meta-analysis.info).

Because we conducted 14 meta-analyses to assess the significance of biomarkers, our Bonferroni-corrected significance level was .004. This provides a stringent approach for preventing false-positive findings, with the cost of reducing statistical power. Because we do not want to draw misleading conclusions about the significance of potential biomarkers, we prefer to err on the side of preventing false-positive results. In contrast, for our analyses of publication biases and heterogeneity, we use an uncorrected α level to ensure that any of these potential problems with the findings could be detected.

RESULTS

The search yielded 19,968 records about "ADHD": 19,500 in PubMed and 468 from HuGeNet. After screening of papers according to the inclusion/exclusion criteria, 210 papers analyzing biochemical markers associated to the ADHD diagnosis and/or therapeutic efficacy were selected.

As reported in Figure 1, a total of 71 studies focused on alterations in the principal metabolites and metabolism enzymes of monoaminergic neurotransmission pathway. We found 87 studies of environmental risk factors divided into heavy metals (18 studies), substance/chemical exposures (16 studies), and nutritional factors (trace elements: 29 studies; essential fatty acids: 24 studies). Biochemical alterations in the HPA pathway were also analyzed (22 studies). A few studies reported on the potential increased ADHD risk with exposure to organophosphates and phthalates, but not to mercury,[5] issues that were not investigated in this review due to the small number of studies.

We use the term "other" for 31 studies investigating other biochemical markers. These include studies of altered levels of growth hormone and thyroid function (15 studies), the oxidative stress cascade (seven studies), cytokine imbalance (three studies), other neurotransmission systems (three studies), neurotrophic factors (BDNF, one study), complement C4-B (one study), and pineal hormone melatonin (one study). These miscellaneous studies were excluded from this review.

The main peripheral biological fluids analyzed in these studies were plasma (26%), serum (25%), urine (24%), saliva (10%), blood (11%), platelets (5%), red blood cells (RBC) (5%), cerebrospinal fluid (CSF, 2%), and hair (0.48%).

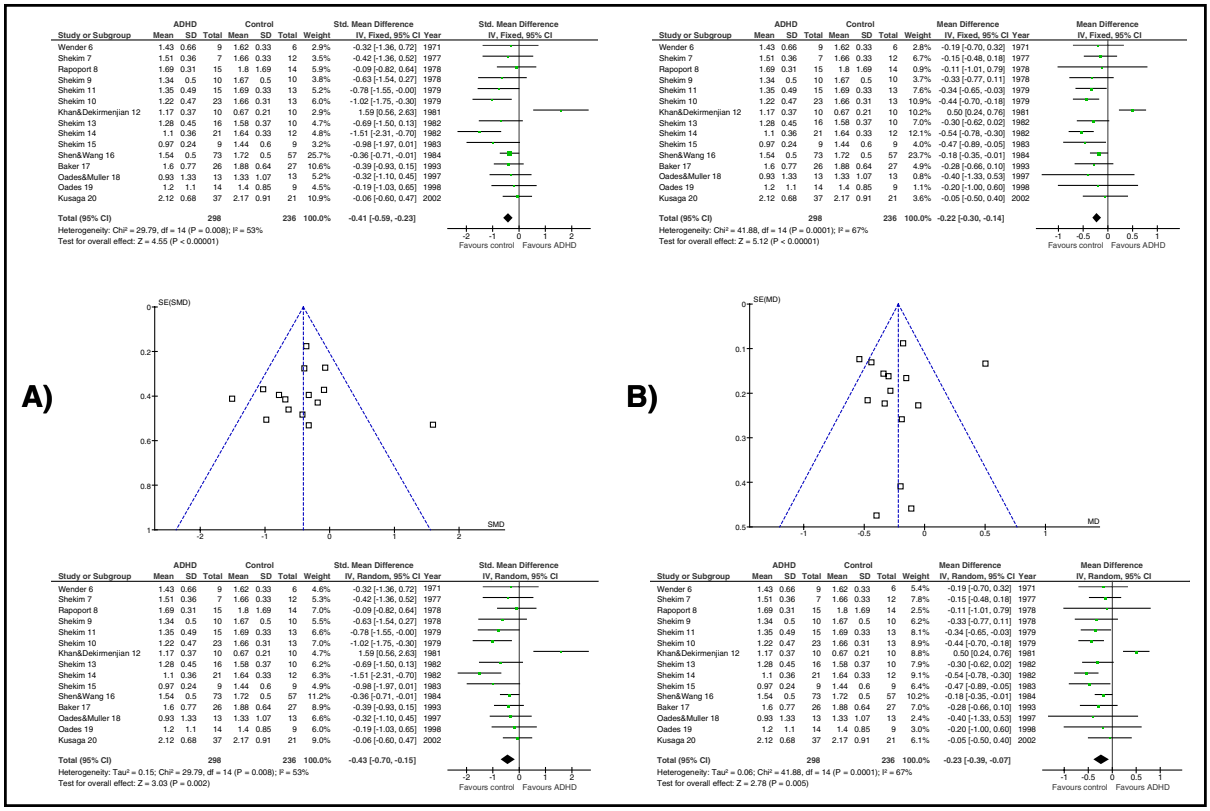
The case-control studies selected for the meta-analysis are described in Table S1 (available online) and in Figure 1. All of the analyses presented below are based on the SMD or Cohen's d. Our analyses using the WMD yielded identical results as regards the significance of effect sizes. Regarding publication biases, the only difference is that the WMD analyses detected publication bias for cortisol studies, whereas the SMD analyses did not. The WMD results are presented in Figure 2,[6-20] Figure 3,[21-25] and Figure 4[26-33] and as supplementary material in Figures S1 through S11(available online). The publication bias results for the WMD/SMD analyses are summarized in the supplementary material (Tables S2 and S3, available online). Furthermore, we presented the fixed effects analyses for the Figures 2 and 3, and as supplementary material in Figures S4, S5, S6, S7, S10, and S11 (available online).

Monoaminergic Neurotransmission Systems, Their Metabolites, and Metabolism Enzymes

The monoaminergic pathways interrogated by the studies we reviewed are described in Figure S12 (available online).

We selected 46 case-control studies and meta-analyzed 28 studies for dopaminergic, noradrenergic, serotoninergic, biogenic trace amines neurotransmission systems, their metabolites and metabolism enzymes (monoamine oxidase [MAO], and dopamine β-hydroxylase [DBH]).

FIGURE 2 Fixed/random forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of urinary 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit; Tau^2 = estimate of the between-study variance in a random-effects meta-analysis.



Dopaminergic Pathway. Urinary levels of Dopamine (DA) and its metabolites dihydroxyphenylalanine (DOPA), and dihydroxyphenylacetic acid (DOPAC) were investigated in several studies. Six studies were available for DA,[8,18,19,34-36] Studies by Hanna *et al.*[34,35] were excluded because they reported data on DA levels during physical, mentally stressful tasks but provided no baseline levels.

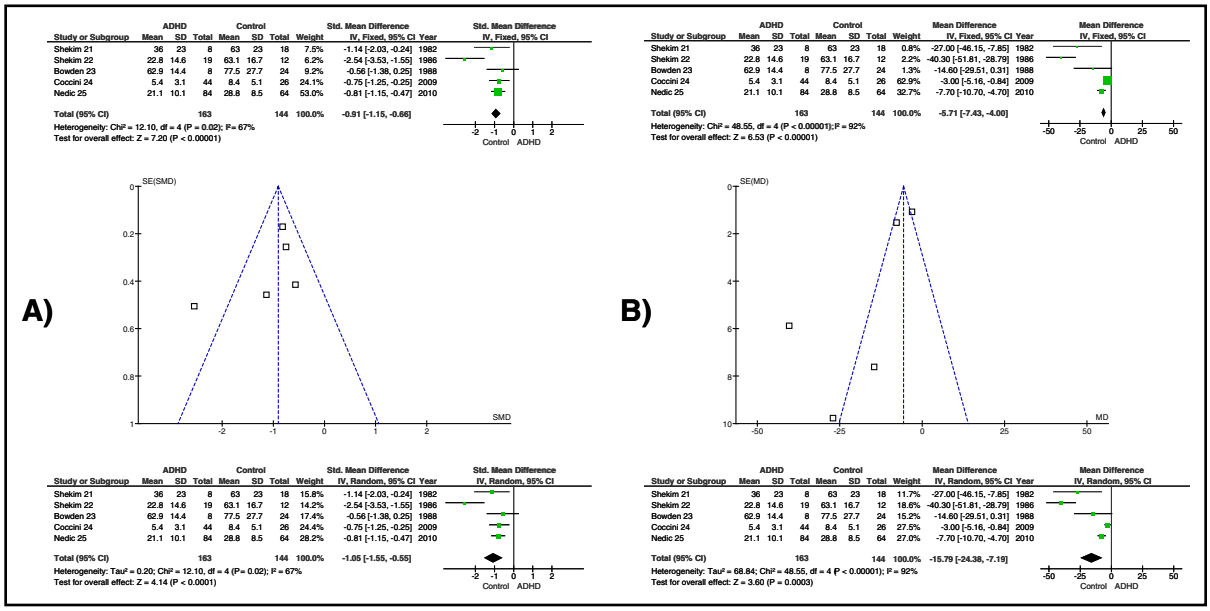
Our meta-analysis found a nonsignificant pooled effect-size of 0.13 (Z = 0.72 p = .47; Figure S1, available online) and no heterogeneity in effect sizes across studies (p = 0.38 I^2 = 3%). Consistent with these findings, for urinary DA, DOPA, and DOPAC urinary levels were found to be similar in ADHD as compared with control subjects.[34,35] One study reported plasma levels of DA to be higher in patients with ADHD,[37] but this finding was not confirmed by Wigal *et al.*[38]

Nine studies assessed the DA metabolite homovanillic acid (HVA) in urine.[6-8,13-15,18-20] Our meta-analysis of these studies indicated no differences between patients and controls, with a pooled effect size of −0.11 (Z = 0.81, p = 0.42; Figure S2, available online) and no heterogeneity in effect sizes across studies (p = 0.08 I^2 = 43%). Since cerebral spinal fluid (CSF) is in direct contact with the brain extracellular space, biochemical changes in the brain are reflected in the CSF. However only two studies were conducted for HVA in CSF yielding mixed results.[39,40]

Noradrenergic Pathway. Our meta-analyses for norepinephrine (NE) included seven studies.[6,8,18,19,36,41,42] Three papers[34,35,43] were excluded: each of these did not report baseline levels of NE, and Pliszka *et al.*[43] did not provide the sample size for the control group.

Our meta-analysis showed higher urinary levels of NE in patients compared with controls (d = 0.41, Z = 2.99, p = .003; Figure S3, available online), with no heterogeneity in effect sizes across studies (p = 0.31, I^2 = 16%). This finding remained significant even after Bonferroni correction. In contrast, our meta-analysis of plasma

FIGURE 3 Fixed/random forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of platelet monoamine-oxidase (MAO) levels. Note: ADHD = attention-deficit/hyperactive disorder; $Chi^2 = \chi^2$ test of goodness of fit; Tau^2 = estimate of the between-study variance in a random-effects meta-analysis.



NE found no differences between patients and controls[19,37,38,44] (d = −0.42; Z = 0.62, p = .54; Figure S4, available online) and substantial heterogeneity in effect sizes across studies (p < .0001, I^2 = 88%). Oades *et al.*[19] reported the analyses not only of catecholamine levels but also of those related to neuropeptide Y (NPY), which frequently colocalizes with catecholamine systems. It participates in the regulation of feeding, circadian rhythms, reproduction and thermoregulation. Oades *et al.*[19] found increased plasma NPY concentrations in children with ADHD compared with controls. Although this finding needs to be replicated, NPY could be a potential biomarker of ADHD, at light of a recent genome-wide copy number variation analysis in which NPY was included in a rare 3Mb duplication on chromosome 7p15.2 to 15.3 and an association of this duplication was found with increased NPY plasma concentrations.[45]

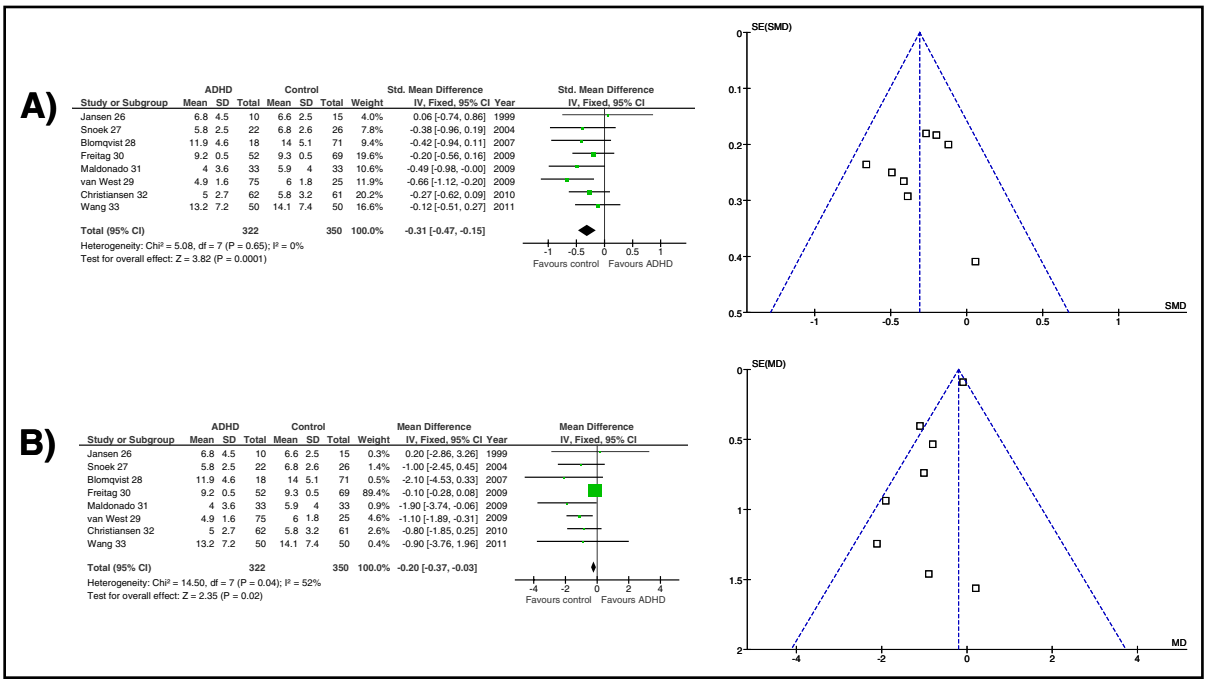
The main metabolite of NE is normetanephrine (NM). The meta-analysis of NM included six studies.[6,7,10,12,17,42] The study excluded was Pliszka *et al.*[43] Baseline differences in urinary NM levels were observed between patients and controls (d = 0.51, Z = 1.98, p = .05; Figure S5, available online), with heterogeneity of effect sizes across studies (p = .02, I^2 = 63%). This finding lost significance after Bonferroni correction.

Another widely studied metabolite of NE is 3-methoxy-4-hydroxyphenylethylene glycol (MHPG). Fifteen studies provided data for our meta-analysis.[6-20] Pliszka *et al.*[43] was excluded. We found significantly lower urinary MHPG levels in patients with ADHD compared with controls (d = −0.43; Z = 3.03, p = .002; Figure 2) and heterogeneity of effect sizes across studies (p = .008, I^2 = 53%). This finding remained significant after Bonferroni correction.

We examined five studies of platelet MAO.[21-25] Platelet MAO levels were significantly lower in ADHD compared with control subjects (d = −1.05; Z=4.14, p < .0001, Figure 3). We found significant heterogeneity in the effect sizes across studies (p = .02, I^2 = 67%). This finding remained significant after Bonferroni correction.

Other metabolites were less studied such as 3,4-dihydroxyphenylglycol (DOPEG) and vanillylmandelic acid (VMA). Concerning DOPEG, two studies[34,35] analyzed its urinary levels during a physical and mental task in patients and controls, but the results were contrasting. For urinary VMA, Wender *et al.*[6] showed no effect of baseline levels on ADHD, whereas Pliszka *et al.*[43]

FIGURE 4 Fixed forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of salivary cortisol levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit.



reported higher levels in ADHD during a mentally physical stressful task.

Dopamine β-hydroxylase (DBH) enzyme, which occurs in the plasma as a stable heritable trait, has been investigated in three studies. No alterations were observed in plasma levels between patients with ADHD and controls.[23,46] However, one study found lower levels in ADHD.[47]

Although lower levels of MHPG are associated with ADHD, stimulant trials show that decreases in ADHD symptoms with treatment are associated with greater reductions in urinary MHPG excretion.[7,9,10,11,13-16,48] Because the findings from these two types of studies are paradoxical, further studies are needed.

The stimulant treatment of ADHD also increases urinary MAO,[21] which is associated with subsequent clinical improvement.[49] Lower platelet MAO activity among patients with ADHD was associated with increased inattention scores[24] as well as with increased impulsivity and short attention span.[22] One study of children with ADHD reported a positive correlation between urinary NE levels and degree of hyperactivity and that treatment of these patients with polyphenol complex (Pyc) normalized NE concentrations, leading to less hyperactivity.[36] Moreover, fenfluramine and dextroamphetamine have also been shown to decrease urinary levels of NE.[48]

Adrenergic Pathway. Six studies were available for the meta-analysis of epinephrine (EPI) in urine.[6,8,19,36,41,42] Hanna *et al.*[34,35] and Pliszka *et al.*[43] were excluded. The meta-analysis found that levels of EPI were not different in patients with ADHD compared with controls (d = 0.41, Z = 1.43 p = .15; Figure S6, available online). High heterogeneity in effect sizes across studies was observed (p = .005, I^2 = 70%). We found similar results for plasma concentrations of EPI, which showed no group difference[19,37,38,44] (d = 0.19, Z=0.48, p = .63; Figure S7, available online) and significant heterogeneity (p = .02, I^2 = 69%).

Metanephrine (M), the main metabolite of EPI, was investigated in five studies.[6,7,10,12,42] We excluded Pliszka *et al.*[43] Urinary M levels were elevated in ADHD (d = 0.45, Z = 2.63, p = .009; Figure S8, available online). We found no significant heterogeneity in effect sizes across studies (p = .32, I^2 = 14%). This finding lost significance after Bonferroni correction.

In summary, in contrast to the significant results obtained for the noradrenergic pathway,

biomarkers in the adrenergic pathway do not provide evidence for utility as biomarkers for ADHD.

Serotoninergic Pathway. Studies of platelet serotonin (5-HT) have given contrasting results. Two studies reported no difference between patients with ADHD and controls,[50,51] whereas Bhagavan *et al.*[52] reported lower levels in patients compared with controls. Moreover, three studies, one in plasma and two in urine, reported negative results.[18,19,53]

5-Hydroxyindoleacetic acid (5-HIAA) has been the most studied 5-HT metabolite.[6,18-20] Our meta-analysis found no alteration in 5-HIAA concentrations between patients than controls (d = 0.29 Z = 1.55, p = .12; Figure S9, available online). We found no significant heterogeneity in effect sizes across studies (p = .21, I^2 = 33%). Consistent with these results, Oades *et al.*[54,55] analyzed 5-HIAA in serum and Shetty *et al.*[39] and Shaywitz *et al.*[40] in CSF, with negative results.

For tryptophan, the precursor of 5-HT, two studies were conducted in plasma and the results showed contrasting results with no alteration in one study[53] and higher levels in children with ADHD in another study.[56] Two studies of serum tryptophan found higher levels in ADHD.[54,55]

In summary, although difference in platelet concentrations of 5-HT were substantially negative between patients and controls and no clear evidence was observed for tryptophan, the 5-HT system could still be a good candidate in light of studies reporting that platelet 5-HT concentrations might index altered 5-HT function in impulsivity.[51]

Biogenic Trace Amines. b-Phenylethylamine (PEA) is considered a "trace amine" because its urinary excretion rate and brain concentration is, compared to catecholamines, very low. The exact role of PEA in humans is not known, but because of its structural similarity to amphetamine, it has been studied extensively.

Although we did not find a sufficient number of studies suitable for a meta-analysis of PEA and ADHD, three studies[20,57,58] confirmed that urinary levels of PEA were significantly lower in patients with ADHD compared with controls. In contrast to these positive results, negative results were observed for the PEA metabolite phenylacetic acid (PAA) in urine[57,58] and in plasma.[58] Negative results were also reported for urinary/serum phenylalanine (a precursor of PEA) and p-tyrosine (a precursor of phenylalanine and dopamine).[55,57,58] Interestingly decreased levels of PEA have also been associated with symptoms of inattentiveness.[59] Administration of D-amphetamine and methylphenidate resulted in a markedly increased urinary excretion of PEA,[20,60] suggesting that ADHD treatments normalize PEA levels.

Environmental Risk Factors: Heavy Metals

From a total of 87 biochemical studies, 34 were case-control studies. Among these, the studies suitable for meta-analysis comprised three studies for heavy metals (lead), 10 for trace elements, and three for polyunsaturated fatty acids (PUFAs; eicosapentaenoic acid [EPA], docosahexaenoic acid [DHA], and arachidonic acid [AA]).

Lead. Although we did not find a sufficient number of studies suitable for a meta-analysis of lead exposure and ADHD, a recent review[61] found that children and laboratory animals exposed to lead show deficits in many aspects of attention and executive function that are impaired in children with ADHD, including tests of working memory, response inhibition, vigilance, and alertness. Higher-level lead exposure has also been associated with a clinical diagnosis of ADHD.[3,62-64]

Manganese. Manganese (Mn) is an essential element, but overexposure can have neurotoxic effects. In particular, exposure to subtoxic levels of Mn has been associated with learning and attention problems, hyperactive behavior, and learning problems with neurofunctional alterations characterized by neuromotor and cognitive deficits and mood changes. A case–control study by Farias *et al.*[65] found that children with ADHD had higher serum Mn levels compared with controls. This finding was consistent with those of previous studies that documented a link between Mn exposure and hyperactive behaviors. Interestingly Mn concentrations were significantly reduced from baseline values following MPH exposure.[65]

Environmental Risk Factors: Heavy Metals: Nutrition

Ferritin (Iron Stores). Seven studies specifically assessing iron status in children with ADHD were available for meta-analysis.[66-72] All of these assessed serum ferritin levels, a peripheral marker of iron status in body tissue including

brain. Excluding Millichap *et al.*[68] for presence of medicated patients, our meta-analysis found significant differences in the serum concentration of ferritin between patients and controls, with lower levels in ADHD (d = −0.86, Z = 2.52, $p = 0.01$; Figure S10, available online) and substantial heterogeneity in the effect sizes across studies ($p < .00001$, $I^2 = 89\%$). This finding lost significance after Bonferroni correction.

Konofal *et al.*[66] showed that low serum ferritin levels correlated with more severe ADHD symptoms and greater cognitive deficits. Similarly low serum ferritin levels predict increased ADHD symptoms severity as determined by the Conners Rating scale,[71] with measures of hyperactivity[73] and with cognitive impairment.[74] Thus low iron stores may be a biomarker for ADHD.

Zinc. Zn is biologically relevant to ADHD because Zn regulates the dopamine transporter, a target of the stimulant medications that treat ADHD. Zn levels have been evaluated in serum,[69,75,76] plasma,[77,78] urine,[79] and hair.[79] All of these studies found lower levels of Zn in ADHD than the controls (Figure S11, available online, and Figures 1 and 2). In our meta-analysis, which excluded Bekaroglu *et al.*[75] because they did not specify whether the patients were on medications, we found significantly lower Zn levels among patients with ADHD (d = −0.88, Z = 3.60, $p = .0003$) and significant heterogeneity ($p = .0002$, $I^2 = 79\%$; Figure S11, available online, and Figure 2). This finding remained significant even after Bonferroni correction.

Several researchers reported a link between Zn levels and the severity of ADHD symptoms.[80] Another report suggested that Zn supplementation might be effective in decreasing ADHD symptoms.[81] Moreover an event-related potentials study showed that Zn-deficient ADHD subjects might have different neurophysiological responses compared with non-Zn-deficient subjects.[77] Recently a significant negative association was reported between Zn levels and parent reported hyperactivity symptoms.[73]

Magnesium. Three studies[65,82,83] reported altered serum magnesium levels in patents compared with controls, but with contrasting results. Lower Mg levels were observed in saliva of ADHD patients.[84]

Polyunsaturated Fatty Acids. A role for polyunsaturated fatty acids (PUFAs) in ADHD was originally proposed following the observation that hyperactive children had physical signs of fatty acid deficiency, including polydispia, polyuria, dry hair, and skin and follicular keratosis. These signs have been found to be at least 30% more frequent in children with ADHD than in controls.

Nine studies of the three PUFAs (EPA, DHA, AA) in RBC were suitable.[85-93] We excluded Germano *et al.*[90] due to insufficient data and Mitchell *et al.*[85] for no formal diagnosis of ADHD. Three studies[86,88,91] were excluded for using medicated patients. The remaining studies[87,89,92] were not enough to conduct meta-analyses. However these studies suggest that levels of EPA, DHA and AA globally did not differ between patients with ADHD and controls.

In serum, two studies were performed.[75,93] However, no comparison was possible because no levels for EPA, DHA, or AA were reported by Bekaroglu *et al.*[75] Irmisch *et al.*[83] and Pivac *et al.*[94] analyzed other lipid/lipoprotein markers.

Although these biomarker studies do not suggest a relation between PUFAs and ADHD diagnosis, a meta-analysis of 10 randomized placebo-controlled trials found that omega-3 fatty acid supplementation demonstrated a small but significant effect in improving ADHD symptoms.[95]

Hypothalamus–Pituitary–Adrenal Axis Pathway

Thirteen case control studies of ADHD interrogated the HPA axis. Of those, nine were suitable for meta-analysis of salivary basal cortisol.[26-33,96] However McCartly *et al.*[96] was excluded for including patients on medication. Our meta-analysis found lower baseline salivary levels of cortisol in patients with ADHD compared with controls (d = −0.31 Z = 3.82, $p = .0001$; Figure 4). This finding remained significant even after Bonferroni correction. We found no heterogeneity in effect sizes across studies ($p = .65$, $I^2 = 0\%$).

The regression results indicated no publication bias in SMD (intercept = −0.55, 95% CI = −4.20 to 3.11, $p = .72$; Table S2, available online), whereas asymmetry was suggested by the WMD funnel plot ($p = .018$ according to Egger's test; Table S2, available online).

Only one study assayed urinary cortisol[97]; the investigators found no differences between patients and controls. Ferguson *et al.*[53] and Ma et al[98] analyzed plasma levels, but the results were contrasting, with negative results in Ferguson *et al.*[53] and lower levels associated with ADHD in Ma *et al.*[98]

In summary, lower baseline salivary cortisol concentrations could be a useful biomarker for

ADHD. Some studies[28] found alterations in HPA axis function in stressed children with ADHD, especially for those exhibiting severe hyperactivity. Finally, single acute doses of stimulants such as D-amphetamine or MPH have been shown to increase circulating cortisol, an effect that appears to be related to the ability of these substances to trigger DA release in the central nervous system (CNS). Similarly, stimulant medication abolished the reduction in cortisol seen in patients with ADHD and oppositional defiant disorder.[99]

One study reported lower salivary concentrations of the neuroactive steroid dehydroepiandrosterone (DHEA) in patients with ADHD compared with controls[96,100] and DHEA levels and DHEA/cortisol ratios were independently correlated with composite scores of CPT distractibility and CPT impulsivity.[96,100] Interestingly MPH and bupropion increase plasma/saliva levels of this neurosteroid.[100,101]

DISCUSSION

By using a statistical method that combines results from all available studies, we provide evidence that peripheral measures of metabolites in blood and urine are different between children with and without attention deficit hyperactivity disorder (ADHD). Our results provide further support for the idea that monoaminergic systems as well as HPA axis are dysregulated in ADHD and that exposure to lead and zinc may be risk factors. They also raise the possibility that peripheral measures may be useful as biomarkers for diagnosis, but more work is needed before using them in clinical practice.

This review sought to assess biomarkers as potential diagnostic markers for ADHD by reviewing and meta-analyzing prior studies assessing peripheral biochemical measures in patients with ADHD and controls. The identification of peripheral biomarkers, which provide molecular signatures of disease, could potentially improve diagnostic classification. Also the identification and validation of biomarkers for a disorder has the potential application as indicators of disease status, course of the illness and potentially as targets to monitor and predict response to therapeutics.

Table 1 provides an overview of the five significant findings that emerged from our meta-analyses after Bonferroni correction for multiple

TABLE 1 Summary of Significant Standard Mean Difference Meta-analyses Findings

Source	Biomarkers Symbol	d	p	Significant after Bonferroni correction?	Significant Heterogeneity?	Publication Bias?	Associated with Drug Response?	Associated with Symptoms Severity?	Associated with Neurophysiological/ Cognitive functioning?
Urine	NE	0.41	.003	Yes	No	No	Yes: ↓	Yes	No
Urine	MHPG	−0.43	.002	Yes	Yes	No	Yes: ↓	Yes	No
Platelet	MAO	−1.05	<.0001	Yes	Yes	No	Yes: ↑	Yes	No
Urine	NM	0.51	.05	No	Yes	No	No	No	No
Urine	M	0.45	.009	No	No	No	No	No	No
Serum	ferritin (iron stores)	−0.86	.01	No	Yes	No	No	Yes	Yes
Serum/plasma/urine	Zn	−0.88	.0003	Yes	Yes	No	No	Yes	Yes
Saliva	Cortisol	−0.31	.0001	Yes	No	No	Yes: ↑	Yes	No

Note: MAO = Monoamine oxidase; MHPG = 3-methoxy-4-hydroxyphenylethylene glycol; M = Metanephrine; NE = Norepinephrine; NM = Normetanephrine; Zn = Zinc.

testing. Four of these significant findings found support in studies of response to ADHD medications, five in studies of symptoms severity and one in studies of neurophysiological or cognitive functioning. Among these significant associations, we found no significant evidence of publication bias by significance testing, although those conclusions could be due to low power in some cases as suggested by the funnel plots. Significant heterogeneity of effect sizes across studies was found for three of the five biomarkers that significantly differentiated ADHD from control subjects. These findings of heterogeneity could reflect differences in study methodology. They could also be due to interstudy differences in the subjects studied as regards demographics, stress exposure, nutritional status or psychiatric comorbidity. Our finding of significant heterogeneity is consistent with the idea that ADHD is a complex, multifactorial disorder that arises from many risk factors, none of which are necessary or sufficient to cause the disorder. For example, genetic studies suggest that ADHD can arise from different combinations of common or rare risk alleles.[102]

The significant meta-analyses for NE, MAO, and MHPG suggest that reduced MAO activity impairs the degradation of NE and leads to lower levels of MHPG in patients with ADHD. Thus, it is possible that urinary "MAO-NE-MHPG" levels could provide a biochemical marker profile for ADHD. It is also possible that dysregulation of the "MAO-NE-MHPG" pathway is a compensatory response to hypo-noradrenergic synaptic activity in ADHD, but the data reviewed cannot clarify that distinction. Low platelet MAO concentrations could impair the degradation of NE and thus lower levels of MHPG.[10] Moreover because these alterations in urinary levels of "MAO-NE-MHPG" in ADHD appear to be corrected by drug treatment, they could be useful biological markers for both diagnostic assessment and the personalization of therapies. These molecules also predict severity of ADHD symptomatology (NE,[36] MAO,[22,24] MHPG[10,11]). Similarly, urinary biogenic trace amine PEA levels could be a biomarker for the diagnosis of ADHD,[20,57,58] for treatment efficacy,[20,60] and associated with symptoms of inattentivenesss.[59]

The meta-analysis support for the MAO-NE-MHPG triad makes biological sense. A significant fraction of urinary MHPG has its origin in the metabolism of NE within the brain. As much as 30% to 50% of the urinary MHPG comes from the metabolism of NE in the CNS in man. Destruction of NE terminals in the CNS of animals has been shown to result in decreased urinary levels of MHPG. Moreover, MHPG excretion in urine has been shown to vary in response to changes in sympathetic activity or stressful situations. According to some authors,[10] reduced MHPG may be considered secondary to decreased activity of MAO enzyme, the intraneuronal enzyme that catalyzes oxidative deamination of monoamines and is thus involved in the metabolism of NE.

The results in Table 1 also implicate serum/plasma/urine Zn and serum ferritin (iron stores) levels as potential biomarkers for ADHD. All studies confirm that lower levels of serum/plasma/urine Zn and serum ferritin (iron stores) (regardless of whether we ignore the Bonferroni correction) were associated with the ADHD diagnosis. Both Zn levels and serum ferritin levels were associated with the severity of ADHD symptoms[66,80,71] as well as behavioral and cognitive functioning.[103] Interacting effects of Zn and iron have been demonstrated in child cognition and behaviour, suggesting that practitioners' dietary recommendations to parents of preschool children should consider not only children's growth and physical health but also their behavioral and cognitive functioning.[103]

The implication of both iron and Zn in ADHD makes biological sense. Both are essential cofactors in the production of DA and NE and both play a pivotal role in oxidant/antioxidant mechanisms. Dysregulation of iron and Zn levels could lead to increased susceptibility to oxidative damage of tissues which is a reasonable hypothesis for the pathophysiology of ADHD. Iron supplementation protects the blood–brain barrier against lead and iron deficiency increases the toxic effects of lead, suggesting a potent neuroprotective effect of iron supplementation on dopaminergic dysfunction due to lead exposure.[104] Although we did not find a sufficient number of studies for a meta-analysis of lead, the available studies suggest that it is a risk factor for ADHD. Because Zn and iron are associated with DA metabolism, it can be speculated that low Zn and iron levels might be associated with impaired dopaminergic transmission in subjects with ADHD.

The implication of environmental risk factors in ADHD is especially important because these are potentially modifiable risk factors. For example, lead is used in many products—including

building materials, paint, pipes and gasoline, due to its high degree of malleability, ductility, and corrosion resistance. Although US efforts to ban the use of lead in paint and gasoline began in the 1970s, contamination persists in soil, dust, and water. Contamination of children's toys, jewelry, imported candies/foods, folk medicines, cosmetics, and some ceramic glazes also occurs.[5]

Table 1 also indicates that low salivary cortisol levels could be a useful biomarker for ADHD. Our meta-analysis shows that baseline cortisol differs between ADHD and control subjects, which is consistent with other findings showing that baseline cortisol is normalized by stimulant treatment.[99] The HPA axis plays an important role in regulating CNS neurotransmitters and behavior, such as attention, emotion, learning, memory and movement. When stimulated, neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotrophin releasing hormone (CRH) into the hypophyseal portal circulation. In the anterior pituitary, CRH induces production of adrenocorticotropic hormone (ACTH), which is released into the systemic circulation to stimulate the formation and release of cortisol from the adrenal cortex. Elevated serum cortisol immediately begins to interact with corticoid receptors to inhibit the stress response via negative feedback. Because we found lower levels of cortisol in patients with ADHD, it is possible that this reflects an impaired ability to regulate stress responses. This may indicate a lower reactivity of the HPA axis in ADHD, which could be due to an elevated threshold for detection of stressors or a subsensitivity of the HPA axis itself.

Few studies were conducted on NPY, DHEA, and Mn. The results of some studies[19,45,65,96,100,101] suggest that these could be additional potential biochemical markers for ADHD, but more studies are needed.

Studies of peripheral metabolites have been viewed with skepticism. It may be argued that urine monoamine levels primarily reflect changes in the peripheral autonomic system and that their measurement has little value for disorders of the brain. However peripheral sympathetic nervous system activity correlates with that in the locus coeruleus and altering peripheral monoamines has been found to induce central effects.[105] Moreover, a review of the literature shows that neurotransmitters excreted in urine may have a place in clinical practice as biomarkers of nervous system function.[106] In support of urinary neurotransmitter assessment, studies have demonstrated that intact neurotransmitters are transported from the CNS to the periphery, via specific blood–brain barrier (BBB) transporters, followed by renal filtration of neurotransmitters with subsequent excretion in the urine.[106] In addition, animal studies have suggested a positive relationship between neurotransmitters excreted in urine and neurotransmitters in the CNS.[106]

Although it remains unclear whether serum/plasma concentrations can reflect CNS activity, there is some evidence for some biomarkers, for instance serum levels of the neurotrophin brain-derived neurotrophic factor (BDNF) reflect alterations in the brain.[107]

Few studies performed assays using plasma, and those that did had small sample sizes and high heterogeneity across studies. In the specific case of NE, contrasting results were obtained from plasma and urine studies. When we combined the results from both sources in the meta-analysis, the results were not significant, and high heterogeneity among studies was observed, due to the plasma results (data not shown).

Saliva is a noninvasively obtained peripheral biological fluid. It has been reported that salivary cortisol correlates closely with plasma free cortisol and it is well established that salivary cortisol levels reflect cortisol secretion.

Methodological procedures are an important source of heterogeneity in biomarker studies. For instance, repeated measurements on each child will reduce variability, but this was not performed in all studies. Moreover, over time, methods for measuring catecholamines have improved in both sensitivity and specificity. Early catecholamine research was hampered by the limitations of colorimetric bioassays that lacked adequate sensitivity and specificity. The fluorometric methods currently available can measure peripheral biomarkers with greater precision. More recently, high-performance liquid chromatography (HPLC) methodology has greatly enhanced the specificity and sensitivity of these measurements and has allowed larger-scale clinical applications. Enzyme-linked-immunosorbent assay (ELISA) and radioimmunoassay (RIA) technologies offer the greatest methodological improvements, allowing higher throughput, increased sensitivity and specificity, and reduced cost.[106] Indeed, we observed substantial hetero-

geneity for those meta-analyses that incorporated studies over a wide temporal range: for instance, this is the case of plasma NE (from 1990 to 2003) and EPI (from 1995 to 2003), urinary EPI (from 1970 to 2007), NM (from 1971 to 2003), and platelet MAO (from 1982 to 2009) levels. This highlights the need for future studies to use the most accurate methods available to heterogeneity and maximize disease associations.

Future studies will have to take into account the deep integration of "omics" sciences such as the "phenomics," "epigenomic," "proteomics," and "metabolomics." In fact, a better understanding of the interaction network of genes, proteins, and biochemical processes in relation to more accurate clinical profiles, by using new high-throughput computational methods, will allow us to identify a list of biomarkers both for the optimization of the diagnostic assessment as well as for the personalization of therapies. Despite the potential contributions of "omics" science, genomewide association studies have not discovered common DNA variants that predispose to ADHD and, although studies of copy number variation have implicated rare variants,[108] these are too rare to be useful as diagnostic biomarkers. Although peripheral studies of mRNA expression may one day provide useful biomarkers of ADHD, such studies have yet to be reported. In addition to assessing biomarkers by assaying gene expression and protein profiles in peripheral tissues, there is much potential for in vitro fibroblast models. Fibroblasts can be first transformed into pluripotent stem cells and then differentiated into specific types of neurons.

Currently, no biomarkers for ADHD have achieved the status of clinical utility as a diagnostic tool. Our review suggests that peripheral metabolites may one day be useful in that regard, but more work is needed to determine if the statistical significance of our findings translate into diagnostic utility. Our meta-analysis of iron and zinc biomarkers, however, suggests that some patients with ADHD with low levels of these nutrients might benefit from supplementation. This would be expected if the low levels played a causal role in ADHD as opposed to being epiphenomena. Children with ferritin levels below 20 ng/mL may benefit from a dietary iron evaluation, followed by a diet that features appropriate amounts of iron supplementation.[109]

With regard to zinc supplementation, a placebo-controlled trial reported that doses up to 30 mg/day of zinc were safe for at least 8 weeks, but the clinical effect was equivocal except for the finding of a 37% reduction in amphetamine optimal dose with 30 mg per day of zinc.[110] In another controlled trial, 6 months of zinc supplementation did not show efficacy for mental health outcomes, but increases in serum zinc levels predicted decreased internalizing symptoms in children at risk for zinc deficiency.

Our conclusions should be tempered by several limitations. Although some biomarkers differentiate patients with ADHD from healthy controls, little information is available about the specificity of these biomarkers for ADHD in comparisons with other disorders. A related problem is that we did not provide meta-analyses of these biomarkers for other disorders, which could have indirectly addressed the issue of specificity. Unfortunately, it was not feasible to review such a huge literature in the space of one journal article. Co-occurring psychiatric disorders or social status could account for case-control differences in biomarkers. Another concern is that older studies had been conducted on patients diagnosed with early, nonstructured diagnostic categories such as minimal brain dysfunction.

Another limitation of our meta-analyses is that they do not take into account study differences in assay sensitivity, method of sample collection, control for confounds such as diet and exercise, time of sample collection, and method for making the ADHD diagnosis. Ideally, such covariates would have been included in a meta-analysis regression, but that was not possible because of the extent of data available. Moreover, it is possible that some of the biomarker results are epiphenomena of ADHD. For example, if ADHD is associated with stressful environments, then the results for salivary cortisol could be due to exposure to these environments rather than ADHD. Likewise, although patients were not receiving treatment at the time of biomarker assessments, it is possible that prior treatment experiences influence biomarker assays. This latter concern is, however, mitigated by the fact that for our significant findings, treatment would be expected to reduce the differentiation of ADHD and control subjects.

An additional limitation is that many of the NE studies were generated by the same group.

This may have biased our estimate of interstudy heterogeneity, and it calls for additional studies to allow a clearer generalization and replication of results. Given these limitations, we can conclude only that the measures that we have reviewed are potentially useful and must await future studies to determine their ultimate clinical utility.

Although the results of our statistically significant meta-analyses are promising, when considering the potential utility of biomarkers, one must address not only the statistical significance of effects, but also whether the magnitude of the effect will translate into a clinically useful measure. The largest significant effect size from our meta-analyses was 1.4. As pointed out by Zakzanis *et al.* [111] an effect size of approximately 3.0 is needed for a clinical test that is suitable for diagnostic purposes. It is possible that a larger effect size would result from a multivariate predictor that uses each of the significant biomarkers in Table 1. Unfortunately, no study has presented the diagnostic accuracy of a panel of biomarkers, so clarifying this point will require additional research.

In conclusion, our review and meta-analyses provide evidence that ADHD is associated with peripheral levels of MAO, NE, MHPG, Zn, ferritin, and cortisol. Although these should not be used for diagnostic purposes in clinical practice, further studies are warranted to replicate these findings, to assess their specificity for ADHD, and to quantify the degree to which they are sufficiently precise to be useful in clinical settings. &

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FIGURE S1 Fixed forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of urinary dopamine (DA) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit.

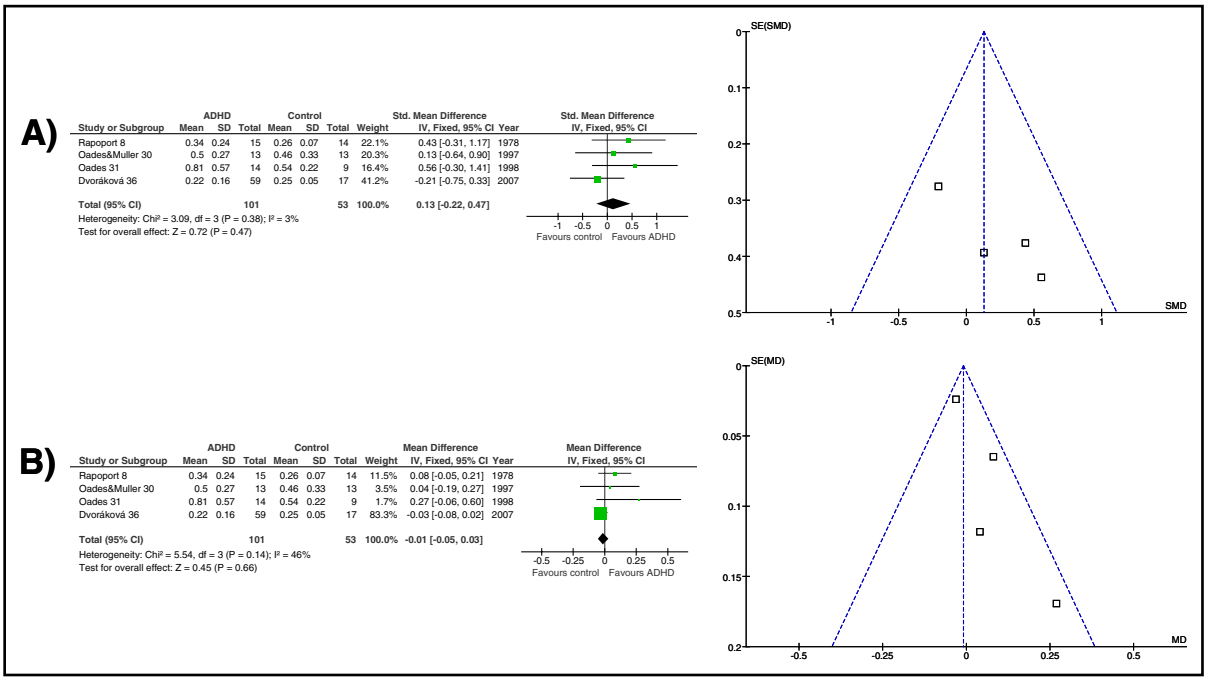

FIGURE S2 Fixed forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of urinary homovanillic acid (HVA) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit.

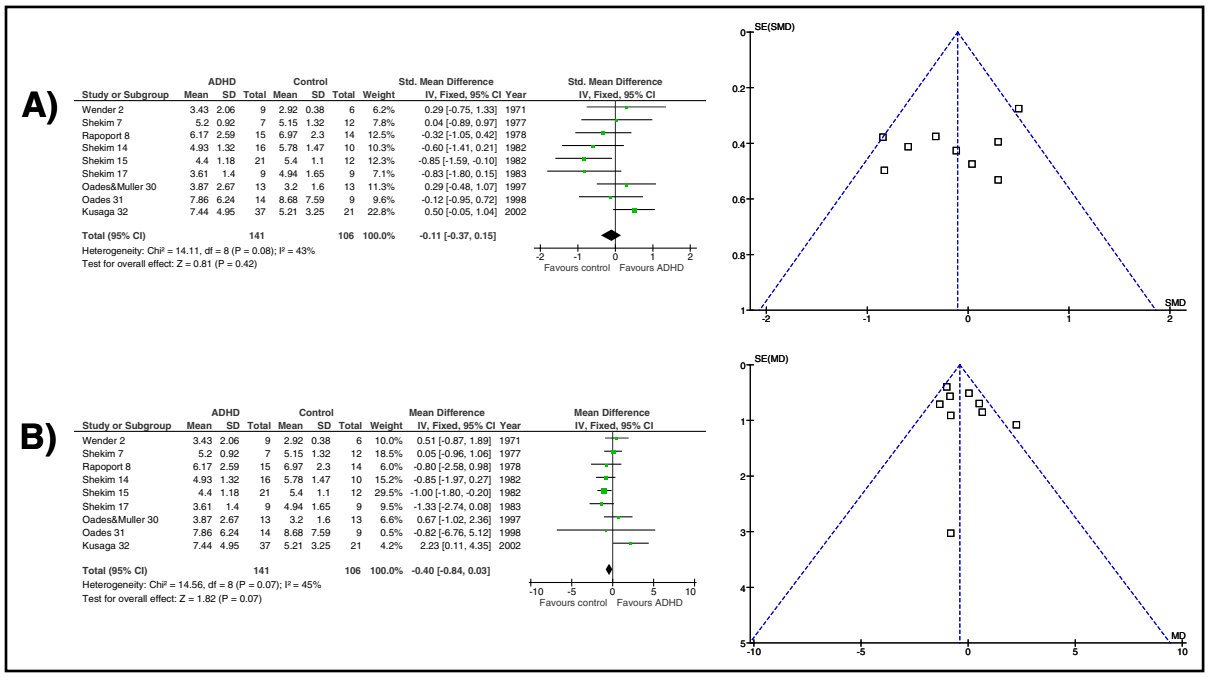

FIGURE S3 Fixed forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of urinary norepinephrine (NE) levels. Note: ADHD =attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit.

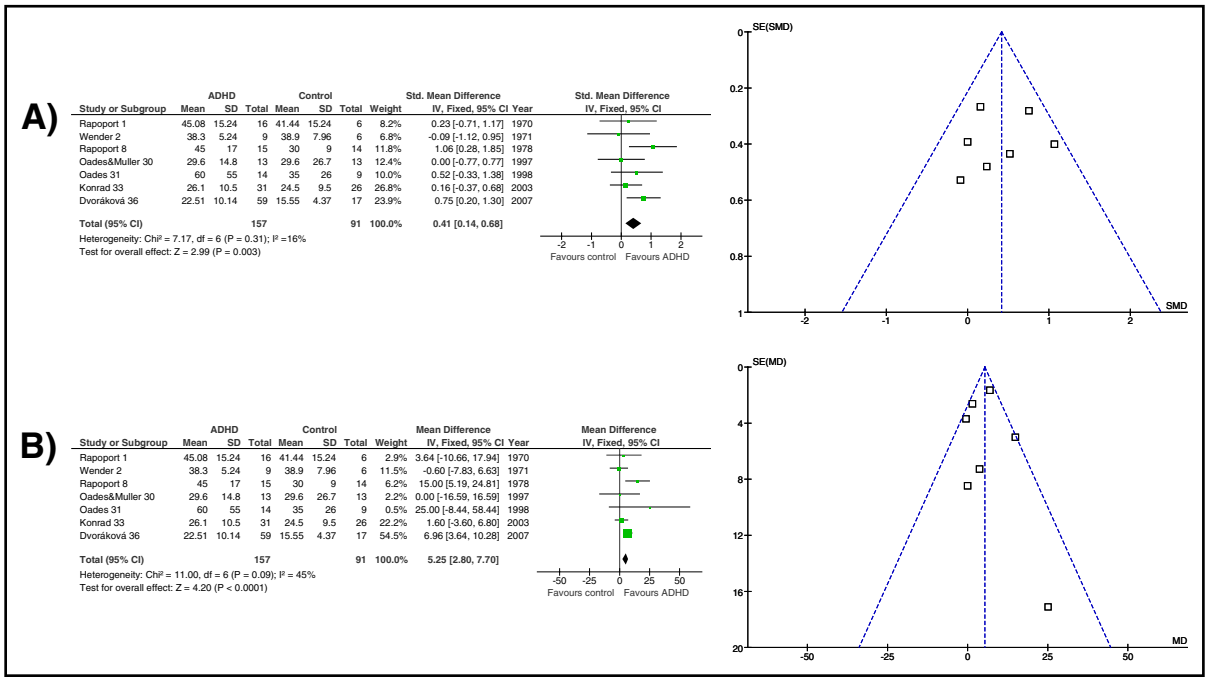

FIGURE S4 Fixed/random forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of plasma norepinephrine (NE) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit; Tau^2 = estimate of the between-study variance in a random-effects meta-analysis.

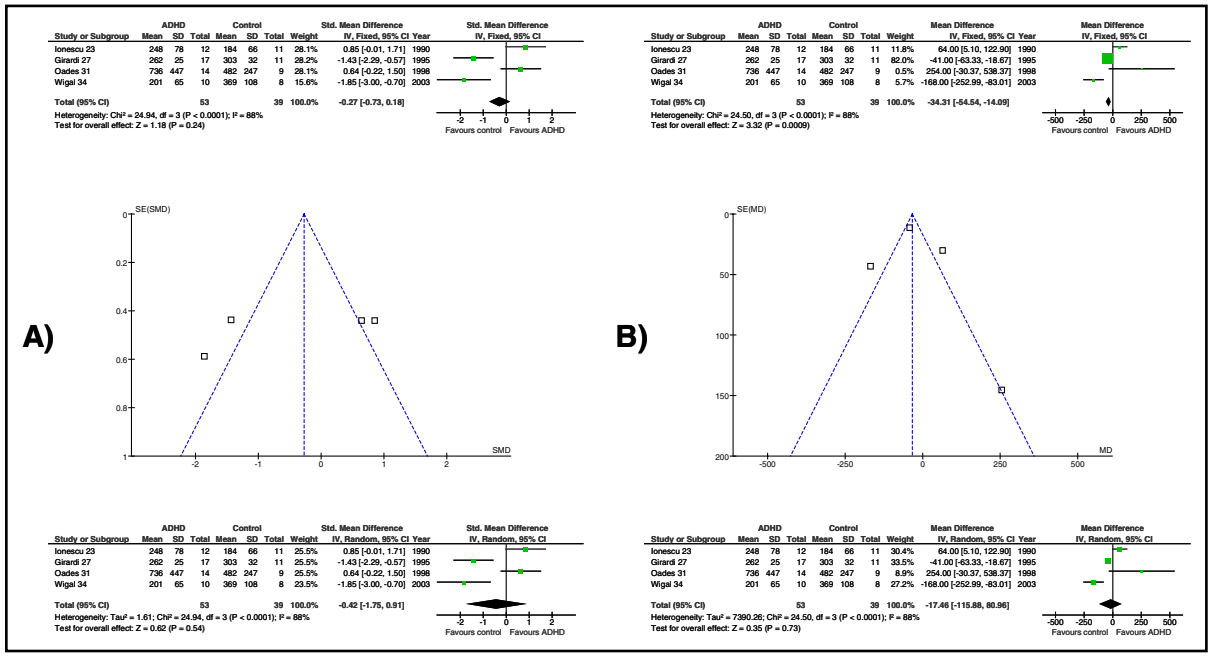

FIGURE S5 Fixed/random forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of urinary normetanephrine (NM) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit; Tau^2 = estimate of the between-study variance in a random-effects meta-analysis.

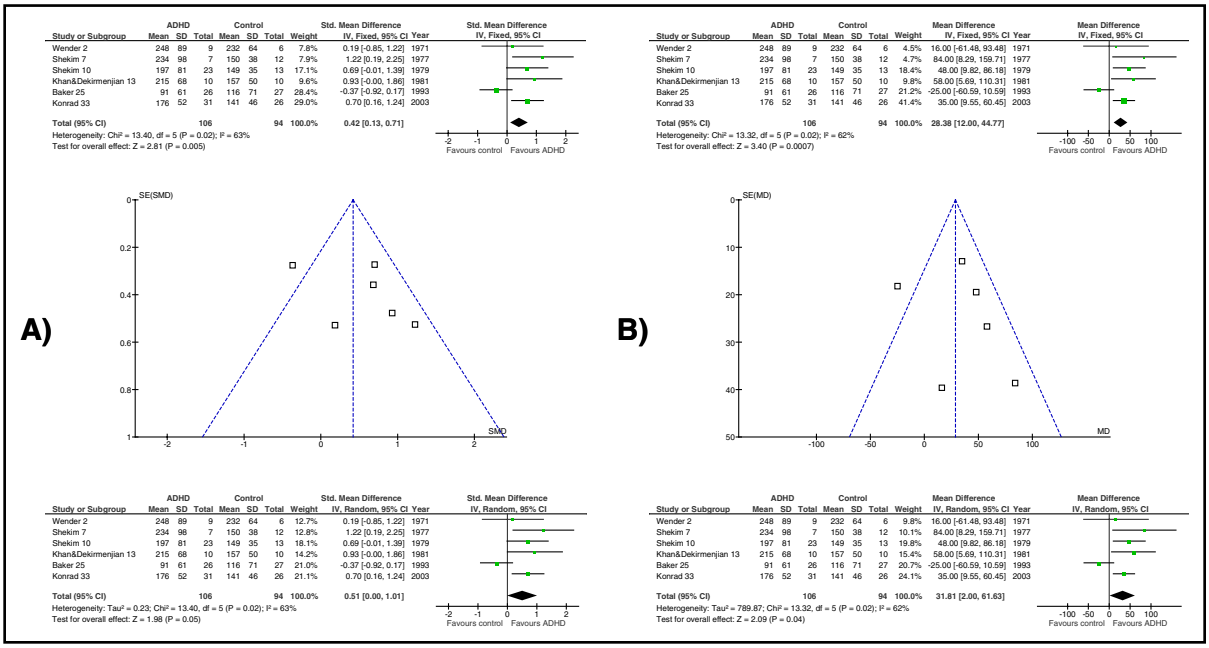

FIGURE S6 Fixed/random forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of urinary epinephrine (EPI) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit; Tau^2 = estimate of the between-study variance in a random-effects meta-analysis.

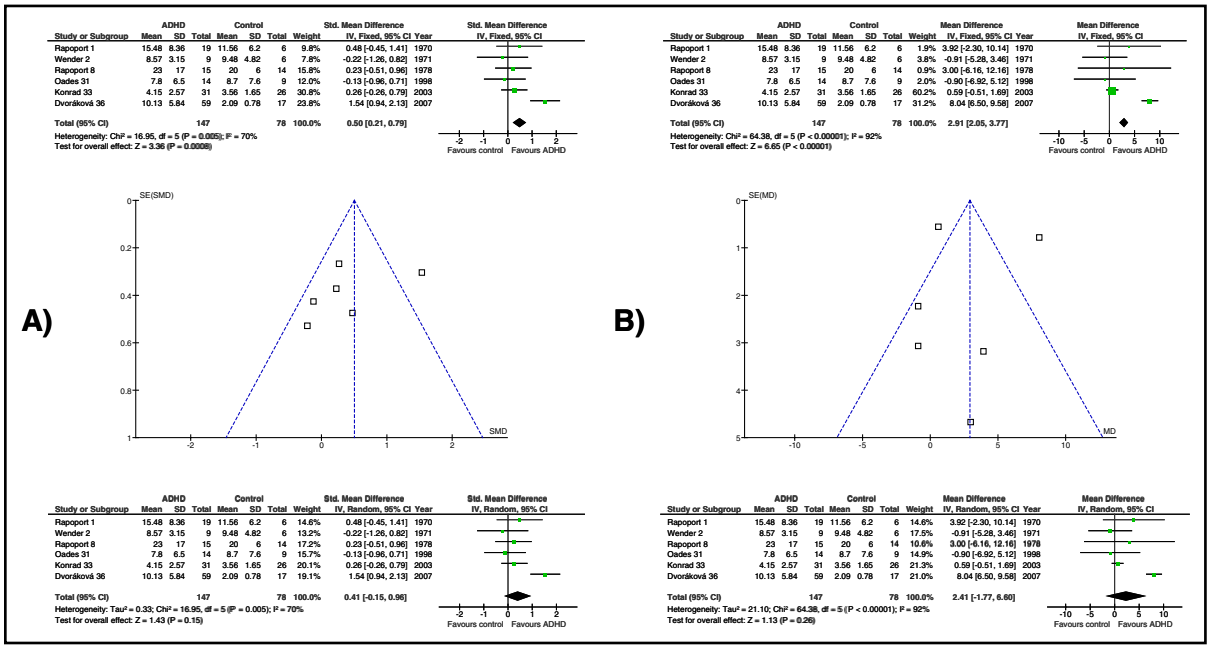

FIGURE S7 Fixed/random forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of plasma epinephrine (EPI) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit; Tau^2 = estimate of the between-study variance in a random-effects meta-analysis.

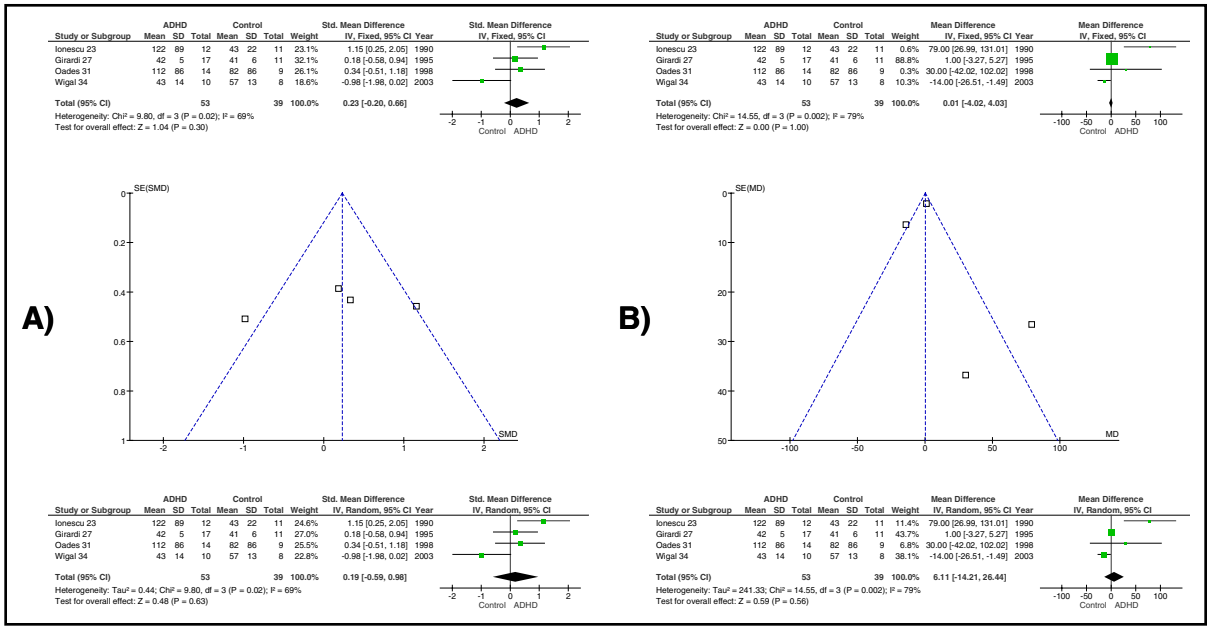

FIGURE S8 Fixed forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of urinary metanephrine (M) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit.

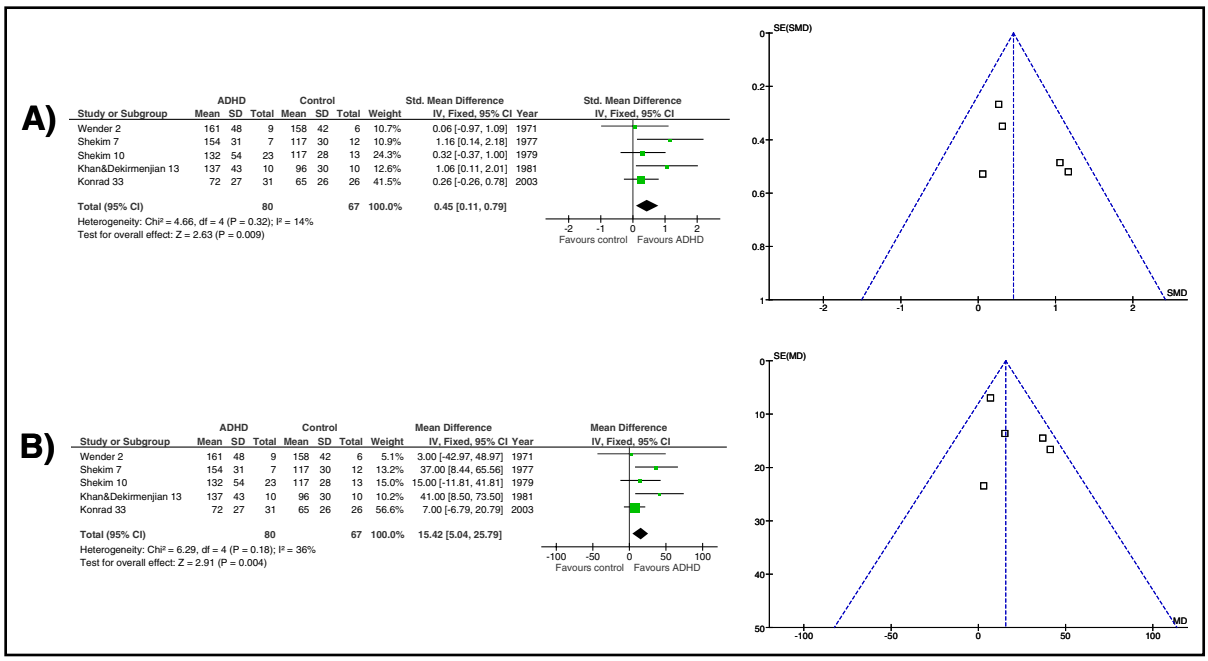

FIGURE S9 Fixed forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of urinary 5-hydroxyindoleacetic acid (5-HIAA) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit.

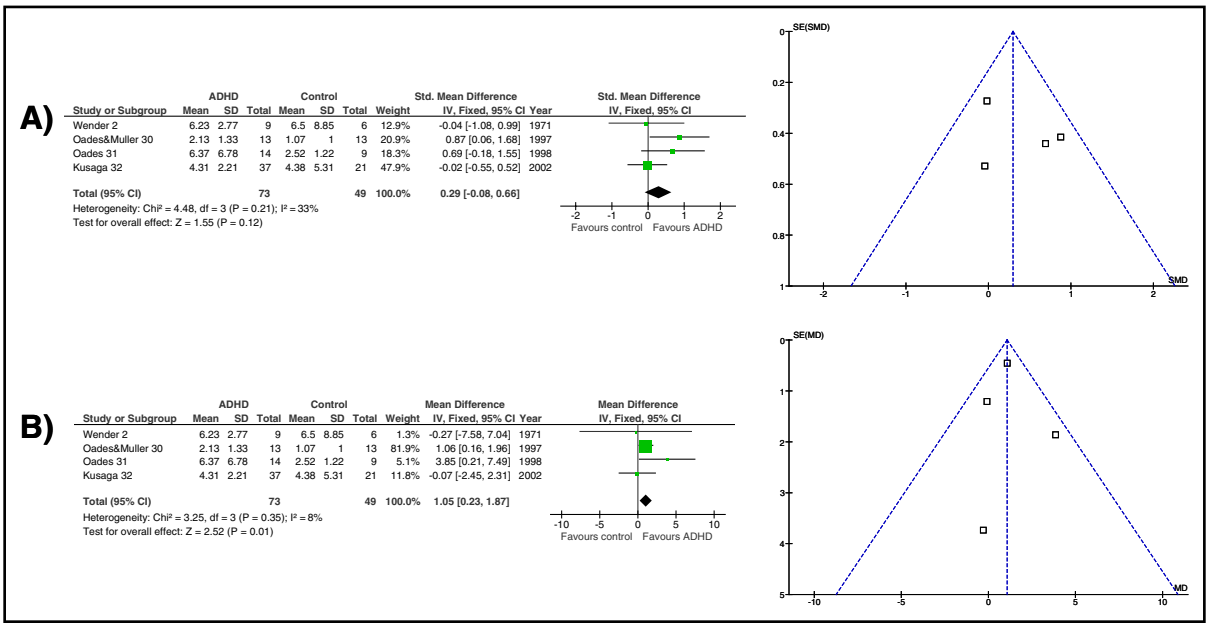

FIGURE S10 Fixed/random forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of serum ferritin (iron stores) levels. Note: ADHD = attention-deficit/hyperactive disorder; Chi^2 = χ^2 test of goodness of fit; Tau^2 = estimate of the between-study variance in a random-effects meta-analysis.

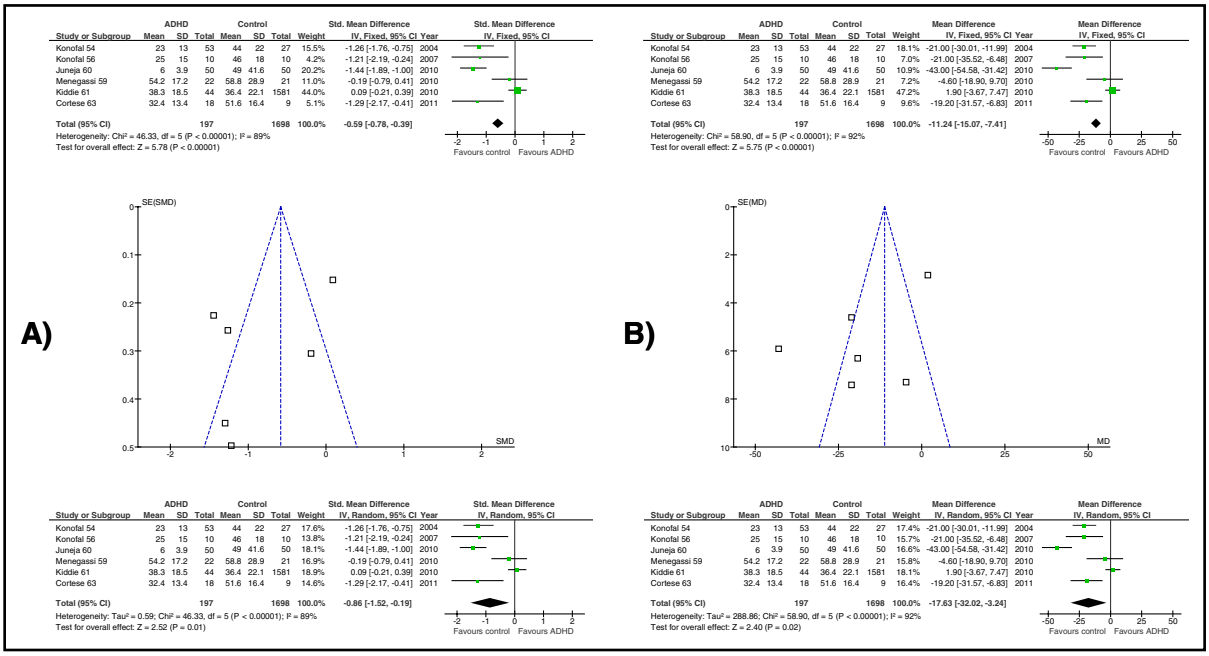

FIGURE S11 (1) Case-control studies reporting lower zinc (Zn) levels in patients with attention-deficit/hyperactivity disorder (ADHD) as compared to controls in several biological sources ($p < .05$ is considered significant). (2) Note: Fixed/random forest and funnel plots for standard mean differences (SMD) (A) and weighted mean difference (WMD) (B) from meta-analysis of serum, plasma, urine, and hair Zn levels. $Chi^2 = \chi^2$ test of goodness of fit; Tau^2 = estimate of the between-study variance in a random-effects meta-analysis

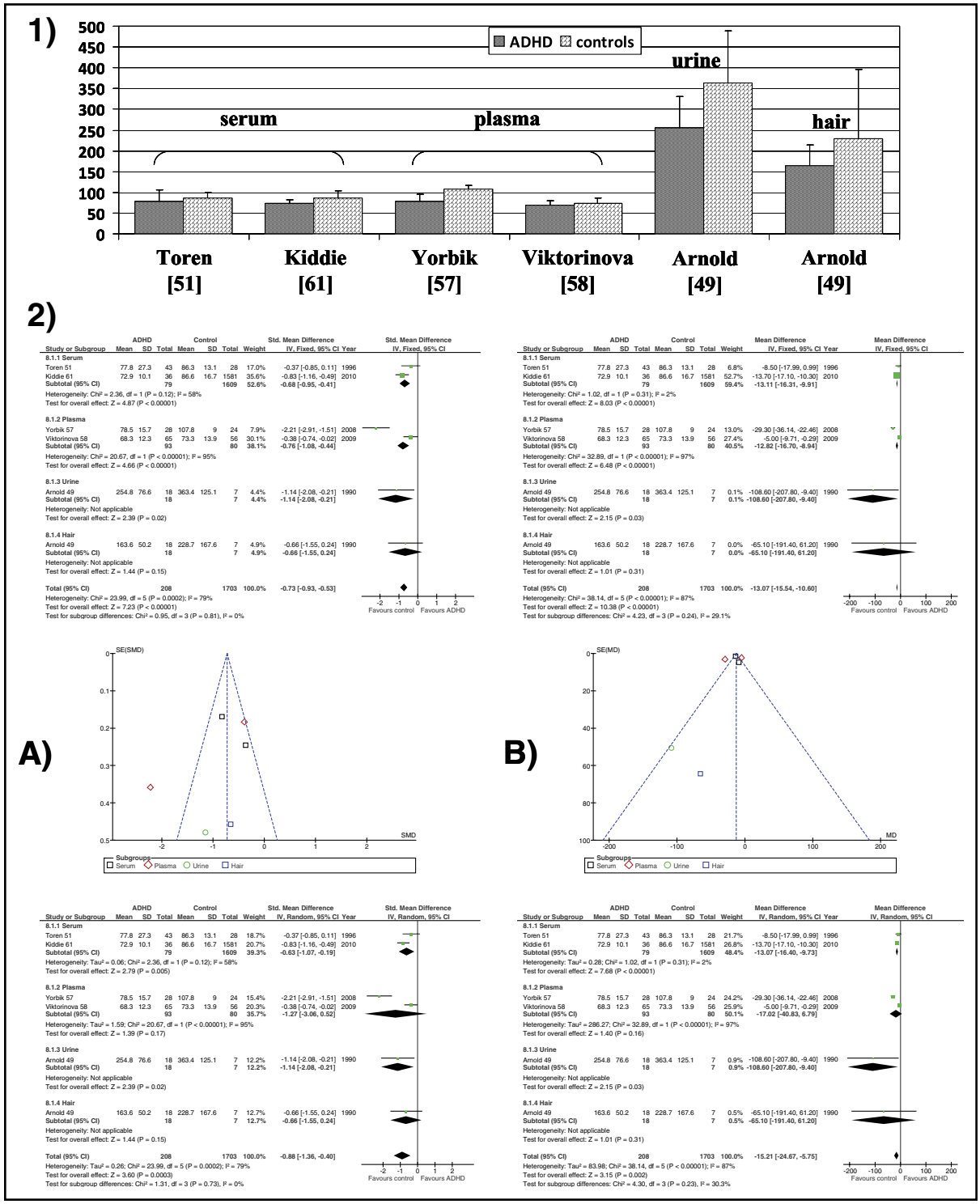

FIGURE S12 Metabolic pathways of Dopamine, Norepinephrine, Epinephrine and Serotonin neurotransmitters. Note: 5-HIAA=5-hydroxyindoleacetic acid; AADC = amino acid decarboxilase; COMT = cathecol-O-methyl-tranferase; DBH = dopamine beta hydroxilase; DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovanillic acid; L-DOPA=dihydroxyphenylalanine; MAO = monoamine oxidase; PLP = pyrydoxal phosphate; PNMT = phenylethanolamine N-methyltranferase; TH = tyrosine hydroxylase; TPH = tryptophan hydroxilase; Trp = tryptophan; Tyr=tyrosine.

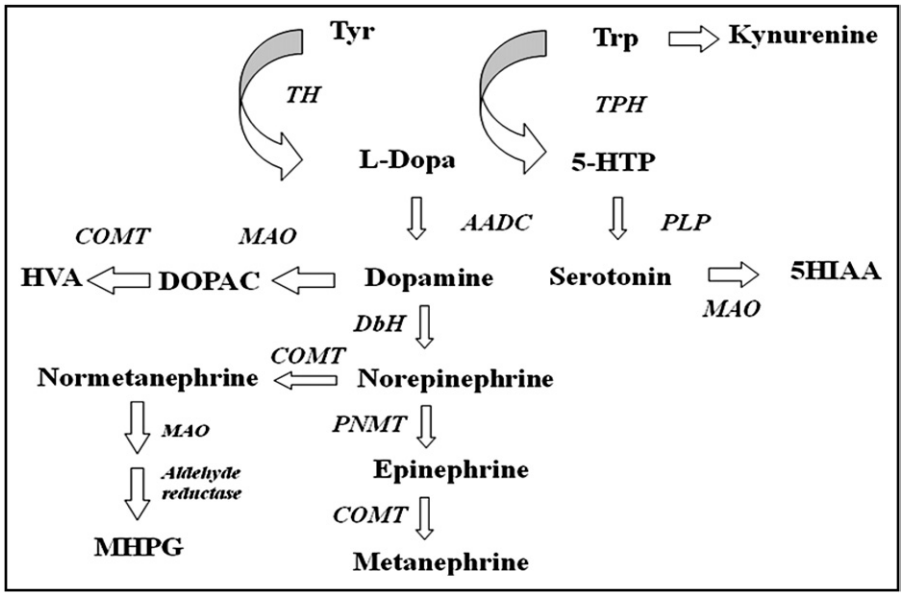

TABLE S1 Summary of Case-Control Studies Selected for Performing, Where Possible, the Various Meta-analyses

Pathway	Authors, Publication Year[Ref]	Population	Biological Fluids	Sample Size[a]	% Male[a]	Age,[a] mean (y)	Biomarkers	Diagnosis	Exclusion	Reasons
Monoamines, Metabolites, metabolism Enzymes	Rapoport *et al.*, 1970[1]	USA	Urine	19:6	100:100	8:8	EPI, NE	Hyperkinetic syndrome	No	
	Wender *et al.*, 1971[2]	USA	Urine	9:6	89:83	9:10	5-HIAA, HVA, NE, EPI, NM, M, VMA, MHPG	Minimal brain dysfunction	No	
	Rapoport *et al.*, 1974[3]	USA	Platelet	35:19	100:100	9:9	5-HT	Hyperkinetic syndrome	Yes	Few studies for 5-HT
	Bhagavan *et al.*, 1975[4]	USA	Platelet	11:11	nr:nr (matched)	8:8	5-HT, PLP	Hyperkinetic syndrome	Yes	Few studies for 5-HT
	Shetty and Chase, 1976[5]	USA	CSF	23:6	83:67	7:10	HVA, 5-HIAA	Hyperkinetic syndrome	Yes	Few studies in CSF
	Shaywitz *et al.*, 1977[6]	USA	CSF	6:26	100:65	8:9	HVA, 5-HIAA	Minimal brain dysfunction	Yes	Few studies in CSF
	Shekim *et al.*, 1977[7]	USA	Urine	7:12	nr:nr	9:10	MHPG, M, NM, HVA	Hyperkinetic syndrome	No	
	Rapoport *et al.*, 1978[8]	USA	Urine	15:14	100:100	10:9	MHPG, HVA, NE, EPI, DA	Hyperkinetic syndrome	No	
	Shekim *et al.*, 1978[9]	USA	Urine	10:10	100:100	nr:nr (matched)	MHPG	Hyperkinetic syndrome	No	
	Shekim *et al.*, 1979a[10]	USA	Urine	23:13	100:100	9:9	MHPG, M, NM	Hyperkinetic syndrome	No	
	Shekim *et al.*, 1979b[11]	USA	Urine	15:13	100:100	9:9	MHPG	Hyperkinetic syndrome	No	
	Ferguson *et al.*, 1981[12]	USA	Plasma	49:11	86:54	9:10	Tryptophan, 5-HT	Hyperkinetic syndrome	Yes	Few studies for 5-HT, tryptophan
	Khan and Dekirmenjan, 1981[13]	USA	Urine	10:10	100:100	9:9	M, NM, MHPG	Hyperkinetic syndrome	No	
	Shekim *et al.*, 1982a[14]	USA	Urine	21:12	100:100	9:9	MHPG, HVA	Hyperkinetic syndrome	No	

TABLE S1 Continued

Pathway	Authors, Publication Year[Ref]	Population	Biological Fluids	Sample Size[a]	% Male[a]	Age,[a] mean (y)	Biomarkers	Diagnosis	Exclusion	Reasons
	Shekim *et al.*, 1982b[15]	USA	Urine	16:10	100:100	nr:nr	MHPG, HVA	Hyperkinetic syndrome	No	
	Shekim *et al.*, 1982c[16]	USA	Platelet	8:18	75:61	nr:nr	MAO	Hyperkinetic syndrome	No	
	Shekim *et al.*, 1983[17]	USA	Urine	9:9	100	13:11	MHPG, HVA	Hyperkinetic syndrome	No	
	Shen and Wang, 1984[18]	China	Urine	73:57	85:86	10:10	MHPG	Minimal brain dysfunction	No	
	Zametkin *et al.*, 1984[19]	USA	Urine	23:28	100	9:9	PEA, PAA, Phenylalanine, Tyrosine	ADD-H	Yes	Few studies on PEA
	Hoshino *et al.*, 1985[20]	Japan	Plasma	10:12	nr:nr	nr:nr	Tryptophan	ADD	Yes	Few studies for tryptophan
	Shekim *et al.*, 1986[21]	USA	Platelet	22:12	100:100	12:12	MAO	ADD-H	No	
	Bowden *et al.*, 1988[22]	USA	Platelet/Plasma	48:24	nr:nr	11:11	MAO, DBH	ADD-H	Yes No	Few studies for DBH for MAO
	Ionescu *et al.*, 1990[23]	Germany	Plasma	12:11	83:54	9:10	EPI, NE, DA	ADHD (*DSM-III*)	No	
	Baker *et al.*, 1991[24]	USA	Urine/plasma	18:26	nr:nr	10:11	PEA, PAA, Phenylalanine, Tyrosine	ADHD (*DSM-III*)	Yes	Few studies on PEA
	Baker *et al.*, 1993[25]	USA	Urine	26:27	81:44	9:10	MHPG, NM	ADHD (*DSM-III-R*)	No	
	Pliszka *et al.*, 1994[26]	USA	Urine	57:nr	98:nr	9:nr	NE, NM, EPI, M, VMA, MHPG	ADHD (*DSM-III-R*)	Yes	Missing sample size of controls; no baseline levels
	Girardi *et al.*, 1995[27]	USA	Plasma	17:11	76:72	11:11	NE, EPI	Attention deficit disorder (ADD)	No	

TABLE S1 Continued

Pathway	Authors, Publication Year[Ref]	Population	Biological Fluids	Sample Size[a]	% Male[a]	Age,[a] mean (y)	Biomarkers	Diagnosis	Exclusion	Reasons
	Hanna *et al.*, 1996a[28]	USA	Urine	12:16	100:100	9:9	DOPA, DA, NE, EPI, DOPAC, DOPEG	ADHD (*DSM-III-R*)	Yes	No baseline levels
	Hanna et al 1996b[29]	USA	Urine	15:16	100:100	11:11	DOPA, DA, NE, EPI, DOPAC, DOPEG	ADHD (*DSM-III-R*)	Yes	No baseline levels
	Oades and Muller, 1997[30]	Germany	Urine	13:13	85:69	10:11	DA; NE, 5-HT, HVA, MHPG, 5-HIAA	ADHD (*DSM-III-R*)	No	
	Oades *et al.*, 1998[31]	Germany	Urine/plasma	14:9	93:56	10:11	NPY, EPI, NE, HVA, DA,5-HT, 5-HIAA, MHPG	ADHD (*DSM-III-R*), ICD-9	No	
	Kusaga *et al.*, 2002[32]	Japan	Urine	37:21	nr:nr	9:8	PEA, MHPG, HVA, 5-HIAA	ADHD (*DSM-IV*, ICD10)	No	For MHPG, HVA, 5-HIAA
									Yes	Few studies on PEA
	Konrad *et al.*, 2003[33]	Germany	Urine	31:26	nr:nr	10:10	EPI,NE, MN, M	ADHD (*DSM-IV*)	No	
	Wigal *et al.*, 2003[34]	USA	Plasma	10:8	nr:nr (matched)	8:9	DA, EPI, NE	ADHD (*DSM-IV*)	No	
	Roessner *et al.*, 2006[35]	Germany	Serum	27:42	nr:nr	10:10	SSAO	ADHD (*DSM-IV*, ICD10)	Yes	One study on SSAO
	Dvoráková *et al.*, 2007[36]	Slovak republic	Urine	57:17	82:47	10-11	EPI, NE, DA	ADHD (*DSM-IV* -TR)	No	
	Roessner *et al.*, 2007[37]	Germany	Urine	42:24	nr:nr	12:24	TIQ	ADHD (*DSM-IV* -TR)	Yes	One study on TIQ
	Coccini *et al.*, 2009[38]	Italy	Platelet	44:26	89:73	9:12	MAO	ADHD (*DSM-IV*)	No	
	Hercigonja Novkovic *et al.*, 2009[39]	Croatia	Platelet	84:30	86:53	9:9	5-HT	ADHD (*DSM-IV*)	Yes	Few studies for 5-HT
	Paclt *et al.*, 2009[40]	Czech republic	Plasma	30:42	nr:nr	9:10	DBH	ADHD (*DSM-IV*, ICD10)	Yes	Few studies for DBH
	Bhaduri *et al.*, 2010[41]	India	Plasma	111:30	100:100	7:9	DBH	ADHD (*DSM-IV*)	Yes	Few studies for DBH

TABLE S1 Continued

Pathway	Authors, Publication Year[Ref]	Population	Biological Fluids	Sample Size[a]	% Male[a]	Age,[a] mean (y)	Biomarkers	Diagnosis	Exclusion	Reasons
	Nedic *et al.*, 2010[42]	Croatia	Platelet	84:64	86:50	9:10	MAO	ADHD (*DSM-IV*)	No	
	Oades *et al.*, 2010a[43]	Germany	Serum	21:21	67:95	9:11	Tryptophan, kynurenine, KA, 3-HK, 5-HIAA	ADHD (*DSM-IV*)	No	
	Oades *et al.*, 2010b[44]	Germany	Serum	20:19	67:95	9:11	Tryptophan, kynurenine, KA, 3-HK, 5-HIAA	ADHD (*DSM-IV*)	No	
Heavy Metals	Nigg *et al.*, 2008[45]	USA	Blood	97:53	64:60	12:15	Pb	ADHD (*DSM-IV*)	Yes	Few studies on lead
	Wang *et al.*, 2008[46]	China	Blood	630:630	69:69	8:8	Pb	ADHD (*DSM-IV* -R)	Yes	Few studies on lead
	Farias *et al.*, 2010[47]	Brazil	Serum	74:35	82:80	8:9	Mn	ADHD (*DSM-IV*)	Yes	One study on Mn
	Nigg *et al.*, 2010[48]	USA	Blood	108:99	71:43	11:12	Pb	ADHD (*DSM-IV*)	Yes	Few studies on lead
Trace Elements										
	Arnold *et al.*, 1990[49]	USA	Urine, hair	18:7	nr:nr	9:9	Zn	ADD-H (*DSM-III*)	No	
	Bekarog˘lu *et al.*, 1996[50]	Turkey	Serum	48:45	69:67	9:9	Zn	ADHD (*DSM-III*-R)	Yes	No info on whether patients were drug free/naive
	Toren *et al.*, 1996[51]	Israel	Serum	43:28	91:86	10:11	Zn	ADHD (*DSM-III*-R)	No	
	Kozielec and Starobrat-Hermelin, 1997[52]	Poland	Serum	116:	81:nr	10:nr	Mg	ADHD (*DSM-III*-R)	Yes	Few studies on Mg in serum
	Chen *et al.*, 2004[53]	Taiwan	Serum	58:52	91:77	8:8	Fe	ADHD (*DSM-IV*)	Yes	No results on ferritin levels
	Konofal *et al.*, 2004[54]	France	Serum	53:27	85:74	9:10	ferritin	ADHD (*DSM-IV*)	No	

TABLE S1 Continued

Pathway	Authors, Publication Year[Ref]	Population	Biological Fluids	Sample Size[a]	% Male[a]	Age,[a] mean (y)	Biomarkers	Diagnosis	Exclusion	Reasons
	Millichap *et al.*, 2006[55]	USA	Serum	68:1053	79:nr	10:10	ferritin	ADHD (*DSM-IV*)	Yes	Patients not drug free/naïve
	Konofal *et al.*, 2007[56]	France	Serum	10:10	90:70	7:7	ferritin	ADHD (*DSM-IV*)	No	
	Yorbik *et al.*, 2008[57]	Turkey	Plasma	28:24	100:100	9:nr	Zn	ADHD (*DSM-IV*)	No	
	Viktorinova *et al.*, 2009[58]	Slovak republic	Plasma	65:54	78:nr	9:9	Cu, Zn, Se, Fe, ferritin, transferrin	ADHD (*DSM-IV*)	No Yes	For Zn No data reported for ferritin levels
	Farias *et al.*, 2010[47]	Brazil	Serum	74:35	82:80	8:9	Fe, Mg, Ca, K	ADHD (*DSM-IV*)	Yes	Few studies on Mg in serum No results on ferritin levels
	Menegassi *et al.*, 2010[59]	Brazil	Serum	22:21	73:71	9:9	Fe, ferritin, transferrin, hemoglobin	ADHD (*DSM-IV*)	No	
	Juneja *et al.*, 2010[60]	India	Serum	50:50	nr:nr	nr:nr	ferritin	ADHD (*DSM-IV*)	No	
	Kiddie *et al.*, 2010[61]	USA	Serum	36:1581	nr:nr	8:8	ferritin, Zn, Cu	ADHD (*DSM-IV*)	No	
	Archana *et al.*, 2011[62]	India	Saliva	20:20	70:70	9:9	Mg	ADHD (*DSM-IV*)	Yes	One study on Mg in saliva
	Cortese *et al.*, 2011[63]	France	Serum	18:9	89:55	11:11	Ferritin, haemoglobin	ADHD (*DSM-IV*-TR)	No	
	Irmisch *et al.*, 2011[64]	Germany	Serum	9:11	100	8:8	Mg	ADHD (*DSM-IV*)	Yes	Few studies on Mg in serum
Essential Fatty Acids										
	Mitchell *et al.*, 1983[65]	New Zealand	RBC	23:20	91:45	10:nr	EPA, DHLA, AA, linoleic acid	Hyperkinetic syndrome	Yes	No formal diagnosis

TABLE S1 Continued

Pathway	Authors, Publication Year[Ref]	Population	Biological Fluids	Sample Size[a]	% Male[a]	Age,[a] mean (y)	Biomarkers	Diagnosis	Exclusion	Reasons
	Mitchell *et al.*, 1987[66]	New Zealand	Serum	44:45	87:82	9:9	EPA, DHA, AA	ADHD (*DSM-III*)	Yes	Few studies in serum
	Stevens *et al.*, 1995[67]	USA	RBC/plasma	46:35	100:100	9:9	EPA, DHA, AA	ADHD (*DSM-III*-R)	Yes	patients not drug free/naïve
	Begaroglu *et al.*, 1996[50]	Turkey	serum	48:45	69:67	9:9	Free Fatty Acids	ADHD (*DSM-III*-R)	Yes	No levels for EPA, DHA, AA; few studies in serum; no info on whether patients were drug free/naive
	Stevens *et al.*, 2003[68]	USA	RBC/plasma	50:24	88:nr	9:nr	EPA, DHA,AA	ADHD (*DSM-III*-R)	Yes	Few studies on PUFAs
	Chen *et al.*, 2004[53]	Taiwan	RBC/plasma	58:52	91:77	8:8	EPA, DHA, AA	ADHD (*DSM-IV*)	Yes	Patients not drug free/naïve
	Joshi *et al.*, 2006[69]	India	RBC	30:29	77:72	8:7	EPA, DHA, AA	ADHD (*DSM-IV*)	Yes	Few studies on PUFAs
	Germano *et al.*, 2007[70]	Italy	RBC/plasma	31:16	90:nr	9:9	EPA, DHA, AA	ADHD (*DSM-IV*)	Yes	No data of EPA, DHA, AA in controls
	Colter *et al.*, 2008[71]	USA	RBC	11:12	82:50	14:14	EPA, DHA, AA	ADHD (*DSM-IV*)	Yes	Patients not drug free/naïve
	Spahis *et al.*, 2008[72]	USA	RBC/plasma	37:35	73:73	9:8	EPA, DHA, AA, Apo, lipids,	ADHD (*DSM-IV*)	Yes	Few studies on PUFAs

TABLE S1 Continued

Pathway	Authors, Publication Year[Ref]	Population	Biological Fluids	Sample Size[a]	% Male[a]	Age,[a] mean (y)	Biomarkers	Diagnosis	Exclusion	Reasons
	Irmisch *et al.*, 2011[64]	Germany	Serum	9:11	100:100	8:8	T/HDL/LDL cholesterol, triglycerides, Lipoprotein Lipase, Phospholipids, Apolipoproteins	ADHD (*DSM-IV*)	Yes	Few studies in lipoproteins
	Pivac *et al.*, 2011[73]	Croatia	Plasma	99:175	nr:nr	9:11	N-glicome	ADHD (*DSM-IV*)	Yes	Few studies in N-glicome
Hypothalamus-pituitary-adrenal axis pathway										
	Kruesi *et al.*, 1989[74]	USA	Urine	15:15	100:100	11:11	Cortisol	ADD-H	Yes	Few studies in urine
	Ferguson *et al.*, 1981[12]	USA	Plasma	49:11	86:54	9:10	Cortisol	Hyperkinetic syndrome	Yes	Few studies in plasma
	Jansen *et al.*, 1999[75]	Netherlands	Saliva	10:15	100:87	10:10	Cortisol	ADHD (*DSM-IV*)	No	
	Snoek *et al.*, 2004[76]	UK	Saliva	23:26	83:77	10:10	Cortisol	ADHD (*DSM-IV*)	No	
	Blomqvist *et al.*, 2007[77]	Sweden	Saliva	18:71	83:66	nr:nr	Cortisol	ADHD (*DSM-IV*)	No	
	Freitag *et al.*, 2009[78]	Germany	Saliva	52:69	79:48	9:10	Cortisol	ADHD (*DSM-IV*)	No	
	Maldonado *et al.*, 2009[79]	Spain	Saliva	33:33	57:45	6:6	Cortisol	ADHD (*DSM-IV* -TR)	No	
	van West *et al.*, 2009[80]	Belgium	Saliva	75:25	84:80	8:9	Cortisol	ADHD (*DSM-IV*)	No	
	Christiansen *et al.*, 2010[81]	Germany	Saliva	62:61	81:80	11:10	Cortisol	ADHD (*DSM-IV*)	No	
	Ma *et al.*, 2011[82]	China	plasma	128:30	100	10:10	Cortisol, ACTH	ADHD (*DSM-IV*)	Yes	Few studies in plasma

TABLE S1 Continued

Pathway	Authors, Publication Year[Ref]	Population	Biological Fluids	Sample Size[a]	% Male[a]	Age,[a] mean (y)	Biomarkers	Diagnosis	Exclusion	Reasons
	McCarthy *et al.*, 2011[83]	USA	Saliva	29:339	66:48	8:7	Cortisol	ADHD (*DSM-IV*)	Yes	Patients not drug free/naive
	Wang *et al.*, 2011a[84]	Taiwan	Saliva	50:50	80:80	8:8	Cortisol, DHEA	ADHD (*DSM-IV*)	No	
	Wang *et al.*, 2011b[85]	Taiwan	Saliva	50:50	80:80	8:8	DHEA	ADHD (*DSM-IV*)	Yes	One study on DHEA

Note: Oades et al., 2000[86] was excluded because the biochemical data on monoamines markers were reported in Oades et al. 1997.[30] Shekim et al. 1987[87] and Eppright et al., 1997[88] were excluded because no response was obtained after contacting the authors for these data. 3HK= 3-OH-kynurenine; 5-HIAA = 5-hydroxyindoleacetic acid; 5-HT = Serotonin; AA = arachidonic acid; ACTH = adrenocorticotropic hormone; ADD = attention-deficit disorder; ADD-H = attention-deficit disorder with hyperactivity; Ca = calcium; CSF = cerebrospinal fluid; Cu = copper; DA = dopamine; DBH = dopamine β-hydroxylase; DHA = docasahexaenoic acid; DHEA = dehydroepiandrosterone; DHLA = dihomogammalinolenic acid; DOPAC = 3,4-dihydroxyphenylacetic acid; DOPEG = 3,4-dihydroxyphenylglycol; EPA = eicosapentaenoic acid; EPI = epinephrine; Fe = iron; HVA = homovanillic acid; K = potassium; KA = kynurenic acid; M = metanephrine; MAO = monoamine oxidase; Mg = magnesium; MHPG = 3-methoxy-4-hydroxyphenylethylene glycol; Mn = manganese; NE = norepinephrine; NM = normetanephrine; NPY = neuropeptide Y; nr = no reported; PAA = phenylacetic acid; Pb = lead; PEA = b-Phenylethylamine; PLP = pyridossal phosphate; PUFAs = polyunsaturated fatty acids; RBC = red blood cells; Ref = reference; Se = selenium; SSAO = semicarbazide-sensitive amine oxidase; TIQ = dopamine-derived tetrahydroisoquinolines; VMA = vanillylmandelic acid; y = years; Zn = zinc.

[a]Patients:controls.

TABLE S2 Rank Correlation Method and Regression Method Tests for Funnel Plot Asymmetry[89,90] for Cortisol, Monoamine-Oxidase (MAO), and 3-Methoxy-4-Hydroxyphenylethylene Glycol (MHPG)

	Cortisol (Saliva)		MAO (Platelet)		MHPG (Urine)	
Current outcome measure	**WMD**	**SMD**	**WMD**	**SMD**	**WMD**	**SMD**
Rank correlation tau-b (continuity corrected)	0	0.0357	−0.5	−0.5	0.1337	−0.0972
Ties	0	0	0	0	1.6885	3.4134
P-Q (SE)	0 (8.0829)	2 (8.0829)	−6 (4.0825)	−6 (4.0825)	15 (20.2073)	−11 (20.2073)
Z	0	0.1237	−1.2247	−1.2247	0.6947	−0.5049
p Value (two-tailed)	1	0.9015	0.2207	0.2207	0.4872	0.6136
Regression method	Egger	Egger	Egger	Egger	Egger	Egger
Regressor weighting	None	None	None	None	None	None
Intercept	−1.3696	−0.5462	−3.9385	−2.1481	−0.1864	−0.2727
95% CI lower limit	−2.4106	−4.2019	−9.1766	−7.9847	−2.7047	−2.8590
95% CI upper limit	−0.3287	3.1094	1.2995	3.6885	2.3320	2.3137
p Value (two-tailed)	0.0181	0.7272	0.0965	0.3261	0.8754	0.8234

Note: P-Q (SE) = difference between number of concordant pairs (P) and number of discordant pairs (Q); SE = standard error; SMD = standardized mean difference; WMD = weighted mean difference.

TABLE S3 Rank Correlation Method and Regression Method Tests for Funnel Plot Asymmetry[89,90] for Epinephrine (EPI), Norepinephrine (NE), Metanephrine (M), Normetanephrine (NM), 5-Hydroxyindoleacetic Acid (5HIAA), Dopamine (DA), Homovanillic Acid (HVA), Ferritin, and Zinc

	EPI (Urine)		EPI (Plasma)		NE (Urine)		NE (Plasma)		M (Urine)		NM (Urine)	
Current Outcome Measure	**WMD**	**SMD**	**WMD**	**SMD**	**WMD**	**SMD**	**WMD**	**SMD**	**WMD**	**SMD**	**WMD**	**SMD**
Rank correlation tau-b (continuity corrected)	0	0	0,1667	0	0	0	0	−0,1667	0,3	0,3	0,1333	0
Ties	0	0	0	0	0	0	0	0	0	0	0	0
P-Q (SE)	1 (5,3229)	−1 (5,3229)	2 (2,9439)	0 (2,9439)	1 (6,6583)	−1 (6,6583)	0 (2,9439)	−2 (2,9439)	4 (4,0825)	4 (4,0825)	3 (5,3229)	−1 (5,3229)
Z	0	0	0,3397	0	0	0	0	−0,3397	0,7348	0,7348	0,3757	0
p Value (two-tailed)	1	1	0,7341	1	1	1	1	0,7341	0,4624	0,4624	0,7071	1
Regression method	Egger	Egger	Egger	Egger	Egger	Egger	Egger	Egger	Egger	Egger	Egger	Egger
Regressor weighting	None	None	None	None	None	None	None	None	None	None	None	None
Intercept	−0,0738	−3,0466	0,9971	−6,3391	0,1414	−0,6666	1,0019	−12,0265	1,5495	2,002	0,9915	2,5509
95% CI lower limit	−7,301	−11,9064	−6,5378	−51,0593	−2,422	−5,4669	−10,6214	−65,1892	−2,6863	−3,2212	−4,4082	−4,2147
95% CI upper limit	7,1534	5,8131	8,532	38,381	2,7049	4,1337	12,6252	41,1363	5,7853	7,2253	6,3912	9,3164
p Value (two-tailed)	0,9787	0,3938	0,6265	0,604	0,8928	0,7357	0,7463	0,433	0,3285	0,3097	0,637	0,3543

	5HIAA (Urine)		DA (Urine)		HVA (Urine)		Ferritin (Urine)		Zinc (Serum, Plasma, Urine, Hair)	
Current Outcome Measure	**WMD**	**SMD**	**WMD**	**SMD**	**WMD**	**SMD**	**WMD**	**SMD**	**WMD**	**SMD**
Rank correlation tau-b (continuity corrected)	0	0	0,5	0,5	0,3056	−0,0278	0	0,1333	0	−0,1333
Ties	0	0	0	0	0	0	0	0	0	0
P-Q (SE)	0 (2,9439)	0 (2,9439)	4 (2,9439)	4 (2,9439)	12 (9,5917)	−2 (9,5917)	−1 (5,3229)	3 (5,3229)	−1 (5,3229)	−3 (5,3229)
Z	0	0	1,019	1,019	1,1468	−0,1043	0	0,3757	0	−0,3757
p Value (two-tailed)	1	1	0,3082	0,3082	0,2515	0,917	1	0,7071	1	0,7071
Regression method	Egger	Egger	Egger	Egger	Egger	Egger	Egger	Egger	Egger	Egger
Regressor weighting	None	None	None	None	None	None	None	None	None	None
Intercept	0,1684	2,0201	1,7796	4,7598	1,311	−2,7929	−5,8268	−4,1458	−1,4637	−2,4128
95% CI lower limit	−4,3012	−9,2539	−0,7613	−1,1446	−1,3886	−8,1028	−14,7175	−12,5753	−6,7116	−9,0418
95% CI upper limit	4,6379	13,294	4,3205	10,6641	4,0106	2,517	3,064	4,2836	3,7842	4,2162
p Value (two-tailed)	0,8861	0,5214	0,095	0,074	0,2885	0,2536	0,1429	0,2438	0,4819	0,3694

Note: P-Q (SE) = difference between the number of concordant pairs (P) and the number of discordant pairs (Q); SE = standard error; SMD = standardized mean difference; WMD = weighted mean difference.