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REVIEW ARTICLE

A review of candidate urinary biomarkers for autism spectrum disorder

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Abstract

Context: Autism is a complex, heterogeneous neurodevelopmental condition with a strong genetic component potentially impacted by various environmental factors influencing susceptibility. There are no reliable laboratory tests available to confirm an autism diagnosis.

Objective: To examine the published literature and identify putative urinary biomarkers of autism.

Methods: A comprehensive literature search was conducted using electronic bibliographic databases.

Results: Putative autism biomarkers were identified that could be categorized according to the key theories that exist regarding the etiology of autism: gastrointestinal factors, immune dysregulation, heavy metal toxicity, neurotransmitter abnormalities, and oxidative stress.

Conclusion: There is scope for specific urinary biomarkers to be useful for identification of autistic metabolic phenotypes.

Keywords: Etiology, gastrointestinal factors, immune dysregulation, heavy metal toxicity, neurotransmitters, oxidative stress

Introduction

Autism is a neurodevelopmental disorder where affected persons have core difficulties with communication and socialization, as well as display restricted and repetitive behaviors (Bacchelli & Maestrini 2006). Autistic disorder (AD), Asperger syndrome (AS), and pervasive developmental disorder–not otherwise specified (PDD–NOS) are collectively termed the autism spectrum disorders (ASD) (Wing 1996). The number of children diagnosed with ASD is reported to have substantially increased globally in recent years and is currently estimated to be 1 in 100–160 children (MacDermott et al. 2007; Baron-Cohen et al. 2009; Kogan et al. 2009).

Recent studies using whole-genome scanning methods, cytogenetics and genetic linkage/association analyses indicate autism has a strong genetic basis (Geschwind & Levitt 2007; Szatmari et al. 2007; Christian et al. 2008; Morrow et al. 2008). In addition, growing evidence points towards gastrointestinal (GI) factors (Horvath et al. 1999; Wang et al. 2009), immunological factors (Careaga et al. 2010), heavy metal toxicity (Grandjean & Landrigan 2006; Kern & Jones 2006; Slotkin et al. 2006), as well as metabolic abnormalities including dysfunctional neurotransmitter systems (McDougle et al. 2005; Zafeiriou et al. 2009) and oxidative stress (Main et al. 2010), all potentially contributing to the etiology of autism.

A common aberration is not consistently seen in all cases of autism suggesting that autism is a group of disorders with each probably having distinctive pathophysiology (Eapen 2011). Currently, ASD is diagnosed on the basis of observed behaviors and assessment of developmental history because there are no reliable, validated laboratory tests available. The combination of abnormal gene expression and environmental stressors can affect biological systems which in turn are reflected in metabolite profiles of bio-fluids (Nicholson et al. 2002). Interest in exploring abnormal metabolite profiles in body fluids, particularly blood and urine, of individuals with ASD has been increasing in recent

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years. Compared with blood, collection of urine samples is relatively simple, convenient, and non-invasive. Although there have been many studies examining potential differences in metabolites in blood, plasma, and serum between individuals with and without ASD, this review focuses on urinalysis.

Identification of altered metabolite profiles in urine from individuals with ASD has the potential to elucidate the important underlying biologic defects thereby facilitating development of better targeted therapies, enable simple and objective diagnosis, identify ASD subtypes allowing for greater individualization of therapeutic decisions, and/or enable monitoring of ASD treatments (Adrien et al. 1989; Bailey & Ulrich 2004; Pardo & Eberhart 2007).

This article reviews the range of putative urinary biomarkers reported to be associated with ASD. The electronic databases, PubMed, Scopus, and Web of Science were searched for this review. References were also hand searched to identify additional citations. Case control studies and case reports were included. Full text copies of all potentially appropriate citations were obtained. The criteria for study inclusion were studies (a) published in English from 1980 to April 2011, (b) conducted in participants diagnosed with autism as described in the ICD-10 (WHO 1992), DSM-III (APA 1980) or DSM-IV (APA 2000), and (c) that described measurement of urinary metabolites. Studies reported prior to 1980 were excluded as the DSM-III was released and included autism as a separate diagnostic category for the first time in 1980 (APA 1980). Search terms used were metabolite/metabolic/compound/substance/biomarker/chemical, and urinary/ urine.

All potential studies identified were independently evaluated for inclusion by two primary reviewers. The primary reviewers were not blinded to the authors, institutions or source of publication at any time during the selection process. Disagreements about the inclusion/exclusion of studies were discussed and consensus achieved. Provision was made for a third reviewer if consensus was unattainable but did not prove necessary.

Retrieved articles were clustered under subheadings that aligned with factors implicated in autism's etiology: GI factors, immunological factors, heavy metal toxicity, altered neurotransmitters, oxidative stress, and "others." Findings are summarized in Tables 1–5 where participant numbers and key results for each study are presented.

Gastrointestinal factors

GI disturbances are common findings in individuals with ASD, although the prevalence described in published studies varies substantially (Buie et al. 2010). Altered GI microbiota (Finegold et al. 2002; Song et al. 2004; Parracho et al. 2005; Finegold et al. 2010) and their fermentation products (Shaw 2010; Yap et al. 2010; Altieri et al. 2011) have been found in ASD compared with controls. Campbell et al. (2009) recently provided genetic evidence to strengthen the link between GI dysfunction and ASD.

There are a number of theories proposed to explain the link between impaired GI function and disturbance of neurological development and/or function resulting in ASD. Inflammatory and immune changes may cause increased intestinal permeability (i.e. leaky gut) (D'Eufemia et al. 1996; de Magistris et al. 2010), which allow increased absorption of chemicals that are neuroactive (Reichelt et al. 1997). Overgrowth of bacteria in the gut capable of secreting neurotoxic chemicals has also been proposed. Moreover, it has been suggested that there may be dysfunction of secretin, a neurotransmitter and digestion control hormone, which has various behavioral effects, or its receptors (Horvath et al. 1999; Sandler et al. 2000; Shattock & Whiteley 2002; Molloy & Manning-Courtney 2003).

Peptides

The roles of the endogenous opioid system are diverse and include roles in GI function, the sensory system, emotion, and cognition (Akil et al. 1998). Enhancement of the function of the endogenous opioid system or administration of opiates to infant animals can result in autistic-like symptoms (Sher 1997). It is hypothesized that exogenous opioid-like peptides, such as those derived from dietary casein and gluten (Reichelt et al. 1997) are implicated in ASD. Previous results of urinary opioid-like peptides in ASD are summarized in Table 1 (Israngkun et al. 1986; Shattock et al. 1990; Reichelt et al. 1997; Pedersen et al. 1999; Hunter et al. 2003; Dettmer et al. 2007; Cass et al. 2008).

Several early studies described different patterns of urinary peptides in individuals with ASD compared with controls (Israngkun et al. 1986; Shattock et al. 1990; Reichelt et al. 1997). However, a recent study did not find significant differences in urinary peptide profiles between children with and without ASD using HPLC–UV and checked the potential peaks using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) (Cass et al. 2008). The limitation of all these studies, however, was that the exact peptides were not isolated, identified, or quantified. Thus, it is unclear whether the specific peptides found to differ (or not) in individuals with ASD compared with controls in these studies were the same in all studies.

The first study to qualitatively investigate a specific urinary peptide found that pyroGlu-Trp-GlyNH_2 was present in the urine of children with ASD (67%) much more commonly than controls (18%) (Pedersen et al. 1999). This study only recorded the area under the curve at 215 nm for each participant using HPLC–UV without referring to any calibration curve. Therefore, it did not allow for a comparison of quantitative results to match with other studies.

Table 1. Results of studies examining urinary peptides in autism compared with controls.

Study	Participants (No.)	Results
(Israngkun et al. 1986)	ASD ($n=13$) Control ($n=10$)	Urinary patterns of individual with ASD were different from controls.
(Shattock et al. 1990)	ASD ($n=25$) Controls ($n=20$)	Urinary patterns of individual with ASD were different from controls.
(Reichelt et al. 1997)	ASD ($n=64$): 21 Italian, 6 American, 13 British, 12 Norwegians and 8 from Finland, Sweden, and Germany. Controls ($n=8$) with neuroleptics from Italy.	Altered urinary peptide patterns and large quantitative differences of urinary peptide profiles were found in ASD (peak area, $p<0.001$) compared with controls.
(Pedersen et al. 1999)	ASD ($n=135$) Controls ($n=126$)	Significantly higher mean levels of the tripeptide like peak area (10.3 vs 1.1 μm^2 under 215 nm trace, $p<0.001$) in ASD than controls.
(Hunter et al. 2003)	ASD ($n=10$) Siblings ($n=10$)	The targeted peptides were not found in the urine from any participants using LC–UV–MS.
(Dettmer et al. 2007)	ASD ($n=54$) Controls ($n=15$)	The targeted peptides were not detected (the limit of detection is 0.25 ng/mL) in urine samples from any participants.
(Cass et al. 2008)	ASD ($n=65$) Controls ($n=158$)	There were no significant differences in the urinary profiles between children with and without ASD.

ASD, autism spectrum disorder.

Two other studies (Hunter et al. 2003; Dettmer et al. 2007) used HLPC–mass spectrometry (HPLC–MS)-based methods to detect several urinary peptides in children with and without ASD. However, none of these peptides were detected subsequently in test samples of children with ASD and controls (Hunter et al. 2003; Dettmer et al. 2007).

An important issue to come out of this research is the importance of the analytical method. The bio-fluid, urine, is a very complex biological matrix and absorption of UV light is rather nonspecific for peptide detection. Hunter et al. (2003) claimed reference peptides spiked into urine could not be seen in UV-chromatograms but were clearly present in single ion chromatograms and concluded that mass spectrometric technique has greater sensitivity and specificity when analyzing peptides in urine.

Indolyl-3-acryloylglycine

Indolyl-3-acryloylglycine (IAG) is a regular constituent of human urine that is reported to be produced by specific gut bacteria. It is proposed that higher levels of urinary IAG are indicative of gut dysbiosis. Shattock & Whiteley (2002) have suggested the planar geometry of indolyl-3-acrylic acid (the acid precursor of IAG), disrupts membrane structures, and in turn increases GI permeability.

Previous studies (Whiteley et al. 1999; Bull et al. 2003; Whiteley & Shattock 2003; Wright et al. 2005; Wang et al. 2009) examining urinary IAG in individuals with ASD (Table 2) have produced conflicting results, which suggest that urinary IAG is not a suitable biomarker for overall ASD diagnosis, but may be useful for distinct subgroups of ASD, such as children with ASD and ongoing GI disturbance (Wang et al. 2009). Interestingly, Wang et al. (2009) also reported that IAG: creatinine in siblings with ASD was intermediate between children with ASD and community controls.

To more conclusively demonstrate that urinary IAG is a biomarker for ASD with GI disturbance, future studies will need to use more objective measures of illness severity for functional GI disorders in ASD.

It has been speculated that levels of urinary IAG may be reduced with a gluten-free diet. Whiteley et al. (1999) investigated urinary IAG levels over a 5-month period in children with ASD implementing a gluten-free diet, children with a gluten challenge and controls. They found participants on a gluten-free diet displayed an improvement in behavioral measures. However, there were no significant changes of urinary IAG between the three study groups and no direct correlation between IAG and ASD behaviors. From these preliminary data, it appears that changes in IAG do not play a role in improvement resulting from a gluten-free diet. Gluten- and/or casein-free diet studies with larger samples and control are required to confirm the preliminary results.

Other urinary compounds of GI origin

A recent exploratory study by Yap et al. (2010) investigated urinary metabolic profiles in children with ASD ($n=39$), their siblings ($n=28$) and healthy controls ($n=34$) using ^{1}H nuclear magnetic resonance (NMR) spectroscopy. They identified a range of differences in the urinary metabolic profiles of children with ASD including higher levels of urinary dimethylamine, and lower levels of both urinary hippurate and 4-cresol sulfate. These compounds are produced by certain GI bacteria (e.g. *Bacteriodes, Clostridia*). Yap et al. (2010) attributed the alterations in the ASD group to an imbalance of GI microbiota.

Further, a study by Altieri et al. (2011) found elevated levels of *p*-cresol in urine of young children with ASD compared with controls. The authors also reported a positive correlation between urinary *p*-cresol and ASD severity. *p*-Cresol is a toxic metabolite of tyrosine catabolism by GI bacteria such as *Clostridia* species and *Pseudomonas stutzeri* (Altieri et al. 2011). Altieri et al. (2011) concluded

Table 2. Results of studies examining urinary IAG in autism compared with controls.

Study	Participants (No.)	Results
(Bull et al. 2003)	ASD ($n=22$) Controls ($n=18$)	Increased median levels of IAG found in ASD compared with controls (942 vs 331 μV/mmol/L of creatinine, $p=0.0002$).
(Whiteley & Shattock 2003)	PDD ($n=427$) Controls ($n=83$)	IAG was identified in more samples from the PDD group than controls (Fisher's exact test, two-side, $p=0.063$). The HPLC results were affected if participants had epilepsy as a co-morbidity and/or medication use.
(Wright et al. 2005)	ASD ($n=56$) including childhood autism ($n=33$), atypical autism ($n=7$) and AS ($n=16$) Controls ($n=155$)	No significant difference between the mean levels of IAG in controls and ASD (i.e. 0. 0508 vs 0.0511 mmol/L, $p=0.625$). Also no difference between levels of IAG in controls and in childhood autism, 0.0433 mmol/L, atypical autism, 0.0637 mmol/L, AS, 0.0444 mmol/L, respectively.
(Wang et al. 2009)	ASD ($n=57$) Siblings (SIB, $n=50$) Community controls (CON, $n=56$)	Higher levels of urinary IAG and IAG: creatinine ratios in children (age adjusted) with ASD *with* ongoing GI disturbance compared with autism *without* GI disturbance ($p=0.006$ and $p=0.001$). IAG: creatinine did not differ between controls (SIB and CON groups) with and without ongoing GI problems.
(Whiteley et al. 1999)	Children with ASD implementing a gluten-free diet ($n=6$) Children with ASD with a gluten challenge ($n=4$) Controls ($n=3$)	A mean reduction in IAG excretion between baseline and post-diet samples in children who had implemented a gluten-free diet compared with two other groups but did not reach significant levels. No direct relationship was found between IAG and autistic behaviors.

AS, Asperger syndrome; ASD, autism spectrum disorder; IAG, Indolyl-3-acryloylglycine; PDD, pervasive developmental disorder.

that follow-up mechanistic studies will have to define the degree of overlap between GI microbiota composition and enhanced GI permeability in individuals with ASD as well as their potential relationship with GI symptoms, abnormal behavior, and personalized response to pharmacological treatments.

Shaw (2010) recently described a higher level of urinary 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) in children with ASD compared to controls. The source of HPHPA was reported to be multiple species of anaerobic bacteria of the *Clostridium* genus in the gut. The author suggested that HPHPA could derive from m-tyrosine, which has been shown to deplete brain catecholamines and causes symptoms of ASD in experimental animals. However, whether HPHPA is produced from m-tyrosine or whether m-tyrosine causes ASD symptoms in humans remains to be demonstrated.

Many children with autism have no or limited expressive language and may not be able to communicate GI discomfort. Thus, these children may react to pain by exhibiting behaviors not obviously related to the GI system (Weber & Newmark 2007). Routine analysis of the specific urinary compounds related to GI disturbance could facilitate early identification of whether children with ASD have or are at risk of GI disturbance and thereby enable appropriate interventions to be commenced which could potentially improve the quality of life of affected individuals.

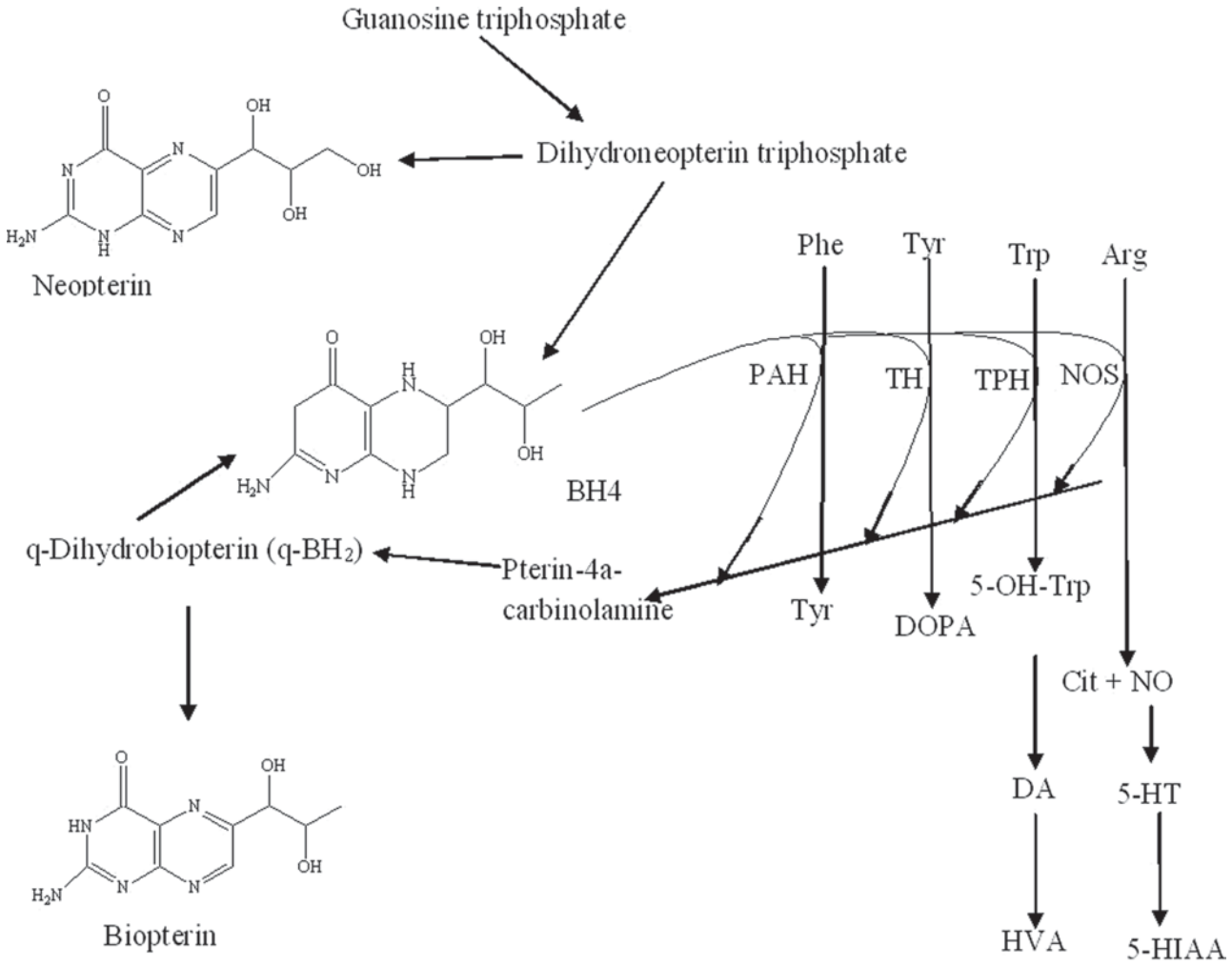
Immune dysregulation

Immunological anomalies involving cytokines, immunoglobulin, inflammation, and cellular activation have been reported in individuals with ASD (Goines & Van de Water 2010), strongly implicating immune dysfunction in the etiology of ASD (Stigler et al. 2009). It has been established that increased levels of neopterin in body fluids often accompanies activation of the cellular immune system (Messahel et al. 1998). Determination of neopterin in body fluids of patients with diverse immune-related diseases has revealed that neopterin may play a key role in diagnostic and therapeutic decisions for such conditions (Fuchs et al. 1993).

A simplified pathway of the biosynthesis of pterins is shown in Scheme 1. Most of these pterins occur in unstable reduced forms in biological systems, while neopterin occurs as D-*erythro*-7,8-dihydroneopterin (Eto et al. 1992). This is important in terms of the analysis of neopterin in urinary samples, as it is possible to pre-treat urine samples to convert unstable reduced pterins to stable oxidized forms.

Three studies have been conducted analyzing urinary levels of neopterin in children with ASD compared with controls (Eto et al. 1992; Harrison & Pheasant 1995; Messahel et al. 1998) (Table 3). Messahel et al. (1998) reported significantly higher levels of neopterin in urine samples (without oxidative pre-treatment) from children with AD compared with controls. Interestingly, and similar to Wang et al.'s findings with IAG (Wang et al. 2009), this study showed that the urinary neopterin:creatinine ratios in siblings were intermediate between ASD and unrelated control values. The raised levels of neopterin in siblings may indicate a common underlying cause within the families due to either genetic or environmental factors.

A second study, without oxidative sample pre-treatment, by Harrison et al.'s (1995) also found that urinary neopterin levels were significantly increased in children with ASD compared with controls. With the use of oxidative pretreatment, however, the same study found that the levels of urinary neopterin between children with ASD and controls were not significantly different. In contrast, a third study, in which oxidative pretreatment was used,



Scheme 1. Biosynthesis of pterins. Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; Arg, arginine; PAH, phenylalanine-4-hydroxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; NOS, nitric oxide synthase; DOPA, 3,4-dihydroxyphenylacetic acid; Cit, citrulline; NO, nitric oxide; DA, dopamine; HVA, homovanillic acid; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole acetic acid. The structures were produced by CS ChemDraw Std (Version 4.5, Cambridge Soft Corporation, USA, 1997).

Table 3. Results of studies examining urinary neopterin in autism compared with controls.

Study	Participants (No.)	Results
(Eto et al. 1992)	ASD ($n=16$) Controls ($n=12$)	Decreased neopterin in ASD compared with controls (0.391 ± 0.041 vs 0.616 ± 0.132 mmol/mol creatinine, $p<0.05$) when samples had oxidative pre-treatment.
(Harrison & Pheasant 1995)	ASD ($n=17$) Controls ($n=17$)	No significantly different levels of neopterin in children with ASD compared with controls (1359.9 ± 1102.5 vs 1283.1 ± 546.4 μmol/mol creatinine), when samples had oxidative pre-treatment but higher neopterin levels in children with ASD than controls when the analyses were carried out on samples without oxidative pretreatment in the same study (i.e. ASD vs controls, 1306.3 ± 786.3 vs 615.1 ± 373.4 μmol/mol creatinine, $p<0.005$).
(Messahel et al. 1998)	AD ($n=14$) Siblings ($n=21$) Controls ($n=16$)	Higher urinary neopterin levels found in AD (no oxidative pre-treatment) versus the unrelated control group (3116 ± 686 vs 908 ± 201 μmol/mol creatinine, $p<0.01$). Urinary neopterin levels in sibling controls (1490 ± 346 μmol/mol creatinine) were intermediate between children with AD and unrelated controls.

AD, autistic disorder; ASD, autism spectrum disorder.

found significantly decreased urinary neopterin in children with ASD compared with controls (Eto et al. 1992).

The apparent discrepancies between these studies are likely due to different methodologies. The ratio between the reduced form, D-*erythro*-7, 8-dihydroneopterin, and oxidized form, neopterin, in collected samples was not reported. This ratio can change with time, unless all samples are collected and prepared simultaneously. Thus, fully oxidative pretreatment of samples before neopterin testing is recommended. Neither study where samples

were subjected to oxidative pretreatment (Eto et al. 1992; Harrison & Pheasant 1995) appears to give evidence of elevated urinary neopterin levels in children with ASD compared with controls.

Another important issue highlighted by this research is the potential importance of age when assessing neopterin levels. In Messahel et al.'s (1998) study, high urinary levels of neopterin were found in children with AD of less than six years old compared with those greater than six years old, while the neopterin concentrations in controls did not change with age and resembled those of children with autism greater than six years old. Some inconsistent results reported in different studies may also be due to the various age ranges in the three studies described above i.e. 6–18 years (Eto et al. 1992), 3–21 years (Harrison & Pheasant 1995), and 3–5 years (Messahel et al. 1998).

Heavy metals' toxicity

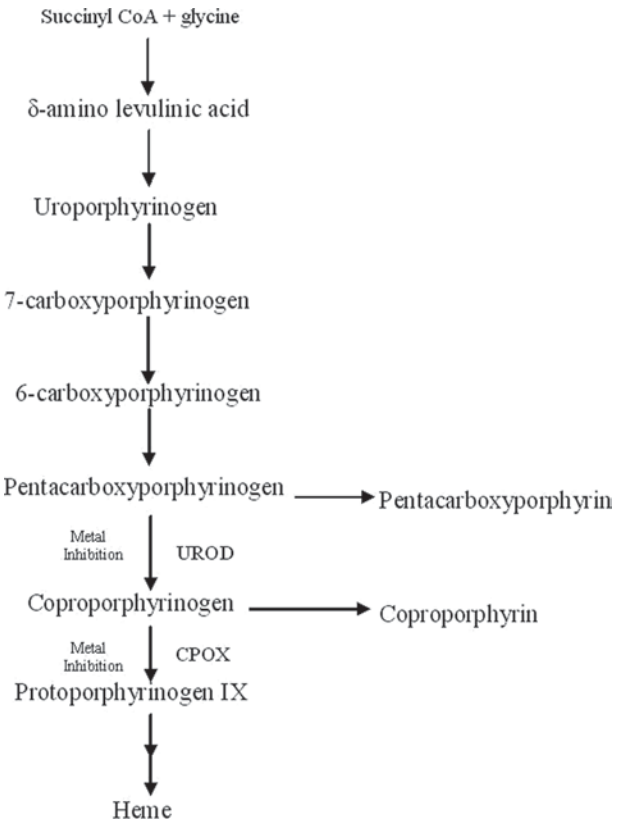
Heavy metals, such as arsenic, lead, and mercury, have been associated with a variety of neurologic deficits and disorders, including lower IQ, Alzheimer's disease, and Parkinson's disease (Zecavati & Spence 2009). James et al. (2004) found a significantly reduced glutathione and oxidized glutathione ratio (GSH:GSSG) in children with AD compared with controls. An impaired glutathione redox ratio is thought to play a role in the etiology of autism by delaying the clearance of heavy metals from the body (Deth et al. 2008). The association between heavy metal exposure and ASD, in particular mercury, has attracted considerable interest (Counter et al. 2002; Holmes et al. 2003; Palmer et al. 2006; Geier & Geier 2007). Mercury has been implicated in immune, sensory, neurological, motor, and behavioral dysfunction resulting in clinical manifestations similar to those defining or associated with ASD. Some studies have suggested that mercury can disrupt neurotransmitter levels and biochemistry (Faustman et al. 2000; Redwood et al. 2001; Bernard et al. 2002) and impact on normal child development.

Heavy metals

It has been suggested that some children with ASD have an increased body burden of mercury which may result from biochemical and genomic susceptibilities within detoxification pathways (Mutter et al. 2005). However, there have been few studies that have directly measured heavy metal burden in children with ASD in urine. Because urinary levels of heavy metals only show what is being excreted out of the body, urine studies are therefore not very useful to reflect the heavy metal burden in the body. A recent study reported an altered urinary heavy metal profile in children with ASD ($n=30$) compared with healthy controls ($n=20$). In particular, urinary chromium levels were significantly higher whereas cadmium and lead were significantly decreased in children with ASD compared with controls (Yorbik et al. 2009). The determinant of lower levels of urinary cadmium and lead in this study is unclear and the authors suggested these findings may be due to the children with ASD having poor heavy metal detoxification mechanisms. This theory has been supported indirectly by studies that have shown decreased levels of heavy metal in the hair of children with ASD (Holmes et al. 2003).

Porphyrins

Porphyrins, such as coproporphyrin, pentacarboxyporphyrin, uroporphyrin, and precoproporphyrin (Bozek et al. 2005) are involved in the formation of heme. Heme is essential for the proper function of many proteins including oxygen transport, energy production, and detoxification. The heme pathway is constantly changing and is active in almost every cell of the body. Any disturbance in the pathway tends to cause rapid and relatively large accumulations of intermediates, such as porphyrins. Also, the enzymes of the pathway are widely distributed in human tissues, and are highly sensitive to the presence of various toxins, creating an accumulation of porphyrins in the pathway. Thus, the level of porphyrin production is useful for assessing the body's capacity to detoxify toxins (Brewster 1988). Heavy metals can inhibit the enzymes uroporphyrin decarboxylase (Woods & Kardish 1983) and coproporphyrinogen oxidase (Woods et al. 2005) (Scheme 2). Subsequent elevation of coproporphyrin and pentacarboxyporphyrin can be detected in urine as a marker of heavy metal levels.



Scheme 2. The heme synthesis pathway and major urinary metabolites: sites of heavy metal inhibition.

Elevated levels of porphyrins are found in urine from both laboratory animals and humans exposed to heavy metals (Bowers et al. 1992). Measurement of urinary porphyrins is an independent and non-invasive method to assess environmental toxicity in children with ASD. Studies that have examined and compared urinary porphyrin levels in children with and without ASD are summarized in Table 4 (Geier & Geier 2006; Nataf et al. 2006; Geier & Geier 2007; Austin & Shandley 2008; Nataf et al. 2008).

Several studies reported atypical porphyrin profiles in children with ASD compared with controls, in particular, significantly higher levels of coproporphyrin were found in children with ASD (Geier & Geier 2006; Nataf et al. 2006; Austin & Shandley 2008). Also, increased urinary levels of pentacarboxyporphyrin in ASD were shown in Nataf et al.'s (2006) and Austin et al.'s (2008) studies (Table 4). The three studies demonstrated aberrant porphyrins in children with ASD. Nataf et al.'s and Geier et al.'s studies have compared the urinary porphyrin levels in different ASD subtypes as well as children with ASD who had and had not received chelation while Austin et al. (2008) did not. As abnormal levels of porphyrins were not found in individuals with AS, it is conceivable that different subtypes have different capacity to detoxify heavy metals. To date, there is insufficient evidence available to conclude whether porphyrins are useful as biomarkers for diagnosis of overall ASD or for subtyping.

Chelation therapy

Chelating agents such as dimercaptosuccinic acid (DMSA) or 2,3-dimercapto-1-propanesulfonic acid (DMPS) are used as a treatment in ASD because they bind to heavy metals and increase their clearance from the body (Weber & Newmark 2007). Additionally, the levels of urinary heavy metals following chelation therapy may be useful to assess heavy metal levels.

Three studies have compared urinary heavy metals levels following chelation therapy between individuals with ASD and controls (Bradstreet et al. 2003; Geier & Geier 2007; Soden et al. 2007). Bradstreet and colleagues (Bradstreet et al. 2003) performed a retrospective

Table 4. Results of studies examining urinary porphyrin in autism compared with controls.

Study	Participants (No.)	Results
(Geier & Geier 2006)	ASD ($n=37$) including subjects who received ($n=18$) and did not receive ($n=19$, 6 AD and 13 PDD–NOS or AS) chelation therapy. Siblings ($n=7$)	Increased median urinary COPRO levels were found in participants with AD (who were naive to chelation therapy) ($n=6$) compared with sibling controls (36 vs 16 μg/ L, $p<0.05$). Decrease in the median levels of COPRO observed in chelated ASD participants compared with Autism participants who were not chelated (19 vs 32 μg/ L, $p<0.05$). Urinary URO, heptacarboxylporphyrin, hexacarboxyporphyrin, and pentacarboxyporphyrin median levels were not significantly different between the total ASD participants and controls.
(Nataf et al. 2006)*	AD ($n=106$) AS ($n=12$) Internal controls ($n=12$) External controls ($n=107$)	Elevations in urinary COPRO were found in children with AD compared with internal controls ($p<0.001$). COPRO: URO was different between AD vs internal controls ($p<0.001$). Similarly for AD vs AS ($p=0.009$). Precoproporphyrin: URO ratio was elevated in AD vs controls ($p<0.001$). Urinary levels of pentacarboxyporphyrin and hexacarboxyporphyrin levels were higher in AD than controls ($p<0.001$ and $p<0.002$). A reduction in urinary COPRO and precoproporphyrin were found following chelation treatment in the AD ($n=11$) subgroup compared with their original porphyrin levels ($p=0.02$).
(Geier & Geier 2007)	ASD ($n=63$) including subjects who received ($n=26$) and did not receive ($n=37$) chelation therapy. Siblings ($n=9$) General population controls ($n=5$)	The mean levels of urinary pentacarboxyporphyrin and COPRO were elevated in unchelated ASD compared to chelated autism participants (1.57 ± 1.04 vs 1 ± 0.85 μg/L, $p<0.05$; 29.97 ± 19.1 vs 16.46 ± 9.51 μg/L, $p<0.05$) and also compared to combined chelated autism plus siblings (1.57 ± 1.04 vs 1 ± 0.77 μg/L, $p<0.05$; 29.97 ± 19.1 vs16.4 ± 8.42 μg/L, $p<0.05$). The mean urinary precoproporphyrin: URO and COPRO: URO in the unchelated ASD participants were increased relative to the general population and were outside the lab reference ranges (26.66 ± 49.19 vs 5.56 ± 1.48 nmol/nmol, $p<0.05$). Unchelated ASD and the general population controls had similar urinary heptacarboxyporphyrin: URO were within the lab reference range.
(Nataf et al. 2008)*	AD ($n=100$) AS ($n=11$) Controls ($n=12$)	Urinary COPRO levels in AD were higher compared with (AS + controls), $p=0.02$. Similarly when COPRO was normalized to creatinine ($p=0.01$).
(Austin & Shandley 2008)	ASD ($n=41$)	A consistent trend in abnormal porphyrin levels was found compared with controls (Minder & Schneider-Yin 1996; Nataf et al. 2006; Geier & Geier 2007) and laboratory reference (Laboratoire Philippe Auguste, Paris, France) i.e. COPRO and pentacarboxyporphyrin levels were 289.61 ± 175.75 nmol/g creatinine, 5.19 ± 2.33 nmol/g creatinine, respectively).

ASD, autism spectrum disorder; PDD–NOS, pervasive developmental disorder not otherwise specified; AS, Asperger syndrome; COPRO, coproporphyrin; AD, autistic disorder; URO, uroporphyrin. *Data presented graphically, exact concentrations not shown.

urinary mercury excretion analysis in 221 autistic children compared with 18 controls after a three-day provocation with DMSA and demonstrated significantly higher levels of urinary mercury in children with ASD compared with controls. However, a limitation of this study is the large imbalance between the number of cases and controls. A case series has reported on 8 children with ASD that showed significantly elevated urinary levels of mercury and/or lead in urine after chelation therapy compared with before treatment (Geier & Geier 2007). It is difficult to interpret this report as this study had no control group(s). Soden et al. (2007) reported similar urinary heavy metal output in children with ASD ($n=17$) compared with controls ($n=4$), but it is likely that the small sample size limited the ability to detect a statistically significant difference.

There has been concern regarding the safety of chelation therapy to assess heavy metal burden. It is notable that a randomized controlled trial designed to examine the safety and efficacy of DMSA for mercury chelation in autism was halted by the US National Institute for Mental Health after an assessment that the study treatment presented more than minimal risk (Mitka 2008). This was partly due to a study in rats designed to assess the effects of chelation with DMSA following lead exposure. This study had the unexpected finding that a single 3-week course of succimer treatment in rats not exposed to lead during their early development produced lasting cognitive dysfunction when assessed over a 7-month period (Stangle et al. 2007). Chelation therapy is therefore inappropriate for routine investigation of heavy metal burden in ASD.

Altered neurotransmitters and neurotransmitter metabolites

Many studies have focused on neurotransmitter abnormalities in individuals with ASD (Garnier et al. 1986; Launay et al. 1987; Minderaa et al. 1987; Barthelemy et al. 1988; Garreau et al. 1988; Barthelemy et al. 1989; Minderaa et al. 1989; Martineau et al. 1991; Martineau et al. 1992; Herault et al. 1993; Martineau et al. 1994; Minderaa et al. 1994; Herault et al. 1996; Croonenberghs et al. 2000; Mulder et al. 2005; Mulder et al. 2005; Mulder et al. 2009) (Table 5). Abnormalities in various neurotransmitters have been implicated in the development of ASD, including serotonin (5-HT), dopamine (DA), noradrenaline (NA), gamma-aminobutyric acid, glutamate, and neuropeptides.

Serotonin and metabolites

5-HT is a monoamine neurotransmitter that plays an important role in the developing brain by directing both neuronal proliferation and maturation (McDougle et al. 2005). Central nervous system (CNS) 5-HT activity has been involved in a range of physiological functions, such as sleep, sensory perception, and appetite, which are often disrupted in ASD (Young et al. 1982). High levels of 5-HT during early development may cause a loss of 5-HT receptors and therefore impact on subsequent neuronal development (Whitaker-Azmitia 2001). Neuroimaging studies suggest altered developmental regulation of 5-HT synthesis may be associated with the pathogenesis of ASD (Chugani et al. 1999). Hyperserotonemia has been consistently reported in people with ASD in more than 25 published studies (Lam et al. 2006).

A number of studies have also reported significantly higher urinary levels of 5-HT in ASD compared with controls (Barthelemy et al. 1989; Martineau et al. 1991; Herault et al. 1993; Herault et al. 1996). Further, a recent study revealed that urinary 5-HT excretion tended to be higher in hyperserotonemic children with ASD than the normoserotonemic group (Mulder et al. 2009). Moreover, Barthelemy et al. (1989) found a significant decrease in urinary 5-HT levels in children with ASD after fenfluramine treatment. Fenfluramine is a 5-HT reducing agent which was used primarily as an appetite suppressant but also showed some benefit in the management of ASD (Aman & Kern 1989). However, the drug was withdrawn from market in 1997 after reports of heart valve disease.

Metabolism of 5-HT is carried out primarily by monoamine oxidase to 5-hydroxyindole acetic acid (5-HIAA) which is excreted in urine (Grahame-Smith 1988). Most studies have shown no significant differences in urinary 5-HIAA between individuals with and without ASD (Minderaa et al. 1987; Herault et al. 1996; Mulder et al. 2005; Mulder et al. 2005). One study reported a trend for higher 5-HIAA excretion in the urine of hyperserotonemic individuals with ASD than controls (Mulder et al. 2009). This study focused on the hyperserotonemic autistic subtype and therefore results do not necessarily conflict with other studies.

Dopamine and metabolites

Dopamine is a catecholamine which acts as a major neurotransmitter in the brain. Generally, the dopaminergic system is thought to affect a wide range of functions, including cognition and attention (Nieoullon 2002), motor function (Niimi et al. 2009), predictive reward signal mechanisms, (Schultz 1998) and immunity (Basu & Dasgupta 2000). Abnormal DA activity is thought to play a key role in the etiology of schizophrenia (Davis et al. 1991). Some animal research has shown that stereotypes and hyperactivity can be induced by increasing dopaminergic functioning suggesting dopaminergic neurons may be overactive in ASD (Miller et al. 2010). Various DA antagonists are often used in the management of ASD (Lewis 1996). The atypical antipsychotic, risperidone, has reasonable evidence for efficacy with studies demonstrating that it can alleviate some ASD related behaviors such as aggression and self-injury (Shea et al. 2004).

Levels of urinary DA have been investigated in several studies. Most studies have reported no significant differences in urinary DA levels between children with and without ASD (Launay et al. 1987; Minderaa

Table 5. Results of studies examining urinary neurochemicals (and metabolites) in autism compared with controls.

Study	Participants (No.)	Results
(Herault et al. 1993)	ASD ($n=23$) Controls ($n=59$)	Higher levels of 5-HT (159.9 ± 17.5 vs 92.8 ± 5.1 nmol/mmol creatinine, $p<0.001$) and NA + A (48.2 ± 5.5 vs 37.2 ± 2.6 nmol/mmol creatinine, $p=0.043$) in ASD compared with controls.
(Herault et al. 1996)	ASD ($n=65$) Controls ($n=35$)	Higher daily (9 a.m.–4 p.m.) urinary levels of 5-HT in ASD than controls (137.2 ± 83.8 vs 95.8 ± 31.9 mmol/mmol creatinine, $p<0.001$). No significant difference in urinary 5-HIAA levels between two groups (i.e. autism vs controls, 4.89 ± 3.76 vs 4.94 ± 2.47 mmol/mmol creatinine).
(Martineau et al. 1992)	ASD ($n=156$): 85 drug free and 71 had medicated[a] Non-autistic participants who had mentally retarded (MR, $n=152$): 111 drug free and 41 had medicated Normal controls ($n=116$)	Higher daily (9 a.m.–4 p.m.) urinary levels of DA were found in MR group than ASD or control groups (mean ± SE, i.e. MR, ASD, and normal, 382 ± 14, 309 ± 11 and 319 ± 12 nmol/mmol creatinine, $p<0.001$). Daily levels of 3MT (i.e. ASD, MR, and controls, 179 ± 12, 200 ± 13 and 140 ± 6 nmol/mmol creatinine, $p<0.005$), HVA (7.9 ± 0.2, 8.1 ± 0.3 and 5.4 ± 0.2 µmol/mmol creatinine, $p<0.0001$) and 5-HT (143 ± 6, 139 ± 6 and 95 ± 5, nmol/mmol creatinine, $p<0.001$) were higher in autism and MR groups than controls. Urinary levels DA were lower in medicated participants with ASD compared to non-medicated participants with autism (290 ± 17 vs 338 ± 14 nmol/mmol creatinine).
(Mulder et al. 2009)	Hyperserotonemic autism ($n=9$) Normoserotonemic autism ($n=10$)	No significant different of 24 h urinary 5-HT and 5-HIAA levels in children with hyperserotonemics and normoserotonemics (63.2 ± 19.2 vs 60.1 ± 21.4 mmol/mol creatinine, 1.79 ± 0.75 vs 1.69 ± 0.86 mmol/mol creatinine, respectively).
(Barthelemy et al. 1989)	ASD ($n=13$) with fenfluramine treatment: 6 had respond and 7 did not	Daily (9 am–4 pm) urinary levels of 5-HT was lower in all participants after drug treatment. HVA were lower in responders compared with non-responders before treatment (6.6 ± 0.7 vs 10.4 ± 0.9 µmol/mmol creatinine, $p<0.005$). Increased HVA in responders after than before treatment (10.2 ± 1.8 vs 6.6 ± 0.7 µmol/mmol creatinine, $p<0.05$ Wilcoxon test) while levels returned to baseline later.
(Minderaa et al. 1987)	ASD ($n=36$): 16 drug free and 20 had medicated[a] Controls ($n=27$)	Two periods time (5 pm–11 pm and 11 pm–8 am) samples were collected. Higher overnight (5.12 ± 1.06 vs 3.44 ± 1.06 µg/mg creatinine, $p<0.01$) and combined urinary levels (4.88 ± 0.87 vs 3.50 ± 1.07 µg/mg creatinine, $p<0.05$) of 5-HIAA were found in hyperserotonemic autism compared with controls. There was a trend ($p<0.1$) to high of overnight (4.16 ± 1.70 vs 3.44 ± 1.06 µg/mg creatinine) and combined (4.07 ± 1.52 vs 3.50 ± 1.07 µg/mg creatinine) urinary 5-HIAA levels in children with unmedicated ASD compared with controls.
(Mulder et al. 2005)	ASD ($n=20$)	24 h urinary 5-HIAA levels (1.74 ± 0.79 mmol/mol creatinine) were similar as previously reported in healthy controls.
(Launay et al. 1987)	Infantile autism ($n=20$) Controls ($n=19$) who had not psychiatric symptoms and bioamine metabolism	No significantly different in overnight urinary compounds were found between autism and controls (DOPAC, 5.13 ± 3.34 vs 4.84 ± 4.37 µmol/mol creatinine; MHPG, 3.79 ± 2.51 vs 5.06 ± 4.01 µmol/mol creatinine; DA, 1.82 ± 1.40 vs 2.23 ± 1.35 10^{-7} mol/mol creatinine; NA, 1.06 ± 0.86 vs 1.39 ± 1.24 10^{-e} mol/mol creatinine and A, 4.97 ± 5.34 vs 5.11 ± 3.92 nmol/nmol creatinine).
(Minderaa et al. 1989)	ASD ($n=36$): 16 drug free and 20 had medicated[b] Control ($n=28$)	Complete evening (5 pm–8 am) urinary levels of HVA were higher in the medicated combined group than unmedicated children with ASD (4.70 ± 2.32 vs 3.33 ± 1.01 µg/mg creatinine, $p<0.05$). Complete evening urinary levels of DA were lower in ASD who had phenothiazines treatment compared with non-medicated autism (125 ± 13.8 vs 192 ± 45.4 µg/g creatinine, $p<0.01$).
(Martineau et al. 1994)	ASD ($n=50$) Controls ($n=50$)	Daily (9 am–4 pm) urinary levels of DA were slightly lower in children with ASD compared with controls. High levels of HVA, 3MT, and NA + A in ASD compared with controls which confirm the previous study by Martineau et al. (1992). No significant difference between urinary DOPAC between two groups.
(Garnier et al. 1986)	ASD ($n=19$) Controls ($n=15$) who had not any psychological disorders	Spot urine (9 am–10 am) sample collected. HVA levels were higher in ASD than controls (5.90 ± 0.41 vs 4.06 ± 0.33 nmol/µmol creatinine, $p<0.02$).
(Minderaa et al. 1994)	ASD ($n=36$): 16 drug free and 20 had medicated[a] Controls ($n=27$)	No significant differences of complete evening (5 pm–8 am) urinary compounds excretion found in all participants (i.e. controls, ASD with drug free, autism with medicated, MHPG, 80.4 ± 46.7, 67.6 ± 20.5 and 70.9 ± 20.8 µg/h; NA, 1.04 ± 0.48, 0.89 ± 0.26, 0.94 ± 0.47 µg/h; A, 207 ± 167, 161 ± 65.9, 188 ± 95.0 ng/h; VMA, 127 ± 38.5, 128 ± 44.6,--).
(Barthelemy et al. 1988)	ASD ($n=8$) Controls ($n=8$)	Total levels of DA (577.35 ± 49.87 vs 1225.63 ± 143.88 ng/mg creatinine, $p<0.01$) and MHPG (1410 ± 100 vs 2040 ± 200 ng/mg, $p<0.05$) were lower in autism, NA (159.25 ± 18.33 vs 93.00 ± 12.93 ng/mg creatinine, $p<0.01$) were higher in ASD.
(Croonenberghs et al. 2000)	AD ($n=13$) Controls ($n=13$)	No significantly different in 24 h urinary 5-HIAA, A, NA and DA between individuals with and without AD.
(Martineau et al. 1991)	ASD ($n=145$) Nonautistic developmental disorders (NADD, $n=141$) Controls ($n=107$)	Daily (9 am–4 pm) urinary levels of 3MT (171 ± 13, 207 ± 15 and 139 ± 6 nmol/mmol creatinine, $p<0.005$), total HVA (7.8 ± 0.2, 8.3 ± 0.3 and 5.4 ± 0.2 µmol/mol creatinine, $p<0.001$) and 5-HT (141 ± 7, 144 ± 6 and 95 ± 5 nmol/mol creatinine, $p<0.001$) were higher in children with ASD and NADD participants than controls.

ASD, autism spectrum disorder; AD, autistic disorder; 3MT, 3-methoxytyramine; 5-HIAA, 5-hydroxyindole acetic acid; 5-HT, Serotonin; A, adrenaline; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MHPG, methoxyhydroxy phenylglycol; NA, noradrenaline; VMA, vanillylmandelic acid.

[a]The autistic and/ or non autistic participants who had medicated with following drugs: anticonvulsants, haloperidol, phenothiazines, benzodiaze; [b]The autistic participants who had mediated with flowing drugs: phenothiazines, haloperidol and anticonvulsant.

et al. 1989; Martineau et al. 1992; Martineau et al. 1994; Croonenberghs et al. 2000) and only a single study found lower DA levels in ASD than controls (Barthelemy et al. 1988). Of particular interest are studies by Martineau et al. (1992, 1994) who found a significantly higher mean level of urinary DA in children with ASD who were not taking DA antagonists compared with those who were taking DA antagonists. A further study reported the various urinary levels of DA with different drug treatments (i.e. phenothiazine antipsychotics, haloperidol and anticonvulsants) (Minderaa et al. 1989) which may be helpful for monitoring the effects of different drugs in ASD. Given various medications lead to alterations in urinary DA, and the information relating to medication use was not identified in the majority of studies (Launay et al. 1987; Barthelemy et al. 1988; Minderaa et al. 1989; Martineau et al. 1992; Martineau et al. 1994; Croonenberghs et al. 2000), it cannot be concluded whether or not urinary DA is useful for ASD diagnosis.

The major metabolites of DA are homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC). Significantly increased level of urinary HVA in ASD compared with controls has been reported (Garnier et al. 1986; Barthelemy et al. 1988; Martineau et al. 1992; Martineau et al. 1994). However, as high levels of HVA are also reported in children with mental redaction (Martineau et al. 1992), it limits the value of urinary HVA as a biomarker for ASD diagnosis.

Noradrenaline

Noradrenaline is synthesized from DA by dopamine β-hydroxylase and released from noradrenergic neurons as well as from the adrenal medulla into the bloodstream. NA plays a critical role in attention, the stress response (i.e. the "fight or flight" response), anxiety, and memory (Amaral & Sinnamon 1977; Fitzgerald 2009), which are frequently observed to be impaired in individuals with ASD.

Previous studies have shown that measurements of NA (i.e. in plasma and urine) are generally well correlated with measurements in the CNS (Roy et al. 1988). A range of neurochemical studies have attempted to examine excretion of urinary NA and/or adrenaline (A) in individuals with ASD compared with controls and have yielded inconsistent findings. Three studies found higher levels of NA and/or A in ASD compared to controls (Barthelemy et al. 1988; Herault et al. 1993; Martineau et al. 1994), while four studies found no differences (Launay et al. 1987; Martineau et al. 1992; Minderaa et al. 1994; Croonenberghs et al. 2000).

It was reported that urinary levels of NA and/or A significantly decreased with age (Martineau et al. 1992). Variations in the mean age of participants varied between studies and, therefore, age may have reduced the significant difference between individuals with and without ASD. For example, the mean age was six years in one study which reported elevated levels of NA and/or A in ASD vs controls (Herault et al. 1993), while it was around 20 years in another study that reported no difference (Croonenberghs et al. 2000).

Oxidative stress

A leading theory implicated in the etiology of ASD is oxidative stress, which results from a complex interplay of genetic and environmental factors. Oxidative stress occurs when reactive oxygen species (ROS) levels exceed the antioxidant capacity of a cell. These ROS target lipids, proteins and nucleic acids (Chauhan & Chauhan 2006) resulting in a risk of neurologic deficits, especially during early life (Zecavati & Spence 2009). Evidence has emerged in recent years supporting the role of oxidative stress in the etiology of ASD, such as increased lipid peroxidation (Ming et al. 2005), altered antioxidant enzymes in plasma (Yorbik et al. 2002), mitochondrial dysfunction (Oliveira et al. 2005), and genetic factors (Cohen et al. 2003; Hovatta et al. 2005).

Biomarkers for lipid peroxidation

A study by Chauhan et al. (2004) reported increased lipid peroxidation in children with ASD compared with their siblings and suggested that increased lipid peroxidation was associated with regressive ASD. Isoprostanes, including 8-isoprostane, are a family of eicosanoids of non-enzymatic origin produced by random oxidation of tissue phospholipids by oxygen radicals. Ming et al. (2005) demonstrated significantly higher urinary levels of 8-isoprostane in children with AD ($n=33$) compared with healthy controls ($n=29$) and were in agreement with Chauhan et al. (2004)*'s* findings. However, Ming et al. (2005) found no significant correlation between the levels of 8-isoprostane and history of regressive AD. More studies on urinary lipid peroxidation metabolites are required to confirm these preliminary results and reveal their usefulness as potential biomarkers for autism diagnosis.

Biomarkers for nucleotide hydroxylation

Urinary 8-hydroxy-2-deoxyguanosine (8-OHdG) is the product of cellular DNA oxidation and hence a biomarker for oxidative damage to DNA (Awad et al. 1996). Ming et al. (2005) also reported a trend for elevated urinary 8-OHdG in children with AD compared with controls but it was not statistically significant.

Methylmalonic acid

Increased vulnerability to oxidative stress could impair vitamin B_{12} metabolism which can potentially cause severe and irreversible damage, particularly in the CNS and thus contribute to the development and clinical manifestation of AD (James et al. 2004). Impaired vitamin B_{12} metabolism can lead to accumulation of methylmalonic acid in tissues and body fluids. A study by Wakefield et al. (1998) describing ileal lymphoid hyperplasia in children with ASD (but retracted 2nd Feb 2010, Lancet) reported that urinary methylmalonic acid levels were significantly higher in children with ASD ($n=8$)

compared to age and gender matched normal controls ($n = 14$). However, there have been no other studies to confirm these results to date.

Overall, there is limited evidence to substantiate the role of oxidative stress in studies where urinary metabolites have been examined. Most studies implicating oxidative stress in the etiology of autism have examined various metabolites in plasma or serum (Main et al. 2010). Preliminary results of these blood studies identify oxidative stress can lead to membrane lipid abnormalities, inflammation, an aberrant immune response, and impaired energy metabolism, all of which can potentially contribute to the clinical manifestations of ASD. As such this area of study requires further investigation, specifically whether there are correlations between blood and urinary metabolites.

Other compounds

Creatine, guanidinoacetate, and creatinine

Creatine deficiency syndrome (CDS) is an inborn error of metabolism where affected individuals share several clinical features with ASD. Abnormal levels of urinary creatine and guanidinoacetate have been reported in people with CDS. A recent study by Wang et al. (2010) determined urinary levels of creatine and guanidinoacetate and found no significant difference between children with ASD ($n = 57$), siblings ($n = 49$), and community controls ($n = 49$).

Creatinine is primarily derived from muscle and its excretion is relatively constant over time. Hence, when other urinary metabolites levels are reported they are usually normalized against urinary creatinine to correct the variations of urinary volume that occur from inter- and intra-day. The results of studies reporting urinary creatinine concentrations in children with ASD have been inconsistent. One previous study highlighted that median levels of urinary creatinine was lower in children with PDDs ($n = 24$) compared with controls ($n = 50$) (Whiteley et al. 2006). Similarly, significantly different urinary creatinine levels were reported in children with and without ASD in a recent study (Wang et al. 2009). On the other hand, Nataf et al. (2008) reported no significant differences in urinary creatinine levels between children with ASD ($n = 217$) and "controls" ($n = 23$) regardless of normalization for age and gender. However, Nataf et al.'s findings should be interpreted with caution as they included children with AS in their control group i.e. 11/23 after they demonstrated that they had urinary creatinine levels that were not significant different to other controls i.e. 12/23 (Nataf et al. 2008).

Inborn errors of metabolism account for less than 5% of autism cases (Manzi et al. 2008). Wang et al. (2010) concluded that measuring urinary creatine, guanidinoacetate, and creatinine as biomarkers for guanidinoacetate methyltransferase or creatine transporter deficiencies during the diagnostic work-up process should be considered for all cases of suspected autism. Early detection of CDS cases in ASD guides the implementation of dietary interventions, which can dramatically improve outcomes for affected individuals.

Nicotinic acid metabolism

The previously mentioned urinary metabonomic study by Yap et al. (2010) reported urinary profiles in children with ASD that reflected altered nicotinic acid metabolism compared with controls. Specifically, they reported increased urinary levels of n-methyl-4-pyridone-3-carboxamide, n-methyl-nicotinic acid, and n-methylnicotinamide and concluded the changes could be attributed to perturbation of the tryptophan–nicotinic acid metabolic pathway in children with ASD.

Amino acids

Yap et al. (2010) also reported altered urinary levels of some free amino acids, including lowered levels of glutamate and higher alanine, glycine, and taurine in children with ASD compared to healthy controls. They suggested that the changes may be due to perturbation in sulfur dependent detoxification and amino acid metabolism in children with ASD.

General discussion

Definitive diagnosis of autism is currently based on DSM-IV or ICD criteria (WHO 1992; APA 2000). Input from a range of professionals in a multi-disciplinary team is essential to confirm a diagnosis of autism (CCD 2001). If an earlier diagnosis was possible or a diagnostic marker could confirm specific pathology of an autism metabolic phenotype, targeted intervention could improve prognosis.

Given the heterogeneity in etiology and/or pathophysiology in autism, it is unlikely that a single urinary marker will be able to discriminate children with and without autism. Thus, the putative urinary biomarkers examined in this review may only be useful for specific subgroups of individuals. The heterogeneity may also account for some of the variability in the results reported between studies. Moreover, the presence of specific comorbidities such as epilepsy or ongoing GI disturbance, may also lead to altered urinary metabolite profiles. The overall effect is that significant differences in certain chemicals may only be evident if autism specific subgroups are identified and investigated i.e. specific subgroups of individuals with autism will show different urinary metabolite profiles. A consistent theme that is emerging in autism research has previously been stated by Buie et al. (2010), "Given the heterogeneity of persons with autism and the many inconsistent research findings regarding autism, it is imperative that the phenotype (biological, clinical, and behavioral features) of future study subjects be well defined."

This literature review covers a range of studies that have various sample sizes, different participant groups, and diverse methods of analysis; all of which can influence findings. Differences in ASD case ascertainments i.e. whether participants have AD, AS, or PDD–NOS may lead to inconsistent results. Similarly, for control groups i.e. whether controls are siblings, unrelated healthy controls, or have other developmental disorders. For instance, significant differences were found between children with ASD and community controls, but not in sibling controls (Messahel et al. 1998). Studies by Martineau et al. (1991, 1992) used children with non-autistic developmental disorders and neurotypical children as two different control groups. Significantly different levels of DA were found in children with non-autistic development disorders compared with controls or ASD. Thus, when a mix of such subgroups is analyzed together this may disguise differences and/or contribute to inconsistent results between studies. Studies with large sample sizes and subjects that are well characterized (age, gender, autism characteristics, autism subtype, comorbidities) will be required to understand which specific characteristics are associated with each urinary biomarker. Furthermore, comparison with multiple, diverse control groups will be useful to help identify how specific the urinary chemical is to autism.

Previous studies reported a range of different urine sample collection periods including 24 h, daily (9 am–4 pm), evening (5 pm–8 am) or first morning urine samples. The collection periods may influence urinary volumes owing to more water intake and exercise during daytime and therefore urinary metabolites' concentrations will vary accordingly. An important direction for future research will be to compare different collection periods and establish which is the most reliable and accurate method of urine collection for the purpose of identifying biomarkers.

It is clear that there is substantial variation in the concentrations of urine metabolites between days for the same individual and due to environmental factors such as dietary variations. Fasting before sampling to avoid the changes caused by differences in dietary intake may be useful but could impose practical difficulties for young children with autism. An alternative would be to keep a food frequency questionnaire record so that nutritional influences may be subsequently investigated. Also, multiple sample collections for each subject may be a viable option to minimize intra-individual variability and the influence of collection period.

Urine is a complex matrix and altered metabolite profiles relate to many factors, such as age, race, and pubertal status. It has been reported that levels of many urinary metabolites are associated with the age of participants and thus age should be considered as a potential confounder. Options may include age restricting participants or adjusting for age during data analysis. The significant effects of pubertal status and race on various biochemical measurements have been established in recent studies (Lam et al. 2006). The soon-to-be released fifth version of Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (APA 2010) has proposed to consider the influence of gender, race, and ethnicity when diagnosing ASD.

A variety of different techniques and protocols have been used for sample collection, storage, and analysis which may also contribute to variation in results. For instance, IAG and neopterin are light sensitive, so samples need to be protected from light (Messahel et al. 1998; Mills et al. 1998). More specific and multi-analytical techniques are better for biomarker studies. For example, a study mentioned the combination of HPLC–UV and mass spectrometric technique may have greater sensitivity and specificity for sample analysis (Hunter et al. 2003). In addition, differences in creatinine levels observed in children with and without ASD suggest it is important to use alternative normalizing reference compounds (Whiteley et al. 2006; Nataf et al. 2008).

Small sample sizes were an issue for many of the studies examined. This may reflect the difficulties in recruiting children generally but specifically recruiting children with autism. Small sample sizes have low statistical power and generalizability is problematic, both limiting interpretation of findings. A common conclusion for many of the studies examined was that although results showed that various biomarkers were potentially useful for diagnosing and/or subtyping, there is a need to independently replicate the results based on a well powered and valid study design before consideration of any clinical implications. A shortcoming of many of these studies is that the actual metabolic pathway implicated is unknown or not investigated. For example, urinary IAG is assumed to be produced by GI microbacteria but this has not been proven. Abnormalities in peripheral measurements of neurotransmitters have been reported in ASD but whether correlations exist with CNS neurotransmitter levels is not clear, limiting interpretation of clinical relevance. It would be valuable to establish a database containing clinical and biological data (including urine samples) to help direct further investigations.

The relationship between what is known about the genetic basis of autism and the urinary metabolite findings is beyond the scope of this review. Further the likely presence of multiple autism phenotypes means interpretation of currently available knowledge could only be speculative. However, further research should be focused towards linking urinary and genetic findings which has potential to reveal etiology-based biomarkers. For example, 5-HT related gene variants have been reported in some individuals with autism (Klauck et al. 1997; Hranilovic et al. 2008) and may contribute to the abnormal levels of urinary 5-HT found in children with autism compared with controls. Further evidence of a genetic role for differences in urinary metabolites is that

for some metabolites, the concentrations measured in siblings are intermediate between ASD and control participants (Messahel et al. 1998; Wang et al. 2009).

Conclusion

Despite a large number of diverse studies focusing on urinary metabolites in autism, there currently is no strong evidence for any single metabolite having significant clinical utility or providing conclusive insight into the etiology or pathophysiology of autism. A number of urinary metabolites are potentially useful and/or insightful but require further validation. Due to the scope of this line of research to aid autism diagnosis as well as the development and selection of treatments, it is important that further studies are undertaken to confirm and expand current understanding of urinary metabolites in autism.

Declaration of interest

The authors report no declarations of interest.

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