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# Age-related change in brain metabolite abnormalities in autism: a meta-analysis of proton magnetic resonance spectroscopy studies

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Abnormal trajectory of brain development has been suggested by previous structural magnetic resonance imaging and head circumference findings in autism spectrum disorders (ASDs); however, the neurochemical backgrounds remain unclear. To elucidate neurochemical processes underlying aberrant brain growth in ASD, we conducted a comprehensive literature search and a meta-analysis of <sup>1</sup>H-magnetic resonance spectroscopy (<sup>1</sup>H-MRS) studies in ASD. From the 22 articles identified as satisfying the criteria, means and s.d. of measure of N-acetylaspartate (NAA), creatine, choline-containing compounds, myo-Inositol and glutamate + glutamine in frontal, temporal, parietal, amygdala-hippocampus complex, thalamus and cerebellum were extracted. Random effect model analyses showed significantly lower NAA levels in all the examined brain regions but cerebellum in ASD children compared with typically developed children (n = 1295 at the maximum in frontal, P < 0.05 Bonferronicorrected), although there was no significant difference in metabolite levels in adulthood. Meta-regression analysis further revealed that the effect size of lower frontal NAA levels linearly declined with older mean age in ASD (n = 844, P < 0.05Bonferroni-corrected). The significance of all frontal NAA findings was preserved after considering between-study heterogeneities (P < 0.05 Bonferroni-corrected). This first meta-analysis of <sup>1</sup>H-MRS studies in ASD demonstrated robust developmental changes in the degree of abnormality in NAA levels, especially in frontal lobes of ASD. Previously reported largerthan-normal brain size in ASD children and the coincident lower-than-normal NAA levels suggest that early transient brain expansion in ASD is mainly caused by an increase in non-neuron tissues, such as glial cell proliferation.

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#### Introduction

Autism spectrum disorder (ASD) is a representative neurodevelopmental disorder that is behaviorally defined by deficits in social reciprocity, impaired verbal communication, and restrictive and repetitive behavior.<sup>1,2</sup> In the background of such atypical behavioral development, previous studies have suggested the existence of atypical brain development in ASD that overall brain size was slightly reduced at birth, dramatically increased within the first year of life, but then gradually plateaued into adulthood.3-5 However, brain size studies cannot provide tissue neurochemical information. Although post-mortem studies demonstrated cytoarchitectonic abnormalities, aberrant minicolumnar organizations and microglial activations in brains of autistic individuals,6 post-mortem studies lack information about the trajectory of brain development. Therefore, the neural mechanisms explaining the aberrant trajectory of brain growth in ASD are yet to be elucidated, although several hypotheses such as excess neuron number have been proposed.<sup>6,7</sup>

<sup>1</sup>H-magnetic resonance spectroscopy (<sup>1</sup>H-MRS) is a noninvasive neuroimaging technique that estimates specific chemical metabolite measures in vivo.8 Previous studies have used <sup>1</sup>H-MRS to quantify glutamine/glutamate (referred to collectively as 'Glx'); N-acetylaspartate (NAA), a marker of neuronal density and activity;9 choline-containing compounds (Cho), a measure primarily reflecting the constituents of cell membranes;<sup>10</sup> creatine and phosphocreatine (Cre), a measure of cellular energy metabolism;<sup>10</sup> and myo-Inositol (ml), a major osmolite, precursor for phosphoinositides involved in the second messenger system.<sup>11</sup> Previous <sup>1</sup>H-MRS findings have yielded some inconsistency such as decreased<sup>12-14</sup> or no difference or increased NAA measure in ASD people compared with typically developed (TD) individuals.<sup>15–18</sup> The statistical power of each single previous <sup>1</sup>H-MRS study is relatively small, and previous studies have not corrected for multiple comparisons. As brain structural studies show an aberrant trajectory of neurodevelopment, it was reasonable to predict that the degree of neurochemical abnormalities indexed by <sup>1</sup>H-MRS may also change according to developmental stages in ASD. However, to date only one longitudinal <sup>1</sup>H-MRS study focusing on lactate level has been reported.<sup>19</sup> Therefore, performing a meta-analysis is one possible solution to realize sufficient statistical power for making conclusion about the neurochemical abnormality of autistic brain and is currently the only way to examine age-related change of <sup>1</sup>H-MRS abnormality of autistic brain.

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To our knowledge, neither a systematic review nor a metaanalysis of <sup>1</sup>H-MRS studies in people with ASD has been reported previously. The current systematic review and metaanalysis were designed to test the hypothesis that the degree of abnormalities in metabolite levels measured with <sup>1</sup>H-MRS would change from childhood to adulthood. Concretely, in case that autistic early brain expansion is mainly caused by increase in neuronal tissue, transient increase in NAA level would be found during childhood but it would not be found in adulthood. On the other hand, if increase in non-neuronal tissue mainly contributes to early brain expansion, NAA level would be remained at normal or even on a decline in childhood but in adulthood.

### Materials and methods

**Data sources.** <sup>1</sup>H-MRS studies that examined metabolite measures in the brains of individuals with ASD and TD control subjects were obtained through the computerized databases MEDLINE, PsycINFO, EMBASE and Web of Science. The search terms used in the systematic screening were *autism, autistic, ASD, Asperger's, developmental disorder, pervasive developmental disorder (PDD)* and *mental development,* which were also combined with *magnetic resonance spectroscopy* and *MRS.* Titles and abstracts of studies were examined to check whether or not they could be included. Reference lists of included articles were also examined to search additional studies to be included.

**Selection of study.** Studies were included if (1) they were brain <sup>1</sup>H-MRS studies published between 1980 and December 2010, (2) they examined people with ASD compared with a TD control group and (3) they reported sufficient data to obtain significant effect sizes; means, s.d. and numbers of participants. The literature search was performed without language restriction. If they did not report sufficient data, we emailed the corresponding and then the last author to obtain them. In cases where neither of them responded, we excluded the study. Two reviewers (YA and HY) performed study screenings independently.

Data extraction. To perform the meta-analyses, we defined a standardized mean difference as the effect size statistic Cohen's d, which is calculated as the difference between the mean of the experiment group and the mean of the comparison group divided by the pooled s.d. In the current meta-analyses, mean measure of NAA, Cre, Cho, mI and Glx in autistic individuals was subtracted from those in TD groups in each volume of interest (VOI) respectively, and divided by the pooled s.d. of both. Data were separated by the mean age of participants to examine the hypothesis the degree of metabolite measure abnormality would change from childhood to adulthood. When the mean age of participants was >20, the study was included in the meta-analysis in adulthood.<sup>15,18,20,21</sup> In a study reporting the age range as from 3 to 5 years with no description of the mean age of participants, we considered the participants to have a mean age of 4 years.<sup>22</sup> In cases of studies reporting more than two measures of metabolites, we determined the priority for extraction as absolute measure then ratio to Cre. Two

reviewers (YA and HY) performed all the data extraction and computation of effect size independently to minimize errors. Meta-analysis of Observational Studies in Epidemiology guidelines<sup>23</sup> were followed in the study.

Identification of brain regions. Our hypothesis focused on the developmental aspect of autistic brain pathology, we classified the sub-regions into frontal, amygdala-hippocampus complex (AHC), temporal, parietal, cerebellum and thalamus, in line with the similarity of developmental background within each sub-region.<sup>24</sup> In the case of a study reporting measures from more than one sub-region in one area (for example, anterior cingulate cortex and dorsolateral prefrontal cortex), these were assigned to the appropriate meta-analysis sub-group (that is, frontal lobe) as two (or more) independent data sets regardless of tissue type, such as gray matter, white matter or both. VOIs in the medial temporal lobe that included the hippocampus or amygdala region were included into AHC sub-group.13,22,25 VOIs in the intraparietal sulcus<sup>20</sup> and temporoparietal junction<sup>20</sup> were assigned to the parietal lobe, while that in the insula<sup>14</sup> was assigned to the temporal lobe. To ensure the meta-analysis was sufficiently powered, brain region measures were included if there were two or more studies reporting more than three VOIs in total. VOIs in TD control subjects who were compared with more than two ASD groups were identified<sup>25,26</sup> and divided into the appropriate number of comparison subgroups to avoid duplicate counting.

Meta-analysis. All meta-analyses were performed using Review Manager ((RevMan) [Computer program], Version 5.1, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011) from the Cochrane collaboration. A random effect model was adapted for the current metaanalysis to control potential heterogeneity such as variation in location of VOI, implementation of tissue segmentation within VOIs, single- vs multi-voxel spectroscopy, echo time, volume of VOI and types of metabolites measure. Cohen's d was calculated and used as effect sizes. As the differences in metabolite levels in ASD compared with TD subjects were predicted to differ between childhood and adulthood, the comparison was examined separately in childhood and adulthood. We employed conservative definitions of significance level determined using Bonferroni corrections, with P < 0.0022 in childhood (= 0.05/23 included metabolites in six regions) and P < 0.0033 in adulthood (= 0.05/15 included metabolites in four regions; Table 2).

**Sensitivity analyses.** The robustness of significant findings from meta-analysis was further tested by sensitivity analysis in specified sub-groups excluding studies with potential confounds. These potential confounds included comorbid epilepsy, medication, presence of mental retardation, field strength of MR scanner, types of MRS measures, segmentation within VOIs and diagnostic tools. The significance level was defined as P < 0.0014 (= 0.05/35 comparisons (7 potential confounds × 5 regions)).

**Meta-regression.** To test the hypothesis that neurochemical abnormalities would change with age, we performed

meta-regression analyses in the combined children–adult sample to examine the relation between participants' mean age and Cohen's *d* for the NAA levels in the frontal lobe, parietal lobe and AHC in which the meta-analysis revealed significant differences between ASD and TD individuals in childhood or adulthood, with sufficient sample sizes to test meta-regression (n > 10).<sup>27</sup> The regression was examined using SPSS 18.0 (SPSS, Chicago, IL, USA). The level of statistical significance was defined, by applying the Bonferroni correction, as P < 0.012 (= 0.05/4 areas).

The current meta-analyses included studies with considerable heterogeneities; such as presence of mental retardation, medication, types of MRS measures, employment of segmentation within the VOI, volume of VOI and comorbid epilepsy (for example, absolute measure or ratio to Cre). To investigate the influence of these potential modifiers, we performed meta-regression analyses for the metabolite measures in which the current meta-analyses showed significant difference between subjects with ASD and TD. The meta-regressions were examined in the childhood and adulthood combined sample to examine in a sufficient number of data sets.<sup>27</sup> The significance level was set at P < 0.05 to strictly assess the effect of heterogeneity.

Assessing between-study heterogeneity. The Cochran Q and  $l^2$  statistics were employed to examine between-study heterogeneity. The significance level was defined as P < 0.10 to conclude the studies were heterogeneous.<sup>28</sup>

**Publication bias.** Publication bias was assessed qualitatively by visual inspection of funnel plots and quantitatively by linear regression analysis for each group and each brain region. Based on previous literature,<sup>27</sup> this calculation was tested with data sets of at least 10.

**Data synthesis.** Twenty-three demographic, clinical and methodological variants, including the number of participants, number of male participants, mean age, range of age, intelligence quotient range, diagnostic criteria or tools, pharmacological status, presence of comorbid epilepsy, sequence for MRS acquisition, utilization of segmentation within VOIs, strength of magnetic field (Tesla), echo time (TE), repetition time (TR), types of MRS measurements (absolute measure or ratio to Cre), type of metabolites reported, location of VOI, size of VOI, and main results of the study were extracted, as shown in Table 1. Total number of participants, studies and VOIs, and mean difference, 95% confidence interval, *P*-value,  $f^2$  score and significance of linear regression analysis of symmetric property of funnel plots calculated from each meta-analysis are shown in Table 2.

### Results

**Study selection.** The literature search described above yielded 244 articles, of which 47 studies were identified as potential candidates for the meta-analysis. Nine articles were excluded because lack of the original data. Ten were excluded because they did not recruit ASD individual. Two were excluded because of not utilizing <sup>1</sup>H-MRS. Two were

excluded because not reporting new data. Thus, 24 studies were included into the database.<sup>12–18,20–22,25,26,28,29,31–39</sup> From the database, one study was excluded from the meta-analysis because they did not report raw data regarding the metabolite measure<sup>28</sup> and one study was excluded because it did not provide sufficient data to calculate the standardized mean difference<sup>36</sup> (Figure 1).

Database. Studies included in the meta-analysis involved 569 participants with ASD and 415 TD control subjects. Table 1 summarizes variables recorded in the systematic review. Among these studies, 17 examined children, <sup>12-14,16,22,25,26,28,29,31-36,39</sup> including 447 with ASD and 285 with TD, while eight studies examined adults,<sup>16-18,20,21,37,38</sup> including 122 with ASD and 130 with TD. Fourteen studies recruited participants with mean intelligence quotient 70 or more,<sup>12,14–17,20,21,25,26,37–39</sup> whereas eight articles did not mention the precise mean or range of intelligence quotient.<sup>13,22,28,29,31-36</sup> Three child studies<sup>22,33</sup> included 19 ASD children with comorbid epilepsy, while only one adult study included one ASD subjects with epilepsy.<sup>37</sup> Five child studies<sup>13,16,25,26,29</sup> and four adult studies<sup>16,17,20,21</sup> included individuals with drugnaïve or no medication only, although medication status was unclear in some studies.<sup>22,28,31-35,38,39</sup> Diagnosis of ASD was determined based on DSM-IV (15 studies),  $^{12,14,18,20,22,25,26,28,29,31,32,36-38}$  DSM-III (one study),  $^{33}$ or ICD-10 (five studies)<sup>15-17,34,35</sup> criteria. Among the studies employing DSM-IV or DSM-III, 10 used Autism Diagnostic Interview-Revised (ADI-R)<sup>12-15,17,20,37-39</sup> or ADI,<sup>16</sup> 10 utilized Autism Diagnostic Observation Schedule (ADOS)<sup>14,16,21,37-39</sup> or Autism Diagnostic Observation Schedule-Generic (ADOS-G),<sup>12,13,20</sup> and nine employed both.<sup>12-14,16,20,21,37-39</sup> For image acquisition, 12 studies utilized point-resolved selec-tive spectroscopy,<sup>15–18,20,21,26,29,31,34,36,37</sup> while six employed <sup>1</sup>H-stimulated echo acquisition mode sequence.<sup>22,28,32,33,35,39</sup> Four studies<sup>12,20,22,32</sup> used 3-tesla MRI scanners, while 20 studies<sup>13–18,21,22,25,26,28,29,31,33–39</sup> utilized 1.5-tesla scanners. Twelve child studies  $^{12-14,16,22,28,31,32,34-36,39}$  and seven adult studies<sup>15–17,20,21,37,38</sup> employed MRS measures with estimation of absolute measure. Seven child studies<sup>12–14,16,25,32,39</sup> and five adult studies<sup>15–17,21,38</sup> utilized tissue segmentation within VOIs.

**Meta-analysis for metabolite measures in childhood and adulthood.** In childhood, individuals with ASD showed significantly reduced NAA levels compared with TD controls in all the brain regions but cerebellum included in the meta-analysis (P < 0.05, Bonferroni-corrected; Figure 2). In contrast, no significant difference was found in other metabolite concentrations between children with ASD and TD controls. Although several metabolites showed differences in levels between children with ASD and TD at the trend level significance (NAA in the cerebellum: P = 0.008; mI in the frontal areas: P = 0.008; Cre in the frontal areas: P = 0.01, in AHC: P = 0.009; in the thalamus: P = 0.04; Cho in the thalamus: P = 0.03), these significant effects disappeared after the Bonferroni correction for multiple comparisons was applied (Table 2).

In contrast to childhood, no metabolites showed a significant difference in metabolites levels between people with

Table 1 Summary c	if included a	irticles															
Study		7	Demogr	aphic char	acter				Meth	golobo	tical c	haracte	¥			Results	
	в И	Mean age years	å	ĨQ°	Diagnostic tools	Medication/ comorbidity	MRS sequence/ sequence/	Tesla/TE/ TR (ms)	Types of MRS	NAA	Cre	Cho n	nl Glx	VOI location	VOI size (ml)	Compared with TD	
	(males)	(range)					segmentation		lifeasures								
<i>Children</i> Chugani <i>et aP</i> <sup>8</sup>	9 (8)	5.7 (3–12)	5	NA	DSM-IV	AN NA	STEAM	1.5 30/2000	Absolute	+	I		I	FL	NA	SN	
								20/2002						с Г	A A Z Z	NAA	
DeVito <i>et al.</i> <sup>12</sup>	26 (26)	9.8 (6–17)	29	≧70	DSM-IV ADIR ADIS-G	Medicated No comorbidity	SEMS Done	3 135/1800	Absolute	+	+	+	+	It. FL	1.2	NAA, GIX ↓	
					D-0074									년 년 년 년	<u></u> vi vi v	NS	
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Endo <i>et al.</i> <sup>25</sup>	38 (32)	12.9 (6–20)	16 8	19.9 (mean)	VI-MSD	No medication	PROBE/SV	1.5	Ratio	+	T	+	1	rt. MT	ω	NAA/Cre ↓	
							POID	0002/86						rt. MPFC CV	ω ω	NS NS	
Fayed and Modrego <sup>29</sup>	21 (18)	7.3 NA	42	AN	NI-MSD	Drug naïve NA	PRESS Undone	1.5 30/2500	Ratio	+	I	+	I.	It. CS	œ	SN	
Friedman <i>et al.</i> <sup>30</sup>	45 (38)	3.95 (3.2–4.5)	13	NA	ADIR ADOS-G	Drug naïve No comorbidity	NA Done	1.5 20/2000	Absolute	+	+	+	l	rt. FL	-	SN	
								272/2000						lt. FL rt. cingulate		NAA, Cre ↓ NAA ↓	
														lt. cingulate rt. caudate lt. caudate		→ → → → → → → → → → → → → → → → → → →	
														rt. putamen It. putamen		NS NA A	
														rt. TH it. TH ineula		NAA ↓ Cre ↓ ml	
														it. insula rt. TL		NS NAA, Cho ↓	
														t. TL MT		NS Cho ↓	
														it. MT at. callosum pt. callosum		NS NS	
														rt. PL It. PL OL		NAA ↓ Cre, ml ↓ ml ⊥	
Fujii <i>et al.</i> <sup>31</sup>	31 (25)	6.1 (2–13)	28	NA	DSM-IV	NA	PRESS	1.5 135/1300	Absolute Batio	+	+	+	I	ACC	4.5	NAA/Cre ↓	
														It. DLPFC rt. DLPFC	3.4 3.4	NAA/Cre ↓ NS	
Gabis <i>et al.</i> <sup>26</sup>	13 (10)	10 (7–16)	80	₩80	DSM-IV-TR	Drug naïve No comorbidity	PRESS	1.5 40/2000	Ratio	+	T	++	1	rt. AHC	4.1	NAA/Cre↓, ml/Cre↑	
														It. AHC	4.1	NAA/Cre↓, Cho/Cre↑_mI/Cre↑	
														СГ	8	Cho/Cre↑, ml/Cre↑	

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Table 1 (Continued)																
Study		Õ	lemogi	aphic chara.	cter				Meth	olobo	gical c	harac	ter			Results
	N <sup>a</sup> (males)	Mean age years (range)	٩	à	Diagnostic tools	Medication/ comorbidity	MRS sequence/ segmentation	Tesla/TE/ TR (ms)	Types of MRS measures	NAA	Cre	Cho	m G	Ix VOI location	VOI size (ml)	Compared with TD
Harada <i>et al.</i> <sup>32</sup>	12 NA	5.2 (2–11)	10	NA	DSM-IV	NA NA	STEAM Done	3 15/5000	Absolute	+	+	+	+++	It. FL + boxticular	<b>თ</b> ი	SN
Hardan <i>et al.</i> , <sup>39</sup>	18 (18)	11.9 (8–15)	16	>70	ADI-R ADOS	NA No comorbidity	STEAM Done	1.5 20/1600	Absolute	+	+	+	+ +		5.8 5.8	NS Cra Cho NAA -
Hashimoto <i>et al.</i> <sup>33</sup>	28 (20)	5.6 (3–12)	25	59.8(mean)	DSM-III-R	NA 6: epilepsy	STEAM Undone	1.5 270/1500	Ratio	+	I	+	I I	ц. н. В	 1∼27	NS
Hisaoka <i>et al.</i> <sup>34</sup>	55 (47)	5.8 (2-21)	51	NA	ICD-10	A A A A	PRESS Undone	1.5 135/1300	Absolute	+	Í.	Ĺ	I I	드 도도도도도 	00000000000000000000000000000000000000	NS NS NAAA NAAA
Levitt <i>et al.</i> <sup>14</sup>	22 (18)	10.4 (5.4–15.7)	20	95.0 (mean)	DSM-IV ADIR	Medicated No comorbidity	NA Done	1.5 272/2300	Absolute	+	+	+	I	н. FL	0.00 1.2	N N
														부가분구 200 200 200 200 200 200 200 200 200 20	<u>, , , , , , , , , , , , , , , , , , , </u>	Cre ↓ Cho ↓ NS
														It: sup AC It: sup AC It: caudate head It: caudate body It: caudate body It: putamen It: putamen It: Tu	<u>, , , , , , , , , , , , , , , , , , , </u>	Cho, Cre ↓ NAA ↓ NS
														╕┽╕┽╕┽ ╤┶╓┙┙ ┲	<u>i q q q q q q</u>	NAA ↓ NS NAA ↓ NAA
Mori <i>et al.</i> <sup>61</sup>	29 (22)	8 (median) (5–15)	19	AN	NI-MSD	NA 8:epilepsy	STEAM Undone	1.5 18/5000	Ratio	+	I	+		lt. MT rt. MT	4.3~6 4.3~6	NS NAA ↓
Mori <sup>22</sup>	70 (56)	NA (3–5)	18	ΨN	VI-MSD	NA 5 epilepsy	MEGA- PRESS Undone	3 68/1500 15/5000	Absolute	+	+	+	+ +	It. CH ACC	4.3∼6 12	J AA ↓ MI ↓
O'Brein <i>et al.</i> <sup>16</sup>	12 (11)	13 NA	10	98 (mean)	ICD-10 ADI, ADOS	No medication No comorbidity	PRESS Done	1.5 35/3000	Absolute Ratio	+	+	+	 +	rt. AHC	9	NAA ↑
Otsuka <i>et al.</i> <sup>35</sup>	27 (21)	NA (2–18)	10	AN	ICD-10	A A N A	STEAM Undone	1.5 18/5000	Absolute	+	+	+	I I	rt. AHC	ن و	NAA U
														E 2 ::	٥	

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N*         Mean generation (maise)         N*         N	Study		L	lemogi	aphic chara'	cter				Meth	olobo	gical	charac	ter			Results
(male)(range)(range)(range)(range)(range)(range)Vasconcolos et al.10 (1) $35$ (maia) MA10MADSM-IVNo conorbidlyPress15 301500Alsolute+++++Vasconcolos et al.11 (12) $292 (21-50)$ 14 $>90$ DSM-IVNo conorbidlyUndone302000Alsolute+++ </th <th></th> <th>Na</th> <th>Mean age years</th> <th>å</th> <th>ß</th> <th>Diagnostic tools</th> <th>Medication/ comorbidity</th> <th>MRS sequence/</th> <th>Tesla/TE/ TR (ms)</th> <th>Types of MRS</th> <th>NAA</th> <th>Cre</th> <th>Cho</th> <th>ml G</th> <th>x VOI location</th> <th>VOI size (ml)</th> <th>Compared with TD</th>		Na	Mean age years	å	ß	Diagnostic tools	Medication/ comorbidity	MRS sequence/	Tesla/TE/ TR (ms)	Types of MRS	NAA	Cre	Cho	ml G	x VOI location	VOI size (ml)	Compared with TD
Vasconcolos et al.**10 (10)9.5 (modian) MA10NADSM·IVMedicatedPress15.3011500Alsolute++ <th></th> <th>(males)</th> <th>(range)</th> <th></th> <th></th> <th></th> <th></th> <th>seginentation</th> <th></th> <th>lifeasures</th> <th></th> <th></th> <th></th> <th></th> <th></th> <th></th> <th></th>		(males)	(range)					seginentation		lifeasures							
AddRs         Ia(12)         29.2 (21-50)         14         >80         DSM-IV         No medication         PRESS         3         Absolute         +	Vasconcelos <i>et al.</i> <sup>36</sup>	10 (10)	9.5 (median) NA	10	NA	DSM-IV	Medicated No comorbidity	Press Undone	1.5 30/1500	Absolute Ratio	+	+	+	 +	AC	8	NS
Address Bernardia et al. <sup>10</sup> 14 (12) $> 23.2$ (21-50)14> 80 $SMHV$ ADDS-GNo medicationPRESS No comorbidity3302000Absolute+++ <th< td=""><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td>It. FL It. striatum It. CL</td><td>ω ω ω</td><td></td></th<>															It. FL It. striatum It. CL	ω ω ω	
Kleinhans <i>et al.</i> <sup>37</sup> 13 (13) $24.5 (15-44)$ 13       >70       DSM-IV       Medicated       PRES       1.5       Absolute       +	<i>Adults</i> Bernardi <i>et al.</i> <sup>20</sup>	14 (12)	29.2 (21–50)	14	> 80	DSM-IV ADIR	No medication No comorbidity	PRESS Undone	3 30/2000	Absolute	+	+	+	+ +	lt. TH	0.5625	SN
Kleinhans et al.3713 (13) $24.5 (15-44)$ 13 $>70$ $DSM.IV$ Medicated $PRESS$ $1.5$ $Absolute$ $+$ <td></td> <td>rt. TH It. TPJ</td> <td>0.5625 0.5625</td> <td>) Lm</td>															rt. TH It. TPJ	0.5625 0.5625	) Lm
Kleinhans et al.3713 (13)24.5 (15-44)13 $>70$ DSM-IVMedicatedPRESS1.5Absolute $+$ <															rt. TPJ It. IPS 4 IPS	0.5625 0.5625	NS
Kleinhans <i>et al.</i> <sup>37</sup> 13 (13) $24.5 (15-44)$ 13         >70         DSM-IV         Medicated         PRESS         15         Absolute         +         <															It. ACC	0.5625 0.5625 0.5625	Gk ↓
Kleinhans <i>et al.</i> <sup>38</sup> $20(18)$ $23.57$ NA $19$ $\gg 80$ $DSM.IV$ NAPRESS $1.5$ Absolute $+$	Kleinhans <i>et al.<sup>37</sup></i>	13 (13)	24.5 (15–44)	13	>70	DSM-IV ADIR ADOS	Medicated 1:Epilepsy	PRESS Undone	1.5 35/3000	Absolute	+	+	+	I I	It. FL	2.7	) AA ↓
Kleinhans <i>et al.</i> <sup>36</sup> 20 (18) $23.57$ NA       19       >80       DSN-IV       NA       PRESS       1.5       Absolute       +															rt. CH CV OL PL	200	SN
Murphy et al. $^{15}$ 14 (14)30 NA1897 (mean)ICD-10Had medicationPRESS1.5Absolute++++C'Brein et al. $^{16}$ 10 (7)35 NA12105 (mean)ICD-10No medicationPRESS1.5Absolute++	Kleinhans <i>et al.</i> <sup>38</sup>	20 (18)	23.57 NA	19	⊗80	DSM-IV ADIR ADOS	NA NA	PRESS Done	1.5 30/2000	Absolute Ratio	+	+	+	 +	rt. AHC	3.4	NS
C'Brein et al. <sup>16</sup> $10$ (7) $35$ NA $12$ $105$ (mean) $ICD-10$ No comorbidityDone $136/2000$ Habsolute $+$ $+$ $+$ C'Brein et al. <sup>18</sup> $10$ (7) $35$ NA $12$ $105$ (mean) $ICD-10$ No medicationPRESS $1.5$ Absolute $+$	Murphv <i>et al.</i> <sup>15</sup>	14 (14)	30 NA	18	97 (mean)	ICD-10	Had medication	PRESS	1.5	Absolute	+	+	+	I	It. AHC rt. MPFC	3.4 8.5	NAA. Cre. Cho ↑
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Oner et al. <sup>18</sup> 14 (14)         24.3 (17–38)         21         >70         DSM-IV         Medicated         PRESS         1.5         Ratio         +         -         +         +         -         +         -         +         -         +         -         +         -         -	O'Brein <i>et al.</i> <sup>16</sup>	10 (7)	35 NA	12	105 (mean)	ICD-10 ADI, ADOS	No medication No comorbidity	PRESS Done	1.5 35/3000	Absolute Ratio	+	+	+	 +	rt. AHC	9	NS
Page et al. <sup>17</sup> 25 (20)         35.6NA         21         >70         ICD-10         Drug naïve         PRESS         1.5         Absolute         +	Oner <i>et al.</i> <sup>18</sup>	14 (14)	24.3 (17–38)	21	>70	DSM-IV	Medicated No comorbidity	PRESS Undone	1.5 270/1500	Ratio	+	I	+	I I	rt. ACC	۲	NAA/Cho ↑
ADIR (72%) No comorbidity Done 35/3000	Page <i>et al.</i> <sup>17</sup>	25 (20)	35.6NA	21	>70	ICD-10	Drug naïve	PRESS	1.5	Absolute	+	+	+	+	rt. DLPFC rt. AHC	<del>-</del> 9	NS Cre. Glx ↑
	)					ADIR (72%)	No comorbidity	Done	35/3000						rt. PL	œ	SN
Suzuki <i>et al.</i> <sup>21</sup> 12 (12) 22 (18–25) 12 >70 ADIR Dng naïve PRESS 1.5 Absolute + + + + A Northead Absolute + + + +	Suzuki <i>et al.</i> <sup>21</sup>	12 (12)	22 (18–25)	5	>70	ADIR ADOS	Drug naïve No comorbidity	PRESS Done	1.5 144/1500	Absolute	+	+	+	I I	It. AHC rt. CH	68	Cho, Cre ↑ NAA ↓

at, anterior; CH, cerebellar hemisphere; CG, cingulate gyrus; Cho, choline-containing compounds; CL, cerebellum, Cre, creatine; CS, centrum semiovale; CV, cerebellar vermis; DLPFC, dorsolateral prefrontal cortex: DSM, Diagnostic and Statistical Manual of Mental Disorders; FL, frontal lobe; ml, myo-Inositol; inf, inferior; ICD, International Statistical Classification of Diseases and Related Health Problems; IPS, intraparietal sulcus; IC, intelligence quotient; MEGA, MEscher – GAwood; MPFC, medial prefrontal cortex: MPL, medial pratrictal lobe; MRS, magnetic resonance spectroscopy; MT, medial temporal; NA, not applicable; NAA, *N*-acetylaspartate; NS, no significant differences; OL, occipital lobe; PRESS, point-resolved selective spectroscopy; PROBE/SV, PROton Brain Exam/Single Voxel; PL, parietal lobe; pt, posterior; SEMS, Scanning Rectron Microscopes; sup, superior; STEAM, <sup>1</sup>H-stimulated echo acquisition mode; TE, echo time; TD, typically developed; TL, temporal lobe; TH, thalamus; TPJ, temporoparietal junction; TR, repetition time. <sup>b</sup>Number of controls. <sup>c</sup>Criteria for IQ or mean IQ of participants.

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Participants	Region	Metabolites	Total number of ASD vs TD subjects	Total number of studies	Total number of VOIs	Standardized mean differences	95% Cl lower	95% Cl upper	P- <i>value</i>	2	Publication bias
Children	Frontal	NAA Cre MI Gix	764 vs 531 561 vs 362 599 vs 378 314 vs 138 Not enough data	8974-	25 22 8 8 22	-0.35 -0.24 -0.07 -0.44 NA	-0.51 -0.43 -0.22 -0.76 NA	-0.2 -0.06 0.08 NA	0.00001 <sup>a</sup> 0.01 0.35 0.008 NA	42% 44% 55% NA	0.226 0.536 0.851 NA NA
	Amygdala-hippocampus complex	NAA Cre MI GIX	245 vs 115 129 vs 46 245 vs 115 128 vs 52 Not enough data	ں ۳ ۵ ۳ ۵	<u>ဗ</u> 4 ဗိ ု ၇ ၀	-0.88 -0.46 0.53 NA	1.31 0.81 -0.53 NA	-0.46 -0.12 0.3 NA	<ul> <li>&lt; 0.0001<sup>a</sup></li> <li>0.09</li> <li>0.59</li> <li>0.22</li> <li>NA</li> </ul>	61% 0% 78% NA	0.385 NA 0.128 NA NA
	Parietal	NAA Cre MI Gix	316 vs 233 178 vs 106 206 vs 131 Not enough data No data	4 0 0 0 0	<u>+</u> 0000	-0.39 -0.33 -0.07 NA	-0.61 -0.69 -0.29 NA NA	-0.17 0.04 NA NA	0.0006 <sup>a</sup> 0.08 0.56 NA	31% 0% NA	0.092 NA NA NA
	Temporal	NAA Cre ml Gix	252 vs 186 142 vs 84 142 vs 84 142 vs 84 Not enough data	∞ <i>∩ ∩ −</i>	0 4 4 4 N	-0.55 -0.09 -0.17 -0.27 NA	0.89 0.44 0.72 0.7 NA	-0.22 0.26 0.38 0.16 NA	0.001ª 0.62 0.55 0.22 NA	62% 33% 55% NA	A A A A A A A A A A A A A A A A A A A A
	Cerebellum	NAA Cre Gix Gix	151 vs 109 79 vs 68 151 vs 109 65 vs 66 Not enough data	ע ט ט <del>–</del>	თ ო თ 4 თ	-0.35 -0.1 0.29 NA	-0.6 -0.44 0.05 NA	-0.09 0.23 -0.23 NA	0.008 0.54 0.33 1.67 NA	0% 11% NA	A A A A A A A A A A A A A A A A A A A A
	Thalamus	NAA Che M GI O	170 vs 98 170 vs 98 170 vs 98 126 vs 58 Not enough data	თ თ თ თ <del>–</del>	0 0 0 <del>4</del> 0	-0.58 -0.38 -0.44 NA	0.89 0.75 0.83 -0.62 NA	-0.28 -0.02 -0.05 0.16 NA	0.0002 <sup>a</sup> 0.04 0.03 0.25 NA	25% 54% NA	A A A A A A A A A A A A A A A A A A A A
Adults	Frontal	NAA Cre Gix Gix	80 vs 101 52 vs 59 80 vs 101 Not enough data Not enough data	404	୦ <del>7</del> ୦ ୦ ୦ ୦	0.1 0.24 0.11 NA	-0.4 -0.15 NA NA	0.59 0.62 NA NA	0.7 0.23 NA NA	62% 5% NA NA	A A A A A A A A A A A A A A A A A A A A
	Amygdala-hippocampus complex	NAA Cre ml Gix	62 vs 56 62 vs 56 62 vs 56 50 vs 44 Not enough data	4440 <del>-</del>	4 4 4 0 <del>-</del>	0.19 0.6 0.39 NA	-0.18 -0.03 -0.25 -0.03 NA	0.55 1.23 1.11 NA	0.32 0.06 0.07 NA	0% 68% 0% NA	A A A A A A A A A A A A A A A A A A A A
	Parietal	NAA Cre ml Gix	99 vs 102 99 vs 102 99 vs 102 73 vs 75 73 vs 75	4 4 4 N N	ちっしょ	-0.37 -0.15 -0.17 -0.47 -0.22	-0.65 -0.43 -0.45 -1.06 -0.55	-0.08 0.13 0.13 0.13 0.13	0.01 0.29 0.12 0.18	0% 0% 0% 0%	A A A A A A A A A A A A A A A A A A A A
	Cerebellum	NAA Cre Cho Gix Gix	38 vs 38 38 vs 38 38 vs 38 No data No data	~~~~	~~~~	-0.7 -0.02 -0.29 NA	1.16 0.52 0.75 NA	-0.23 0.49 0.16 NA NA	0.004 0.95 NA NA	19% NA NA	A A A A A A A A A A A A A A A A A A A
Abbreviations: *Statistically si	ASD, autism spectrum disorder; Cl, cc gnificant after Bonferroni-correction.	GIX Infidence interva	No data Il; Cho, choline-containing	0 compounds: Cre,	0 creatine; ml: myo	NA -Inositol; NA, not appl	NA licable; NA	NA A, <i>N</i> -acety	NA lasparta	te; T	te; TD, typica

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Figure 1 Process of study selection.

ASD and TD in adulthood. The NAA levels in the parietal lobe and cerebellum showed a trend for decreased measures in adults with ASD compared with those with TD (P=0.01 in the parietal lobe and P=0.004 in the cerebellum), although these differences did not reach statistical significance after the Bonferroni correction was applied (Table 2).

In addition, it was confirmed that Bonferroni corrections for multiple comparisons were not too strict, because correction using the Student–Newman–Kuels procedure did not change the results both for childhood and adulthood.

Sensitivity analyses. All sensitivity analyses performed in the specified-subgroups with more homogeneous quality showed significant reductions in NAA measures in the frontal lobe of children with autism (P < 0.05, Bonferroni-corrected). These results demonstrated the robustness of reduced frontal NAA level during childhood even after considering methodological and participant's heterogeneity such as comorbidity of other neuropsychiatric diseases and intellectual disability, medication status, diagnostic methods, field strength of MR scanner, types of MRS measures and implementation of segmentation within VOI. In the other areas, some sensitivity analyses revealed that the significant effect of low NAA level disappeared in some subgroups. In the AHC, parietal cortex, temporal regions and thalamus, the significance of NAA reductions was preserved in the most subgroups, such as ASD individuals with no comorbid epilepsy, no medications and acquisition of MRS in a 1.5-tesla scanner (Supplementary Table 1).

**Meta-regression.** The current meta-regression revealed a significant inverse effect of mean age of participants on NAA measures in the frontal lobe (P=0.009), but in the AHC or

parietal cortex (Figure 3). Even after excluding heterogeneity of participants and methodologies in the included study, meta-regression analyses in specified subgroups further demonstrated the significant effects of mean age in the frontal lobe. These analyses were performed in studies with implementation of segmentation within VOIs (P<0.001), with multi-voxel MRS (P=0.021), with 1.5-tesla scanner (P=0.004), with participants without comorbid epilepsy (P=0.001), without medication (P=0.006), without mental retardation (P=0.032) and without participants diagnosed not using ADI-R or ADOS (P=0.006).

The meta-regression revealed significant effects of the type of MRS measures and the employment of segmentation within VOIs on the NAA levels in AHC (P<0.05). However, no potential modifiers significantly affect the NAA levels in the frontal and parietal regions (Supplementary Table 2).

Between-study heterogeneity. No significant heterogeneity was detected in all the metabolites in any regions but in ml measure in AHC and cerebellum during childhood ( $\ell = 78\%$  and 80%, respectively) (Table 2).

**Publication bias.** The linear regression test showed significant publication bias was not detected in most metabolites (5/6) but in the parietal NAA of children (P<0.1; Table 2).

## Discussion

To our knowledge, this is the first systematic review and meta-analysis of <sup>1</sup>H-MRS studies in people with ASD. A total of 22 studies were integrated in the meta-analyses, of which 1476 <sup>1</sup>H-MRS measures from 31 data sets at maximum, 844 measures from ASD and 632 measures from TD individuals.

The current meta-analysis demonstrated that NAA levels in the frontal, parietal, temporal lobule, AHC and thalamus were significantly lower in ASD children compared with TD controls. In contrast, no significant difference in the any metabolite levels was found in adulthood. Importantly, our meta-regression analysis provides the first evidence that the degree of lower frontal NAA levels linearly declined with aging from childhood to adulthood in ASD people. The systematic review showed obvious methodological heterogeneities across studies, including in comorbid epilepsy, psychotropic medications, mental retardation, field strength of MR scanners, types of MRS measures, utilization of segmentation within VOIs and diagnostic tools. However, the sensitivity analyses further emphasized the robustness of current findings, especially regarding the lower-than-normal frontal NAA level in ASD children, since the potential confounds, heterogeneity between studies and publication bias did not significantly affect the findings.

The finding of robustly lower-than-normal frontal NAA during childhood seems to be consistent with the importance of this area in the pathophysiology of ASD indicated by previous functional imaging studies have repeatedly reported dysfunctional prefrontal cortices during psychological tasks requiring theory of mind,<sup>47</sup> social perception<sup>48,49</sup> and self-referencing.<sup>43</sup> The presence of early brain enlargement especially in frontal lobe<sup>50</sup> shown on structural MRI in autism



**Figure 2** Forest plot of frontal *N*-acetylaspartate (NAA). Standardized mean differences for NAA measures in frontal lobe between subjects with autism spectrum disorders (ASDs) and those with typical development (TD) in child and adulthood. The forest plot displays standardized mean differences and 95% confidential intervals (CIs).

is well established.<sup>3–5</sup> Previous post-mortem studies have also shown several deviations in various histological tissues including glial cells, von Economo neurons and neuronal organization in frontal cortices of ASD.<sup>6</sup>

The present linear regression analysis demonstrated a strong inverse correlation between decrement of frontal NAA and mean age in ASD subjects. Previous brain structural findings revealed abnormal trajectory of brain growth, such that the overall brain size in autism was slightly reduced at birth, dramatically increased within the first year of life, then gradually plateaued in adulthood.<sup>3–5</sup> As the current meta-analyses included studies involving participants with mean ages between 4 and 35, the period of which the meta-analyses covered corresponds to the phase of gradual dissipation of early brain overgrowth in ASD. It is notable that the decrease and subsequent recovery in NAA level was found during the period showing a significant increase and subsequent normalization in brain size in ASD, because

these overlapping effects were inferred on the basis of independent data.

The current neurochemical findings could provide some insight into the histochemical background of the abnormal trajectory of autistic brain growth. Although the histological background is yet to be uncovered, several hypotheses have been formulated.<sup>6,7</sup> These hypotheses could be divided into two major categories. The first is abnormalities associated with neurons: for example, excessive numbers of neuron, synapse, or minicolumns and excessive and/or premature growth of axon, dendrite, or neuron cell bodies. The second is abnormalities associated with glial cells, such as excessive numbers of glia, activated and enlarged glia, and excessive and/or premature myelination. NAA is localized mainly in the cell bodies, axons, dendrites and dendritic spines of mature neurons, and is considered to function as a marker of functional and structural neuronal integrity.9 Although recent studies have suggested NAA expressed



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Figure 3 The relationship between effect sizes for reduced *N*-acetylaspartate (NAA) and ages of study participants. Effect sizes from each comparison of VOIs are plotted by the mean age of participants with autism spectrum disorders of the study. The line of best fit shows a gradual but substantial decrease in NAA reduction. No data were obtained from individuals before the age of 4 years.

by oligodendrocytes,<sup>51</sup> NAA is less distributed in glia.<sup>52</sup> Therefore, since brain enlargement because of increase in neuron number should heighten NAA level in autistic brain, it is more likely that reduced NAA measures during childhood reflect decreased neuron density induced by increasing brain volume because of a factor other than associated with neurons.

Considered together, among the two possible explanations for early brain expansion in ASD, increased cell bodies, axons, dendrites and dendritic spines of neurons seems less likely than abnormalities associated with glia. Glial cells (for example, astrocytes) have been shown to increase in volume after birth.53 As glial cells initially occupy large percentage of brain volume,54 increased glial cell volume may be a major factor in brain enlargement without a significant increase in the NAA measure of <sup>1</sup>H-MRS. Previous post-mortem studies have reported glial abnormalities that can contribute the abnormal volume increase, such as microglial and astroglial activation and increased microglial density, in the prefrontal cortex in ASD.<sup>55,56</sup> Thus, it is reasonable to hypothesize that proliferation of glial cells diluting the density of neurons is a major factor in the abnormal brain overgrowth and decreased NAA levels observed in children with ASD.

The hypothesis that proliferation of glia could cause brain expansion with decreased neuron density and low NAA is consistent with observations in some neurological and genetic diseases. Previous studies have demonstrated decreased NAA because of abnormal synthesis of glia in patients with glioma.<sup>57</sup> Some other diseases manifesting macrocephaly, for example, neurofibrosis type 1 demonstrates decreased NAA measures. It was concluded that the reduced NAA measures in neurofibrosis type 1 was caused by increased brain volume because of excess myelination.<sup>58</sup>

Proliferation of glia can also explain the transient brain expansion during the neonatal period and infancy, and is consistent with the subsequent preservation of behavioral dysfunction in the period of gradually normalizing for brain expansion in ASD. Transient microglial cell proliferation and subsequent irreversible dysfunction could occur after inflammation or hypoperfusion.<sup>59</sup> Existence of inflammation or hypoperfusion during neonatal and infancy in ASD has also been suggested by several lines of evidence,<sup>55,56</sup> including decreased serum levels of adhesion molecules and a correlation with head circumference at birth.<sup>60</sup> Diminishing of transiently increased glia after early infancy is consistent with the normalization of transiently expanded brain size and decreased NAA during the overlapped time period. However, because we could not find the MRS study involved the participants with the age <1, abnormal brain growth during this period is out of the finding of the current metaanalysis.

Several methodological considerations and limitations of our study should be considered. First, because of the nature of meta-analysis, we can make statistical analysis only at the level of studies. There is no way to confirm whether the participants of included studies actually exhibited enlarged brains during childhood. Second, although inverse effect of age on frontal NAA was shown robustly by the metaregression analysis performed with the child-adult combined group, it remains unclear whether NAA levels in adults with ASD are equal to, or exceed, levels in TD, because of the relatively small number of included studies in adulthood. Third, because the most included studies utilized 1.5-tesla instead of 3-tesla scanner, it might be insufficient to collect reliable data about several metabolites, which could not be reliably evaluated with insufficient strength of magnetic field such as GIx. Fourth, the included studies display considerable heterogeneity such as variations in the type of metabolite measures and implementation of segmentation. The use of a ratio to Cre is based on the hypothesis that there is no difference in Cre levels between ASD and TD, which is shown to be questionable in the current meta-analysis. Not implementing segmentation is also based on the hypothesis the same proportion of cerebrospinal fluids in each VOI between cases and controls, while structural differences between ASD and TD have been repeatedly demonstrated. Furthermore, the abnormalities in metabolites level of ASD were reported to be different between those in gray matter and white matter.<sup>30</sup> We employed the random effect model and the sensitivity analyses in more homogeneous subgroups to control the heterogeneity, and the main findings were preserved when the heterogeneity was controlled. However, the current results were partially affected by such heterogeneity, and should be interpreted carefully. Fifth, although we categorized locations of VOIs into six brain areas with the similar developmental origin,<sup>24</sup> the classification might be criticized to be over-simplified considering the functional variability within each sub-areas.

In conclusion, the current meta-analysis robustly showed a significant frontal NAA reduction in ASD children compared with TD, and further demonstrated a significant linear correlation between older age and a smaller magnitude of NAA decrease. This NAA reduction then disappeared in adulthood. Taken together with previous findings suggesting early brain overgrowth and subsequent normalization during the same time period, the current findings support the hypothesis that abnormal brain enlargement in ASD is mainly caused by increases of non-neuron tissues, such as glial

cell proliferation. The current systematic review and metaanalysis emphasized the importance and implication for future research. Future longitudinal and large-scale original study with sufficient statistical power that is free from the methodological limitations is required to test the current hypothesis.

#### **Conflict of interest**

The authors declare no conflict of interest.

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Author contributions. YA and HY performed study screenings independently. In the case of discrepancies, a consensus was reached by means of discussion with the third reviewer (KK). YA performed all the data extraction and computation of effect size twice to avoid carelessness mistake. HY further performed data extraction and computation of the effect sizes independently. YA and HY wrote paper.

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