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Arch. Dis. Child. 2008;93;832-837; originally published online 5 Feb 2008; doi:10.1136/adc.2007.122937

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Accepted 9 October 2007
Published Online First 5 February 2008

ABSTRACT

Objective: To test the hypothesis that measles vaccination was involved in the pathogenesis of autism spectrum disorders (ASD) as evidenced by signs of a persistent measles infection or abnormally persistent immune response shown by circulating measles virus or raised antibody titres in children with ASD who had been vaccinated against measles, mumps and rubella (MMR) compared with controls.

Design: Case–control study, community based.

Methods: A community sample of vaccinated children aged 10–12 years in the UK with ASD (n = 98) and two control groups of similar age, one with special educational needs but no ASD (n = 52) and one typically developing group (n = 90), were tested for measles virus and antibody response to measles in the serum.

Results: No difference was found between cases and controls for measles antibody response. There was no dose–response relationship between autism symptoms and antibody concentrations. Measles virus nucleic acid was amplified by reverse transcriptase-PCR in peripheral blood mononuclear cells from one patient with autism and two typically developing children. There was no evidence of a differential response to measles virus or the measles component of the MMR in children with ASD, with or without regression, and controls who had either one or two doses of MMR. Only one child from the control group had clinical symptoms of possible enterocolitis.

Conclusion: No association between measles vaccination and ASD was shown.

What is already known on this topic

- Public concern about a putative link between mumps, measles and rubella (MMR) vaccination and autism spectrum disorders (ASD) has resulted in lower uptake of MMR vaccine.
- Epidemiological studies have shown no link between MMR and ASD.

What this study adds

- There is no difference between ASD cases and controls in circulating measles genome or measles antibody concentrations.
- There is no evidence of an altered persisting immunological response following either one or two MMR vaccinations in ASD cases with and without a history of regression.
- There is no evidence of increased enterocolitis in the ASD group with regression.
- In this cohort, children were less likely to receive the second MMR vaccination after diagnosis of a developmental problem.

Recent studies of the prevalence of autism spectrum disorders (ASD) have found rates between 6 and 12 per thousand, significantly higher than previous estimates, depending on the strictness with which the diagnostic criteria are applied.[1–3] Although widening of the diagnostic concept, improved ascertainment, and other methodological aspects of more recent studies are likely to be major reasons for the increased rate, and despite the fact that autism is known to have a strong genetic basis, concerns about environmental risk factors for an increased prevalence have inevitably been raised.

In 1998, a report of a small case series of 12 children and no control group suggested that measles, mumps and rubella (MMR) vaccination might be linked to the development of ASD.[4] A subsequent larger case series described a condition referred to as "autism enterocolitis", which was postulated to be associated with MMR vaccination and specifically with regression in autism.[5]

Several epidemiological studies[6–8] found no association between MMR vaccination and ASD; however, fear about MMR vaccination resulted in a reduction of uptake of the combined MMR vaccine, from 92% in 1995–96 to 80% by 2004,[9] risking exposure of the population to a measles epidemic and outbreaks in susceptible groups.[10] There continues to be an impact on parents of children with autism[11] and general public concern about the risks, which is reflected in parental decisions about MMR vaccination.[12 13]

Raised concentrations of measles antibodies have been reported in autism.[14] Two laboratories have reported the detection of measles virus, one by conventional reverse transcriptase (RT)-PCR in three cases of autism[15] and another by real-time TaqMan PCR,[16] the latter in intestinal samples of 75/91 patients with ASD compared with 5/70 control patients. The origin and characterisation of the fragments of measles virus genome described in these studies have not been established, and concerns about the scientific methods used have been widely expressed. Two recent studies have failed to find measles virus genome by real-time PCR in children with ASD compared with controls in peripheral blood mononuclear cells (PBMCs) rather than gut mucosal samples.[17 18]

We took advantage of a new geographically defined study of the prevalence of ASD (Special

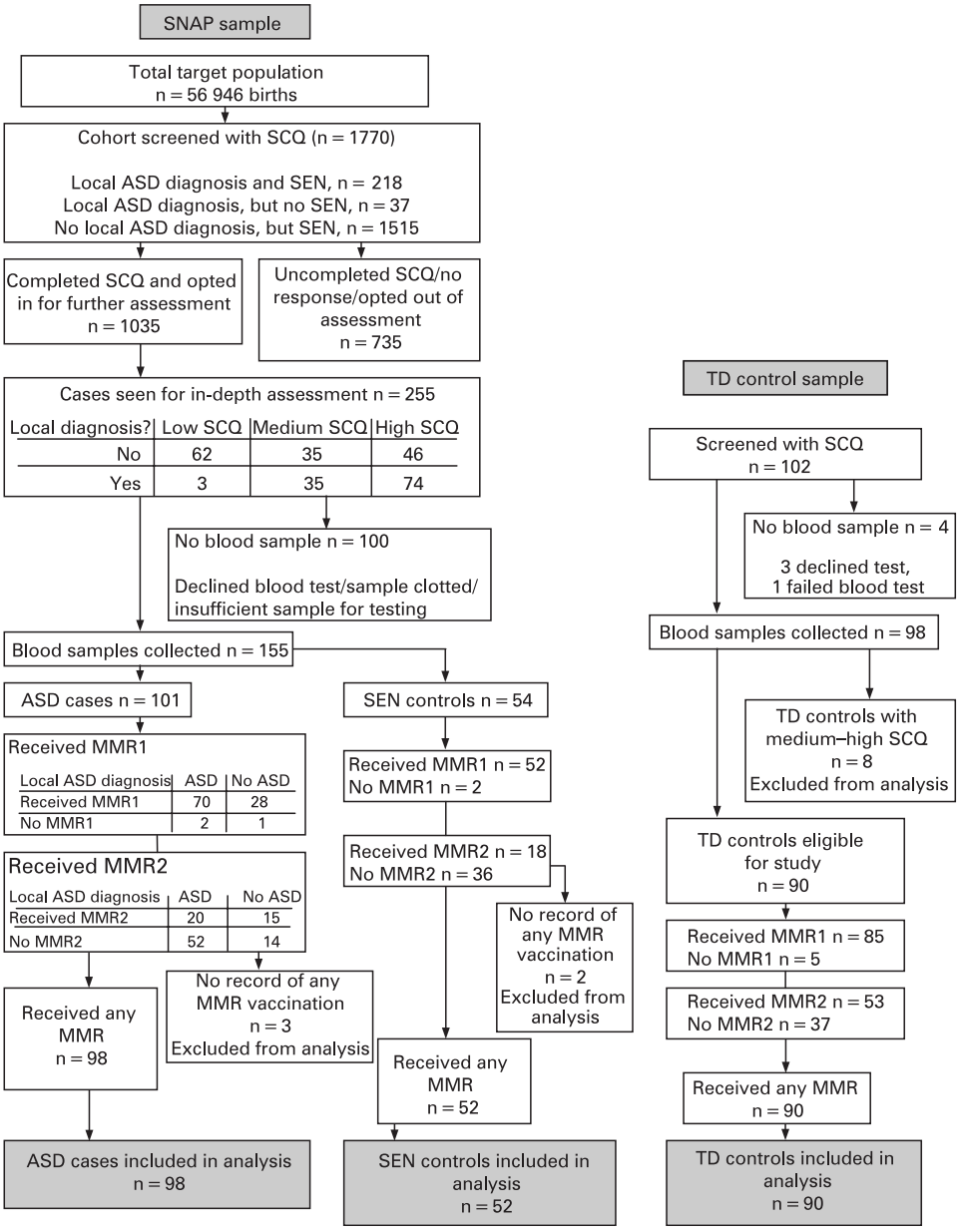

Figure 1 Flow chart of stratification of patients. ASD, autism spectrum disorders; MMR, measles, mumps and rubella (MMR1 and MMR2, first and second stage vaccinations); SCQ, Social Communication Questionnaire; SEN, special educational needs; SNAP, Special Needs and Autism Project; TD, typically developing.

Needs and Autism Project; SNAP),[1] to test the hypothesis that measles vaccine was involved in the pathogenesis of ASD, as evidenced by signs of a persistent measles infection or abnormally persistent immune response shown by circulating measles virus or raised antibody titres in MMR-vaccinated children with ASD compared with controls—in particular, in children with ASD and a history of regression. Measles virus replicates in a range of cells during infection, including the upper respiratory tract, intestinal cells, several T cell lineages and macrophages. Replication occurs for similar periods in these different sites. An earlier study had suggested detectable virus using PCR in PBMCs from children with ASD.[15] We used PBMCs in this study as a proxy for gut mucosal cells, which were not obtained for ethical reasons.

METHODS

Participants

The population studied was a cohort of 56 946 children born between 1 July 1990 and 31 December 1991 from 12 districts in the South Thames region of the UK. At age 9–10 years, children with a statement of special educational needs (SEN) (1733; 218 of whom had a local ASD diagnosis) or a local diagnosis of ASD but no SEN statement (37) were screened using the Social Communication Questionnaire (SCQ).[19] Stratification by local diagnosis and high, medium and low SCQ score was used to derive a subset (255) who received an in-depth diagnostic assessment (see fig 1 for a flow chart of the process, and Baird *et al*[1] for further explanation). The diagnostic assessment included standardised clinical observation (Autism Diagnostic Observation Schedule-Generic (ADOS-G))[20] and parent interview assessments of autism symptoms (Autism Diagnostic Interview-Revised (ADI-R)),[21] language and IQ, psychiatric comorbidity, and a medical examination (table 1).

Children were classified using International Classification of Diseases-10th revision (ICD-10) research criteria such as childhood autism, other ASD or no ASD by clinical consensus using all sources of information. The ASD group was divided into a "broad ASD" and "narrow autism" group, the latter

Table 1 Sample descriptive statistics: autism symptoms and IQ

	TD (n = 90)	SEN (no ASD) (n = 52)	Broad ASD (n = 66)	Narrow autism (n = 3232)
SCQ score	4.26 (3.59)	9.03 (7.54)	22.03 (6.88)	28.03 (5.06)
ADI-comm	NA	5.37 (3.84)	14.73 (5.57)	18.09 (3.32)
ADI-soc	NA	5.27 (4.85)	19.70 (6.66)	24.69 (3.53)
ADI-rep	NA	1.23 (1.35)	6.00 (3.17)	7.59 (2.17)
ICD-10 sym	NA	1.62 (1.25)	7.21 (2.18)	10.31 (1.64)
ADOS-comm	NA	0.96 (1.10)	2.05 (1.35)	5.59 (2.09)
ADOS-soc	NA	2.84 (2.33)	5.27 (3.10)	10.59 (1.93)
ADOS-rep	NA	0.60 (0.77)	1.74 (1.64)	3.66 (2.12)
IQ	NA	78.46 (20.21)	78.94 (22.49)	63.84 (17.67)
Age (years)	12.2 (0.33)	12.7 (0.89)	11.6 (0.90)	11.7 (0.90)

Values are mean (SD).
SCQ, Social Communication Questionnaire; NA, not available; ADI-comm, Autism Diagnostic Interview-Revised Communication domain algorithm score (4–5 years); ADI-soc, Autism Diagnostic Interview-Revised Reciprocal Social Interaction domain algorithm score (4–5 years); ADI-rep, Autism Diagnostic Interview-Revised Repetitive and Stereotyped Behaviours domain algorithm score (4–5 years); ICD-10 sym, ICD-10 symptom count (0–12); ADOS-comm, Autism Diagnostic Observation Schedule-Generic Communication domain algorithm score; ADOS-soc, Autism Diagnostic Observation Schedule-Generic Social domain algorithm score; ADOS-rep, Autism Diagnostic Observation Schedule-Generic Repetitive domain algorithm score.

defined as meeting autism criteria on the ADI-R, the ADOS-G and clinical consensus of ICD-10 childhood autism, and the former as all other cases meeting clinical consensus of any ASD. The total number of ICD-10 autism symptoms was recorded. Those who experienced "regression" were divided into a "definite language regression group" defined as the loss of five or more words used communicatively during a 3-month period, and a "lower level regression" group, who had not achieved the five-word stage at the time of regression but had reported regression of words or skills in social communicative or play behaviour. The "no ASD" group had a variety of diagnoses, learning difficulties, specific language or literacy impairments, attention deficit/hyperactivity disorder, cerebral palsy, deafness and visual impairment.

After consent had been obtained, of the 255 children seen for an in-depth assessment, sufficient blood suitable for analysis was collected from 101 with an ASD diagnosis (mean (SD) age 11.6 (88) years) and 54 SEN controls with a non-ASD diagnosis (mean age 12.7 (88) years). The age span reflects the time scale of the diagnostic project.

A further 98 typically developing (TD) controls, born at the same time, attending two mainstream schools within the same geographical area, who did not have a SEN statement and who consented to venepuncture, were recruited. The SCQ was used to screen out possible cases of autism, and eight cases were subsequently excluded from analysis on the basis of scores of at least 15, the cut-off recommended for identifying likely cases of ASD.[19] The mean (SD) age of the 92 TD controls was 12.2 (0.33) years.

Gastrointestinal symptoms reflecting the presentation of gastrointestinal symptom constellations in general clinical paediatric practice were assessed using a 22-item questionnaire completed by the main caregiver. Current (in the last 3 months) and past symptoms were elicited. A "possible enterocolitis" group was constructed from the presence of two or more of the following five current gastrointestinal symptoms—current persistent diarrhoea (defined as loose/watery stools three or more times a day for >14 days); current persistent vomiting (occurring at least once a day or more than five times in a week); current weight loss; current persistent abdominal pain (three or more episodes severe enough to interfere with activity); current blood in stool—plus past persistent diarrhoea for >14 days duration and excluding current constipation.

Vaccination

Information about MMR vaccination (fig 1) was obtained for all children using district records, parent records, and information from general practitioners. A total of 235 children had received the first MMR vaccination: 98 (97% of the group) with ASD, 52 (96%) SEN controls, and 85 (94%) TD controls. Stage 2 MMR vaccination (first introduced in 1996) was received by 106 children: 35 (36%) children with ASD, 18 (35%) SEN controls and 53 (62%) TD controls. Five children with no evidence of at least one MMR vaccination were excluded from the analysis.

Studies show 95% seroconversion for measles after the first MMR vaccination, with the second dose of MMR converting most of those not converted with the first vaccination and inducing only a transient rise in antibody proportional to the earlier response in earlier responders.[22] Thus it is justifiable to include every child who had had at least one MMR vaccination in a case–control comparison of vaccinated children: 98 ASD cases (32 narrow autism; 66 broad ASD), 52 SEN controls and 90 mainstream (TD) controls. However, for completeness, children who had had only one MMR and those who had had two MMR vaccinations were analysed separately, and the results were then combined. For some analyses, the SEN no-ASD controls and TD controls were compared separately and then in combination to form a total control group of 142. The 98 ASD cases were analysed as broad ASD and narrow autism separately and in combination.

Laboratory tests

Clotted and anticoagulated blood samples (in EDTA) were couriered to the laboratory on the day of collection for processing. Serum was separated and stored at −20°C until tested for antibody. Samples were processed using the Amplicor kit and then stored at −70°C until tested for measles virus. Samples were batched, and the laboratory was blind to case–control status.

Table 2 Primers and probes for the real-time assay for the H gene

Gene	Primer	Position	Sequence (5′–3′)	Product
H gene	Forward	117–140	GGCTGTTCTGTTTGTCATGTTTGT	68
	Reverse	161–184	GATGAAGTCTAATGCCTGCAATGG	
	Probe	141–156	CAACCCGATCAAGCTC	

Genome detection

Detection of measles virus in the EDTA sample was conducted on peripheral mononuclear cells after concentration using the Amplicor whole blood preparation kit (Roche, Burgess Hill, West Sussex, UK). Satisfactory EDTA samples for this were available from 94/98 ASD cases and 130/142 SEN and TD controls. Samples were tested for the presence of measles genome after extraction of RNA using the Magnapure extractor. Three RT-PCR assays were used: published assays for M gene[23] and N gene[24] RT-PCRs, and an RT-PCR for the H gene using AB1 PRISM 7000 sequence detector platform (TaqMan) was developed.[25] Assays were run for 40 cycles, and data analysed according to the manufacturer's instructions. The primers and probes in table 2 were used for the real-time assay. The sensitivity of the assay was determined to be two genome copies. Samples were tested to ensure that they were adequate by using a β_2 microglobulin housekeeping gene PCR with a sensitivity of 10 genome copies per reaction mixture.

Antibody studies

Serum samples were tested for measles IgG antibody by the plaque reduction neutralisation test (PRN). This test was chosen because recent evaluation has demonstrated its greater sensitivity over commercially available enzyme immunoassay tests.[26] Measles antibody was quantified in international units to control for variation using the international reference standard serum for the PRN.[27]

Statistical analysis

All summary statistics and analyses of antibody response are based on $\log_{10}$-transformed mIU/ml and undertaken in Stata V9. Having tested for homogeneity of variance (Bartlett test), we report analysis of variance *F* and Scheffé tests for the four-group comparison of TD, SEN (no-ASD), broad ASD, and autism. In addition, in view of the variety of specific alternative hypotheses proposed, we report further pairwise comparisons of combined groups, and findings for a group defined by regressive autism and a linear trend test over the quintiles of the ICD-10 autism symptom score. Results are also reported for these additional analyses using Wilcoxon rank-based tests[28] that enable the inclusion of subjects with no detectable antibody response (coded as 0). p Values from these rank-based tests are denoted p*. All p values and 95% CI for these additional tests are nominal with no correction for multiple testing.

RESULTS

Table 1 shows descriptive statistics of the TD, SEN, broad ASD and narrow autism groups.

Measles virus assays

Samples from all cases contained β_2 microglobulin gene detectable by PCR. All samples were negative in the measles H gene RT-PCR assay. Fifty-six samples (based on availability of sufficient nucleic acid) were also tested and were negative in the N gene RT-PCR. One case sample and two control samples were reactive in the M gene PCR. These PCR products were sequenced: a genotype C2 measles strain was characterised in one case (narrow autism but no regression history) and a measles vaccine strain and a D6 strain in two TD mainstream controls. These sequences were unlike any previous isolates seen in the laboratory. The results were not repeatable; the three reactive samples were negative when retested in the M gene PCR.

Antibody response to measles

Eight subjects (one TD, five SEN (no-ASD), one broad ASD and one narrow autism) who had received MMR vaccination had no detectable measles IgG antibody by PRN, suggesting that the attenuated measles virus did not replicate and triggered no immunological response. There was no difference in mean $\log_{10}$ (mIU/ml) measles titre between those with one or two MMR vaccinations (difference = 0.00, 95% CI −0.12 to 0.11, p = 0.94, p* = 0.62).

Figure 2 shows the similarity of distributions of measles PRN responses by group, combined by MMR number. The plots give

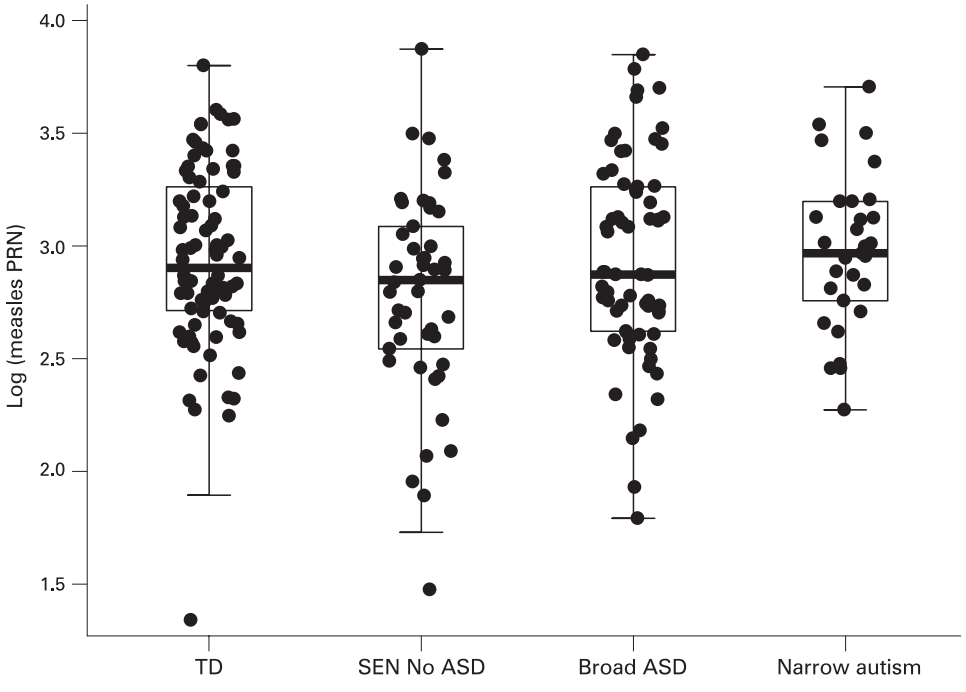

Figure 2 Measles plaque reduction neutralisation test (PRN) responses for the typical/mainstream (TD), special educational needs (SEN), (broad) autism spectrum disorders (ASD) and narrow autism groups ($\log_{10}$ (mIU/ml) in measles PRN). The box indicates the interquartile range, and the thick black line the median of each distribution (geometric means: TD, 2.95; SEN (no-ASD), 2.79; broad ASD, 2.94; narrow autism, 2.98). Whiskers extend to the highest and lowest observed values or, if less extreme, 2.5 times the interquartile range.

no indication of extreme titres in the ASD and autism groups that fall outside of the distribution among the controls. The overall difference of means test indicated no significant differences ($F_{3,223}$, p = 0.13), with the most significant of the six Scheffé paired comparisons giving p = 0.23. The corresponding tests for those with a single MMR were $F_{3,126}$, p = 0.20 with most significant Scheffé p = 0.20, and for those with two MMR were $F_{3,93}$, p = 0.66 with most significant Scheffé p = 0.74.

The combined control group mean $\log_{10}$ titre was not significantly lower than that for the narrow autism group (difference = 0.05, 95% CI −0.08 to 0.18, $F_{1,194}$, p = 0.45, p* = 0.26), the ASD group (difference = 0.08, 95% CI −0.07 to 0.25, $F_{1,160}$, p = 0.29, p* = 0.26), or the combined autism/ASD group (difference = 0.06, 95% CI −0.05 to 0.17, $F_{1,225}$, p = 0.27, p* = 0.26). This comparison of the combined case and control groups had 80% power to detect a mean titre difference of 45% (or 0.16 $\log_{10}$(mIU/ml)). Within the autism groups there was no trend of PRN response over ICD-10 symptom quintiles (p = 0.99; p* = 0.63).

Regression was reported in 23 children with ASD, but PRN titres were not significantly higher in these than in combined controls (difference = –0.12, 95% CI −0.30 to 0.06, $F_{1,162}$, p = 0.18, p* = 0.33).

"Possible enterocolitis", as defined above, was found in only one child who did not have ASD or regression. He had current and past diarrhoea and abdominal pain and was in the combined control group. No child had a previous diagnosis of inflammatory bowel disorder.

DISCUSSION

No difference was detected in the distribution of measles antibody or in measles virus in ASD cases and controls whether the children had received the first, second or both MMR vaccinations. This remained true when the analysis was restricted to ASD cases with a history of regression. Only one child had symptoms of possible enterocolitis, and this child was in the control group.

This is one of three virological case–control studies that have failed to demonstrate any association between measles vaccination and ASD using well-validated techniques.[17 18] In the study of D'Souza *et al*,[18] children were 26–30 months from vaccination, in contrast with ~9 years in this study, with identical conclusions. The report from D'Souza *et al* also describes an exhaustive validation of the molecular detection methods used in the only study to detect measles genome in ASD cases,[16] showing that the methods used can generate false-positive results.

The strengths of this study are that the patients with ASD were from a well-characterised community, not clinic, derived sample. The sample is the largest reported. Regression was clearly defined. The diagnostic process allowed a "dose–response" of ICD-10 symptoms to antibody titre to be analysed. All children had a well-documented vaccination history. A highly sensitive methodology was used for assay of measles antibody. The laboratory techniques used to collect, extract, store and test samples for measles genome used well-established, block-based RT-PCR assays, which have been shown to be highly sensitive in an international comparative study.[24] Laboratory analysis was conducted blind to case–control status. A real-time RT-PCR was also used.[25] This platform was used in earlier studies, and, although of comparable sensitivity to nested conventional PCR, risk of contamination is reduced. PBMCs were used to look for measles by RT-PCR because they are a site of viral replication in acute measles infection and they have been reported to contain measles genome, detected by RT-PCR, in a small number of autism children.[15]

There are two possible explanations for the finding of one RT-PCR-reactive sample in 98 cases of ASD and two in the 90 TD children. Immunity to measles is not always complete,[29] and measles genome has been detected in the PBMCs of asymptomatic people during measles epidemics.[30] C2 and D6 measles genotypes were detected in the UK population before 2002. The finding may also be due to laboratory cross-contamination, which can be problematic with RT-PCR assays.

Limitations of the study

Subjects in the TD group were not randomly selected from the whole population for reasons of time, convenience and cost. Parents were informed that the study was about MMR vaccination, and it is possible that a biased group responded to the request to participate. Satisfactory blood samples were not obtained in 100 children, both ASD cases and SEN controls, for a variety of reasons, including refusal by the young person concerned and haemolysation during transport. We did not obtain gut mucosal samples for ethical reasons; PBMCs were used for measles genome assay, justified as a site of known viral replication and an appropriate proxy for gut mucosal cells. Gut symptoms were elicited, but the children were too old for accurate reporting of retrospective gut symptoms confidently contemporaneous with MMR vaccination. A clinically relevant definition of enterocolitis based on persistent symptoms was therefore used for this paper.

It is of public health relevance that there is a differential uptake of MMR2 across the groups, with both ASD and SEN control groups having lower uptake and hence less exposure to measles virus. This may reflect parental concern about vaccination following a diagnosis of developmental abnormality. Only 29% (20/70) of children who had a local diagnosis of ASD received MMR2 compared with 50% (14/28) of those who had no local ASD diagnosis.

Acknowledgements: We thank the parents and children who participated, and Dr Sameena Shakoor and Ms Beryl Packman, as well as other colleagues, for help with data and sample collection.

Funding: The study was funded by the Department of Health, the Wellcome Trust, the National Alliance for Autism Research (NAAR) and Remedi. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation or writing of the report. The corresponding author had full access to all the data in the study and final responsibility for the decision to submit for publication.

Competing interests: MA and DB have given unpaid advice to lawyers in MMR and MR litigation. GB has acted as an occasional expert witness for the diagnosis of autism. AP receives royalties from SCQ and ADOS-G instruments. PBS has acted as an expert witness in the matter of MMR/MR vaccine litigation. All other authors have no conflicts of interest.

Ethics approval: South Thames MREC 00/1/50; Kent & Medway LREC WK153/8/02.

GB, ES, TC and DB obtained funding. DB, MA, BT and LJ were responsible for the laboratory tests. TL, SC and DM collected data and samples. PS was responsible for gastrointestinal assessment. AP had overall responsibility for the statistical analysis. All authors contributed to the paper.

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Archivist

Chronic headache after head injury

Headache is very common in children and head injury is fairly common. The reported prevalence of chronic post-traumatic headache (CPTH) after head injury has varied from 23% in one study to 3% in another. No correlation has been found between CPTH and the severity of head injury. The type of headache in CPTH has usually been described as tension headache, migraine or mixed headaches. A study in Stirling, Scotland (Charlotte Kirk and colleagues. *Developmental Medicine and Child Neurology* 2008;**50**:422–5) has provided more information.

Over a 15-month period in 2003–2005, a total of 190 children aged 3–15 years were admitted to Sterling Royal Infirmary with head injury (estimated prevalence of 299 per 100 000 children per year). Among the 117 children with follow-up data, the head injury was classified as minor (closed injury, no loss of consciousness, Glasgow Coma Score 13–15) in 93 and significant (loss of consciousness for >30 min, Glasgow Coma Score <13, post-traumatic amnesia for >48 h) in 24. Eight of the 117 children developed headache for the first time, beginning within 14 days of head injury and persisting for longer than 3 months, fulfilling criteria for the diagnosis of CPTH. Only one of these children had had a significant head injury. The headaches occurred up to twice a week and were severe in the first 6 months, but they stopped within 3–27 months (mean 13 months) in seven children (one child had continuing headaches at 22 months after which she was lost to follow-up). The type of headache was episodic tension headache in five children, migraine without aura in two, and migraine with aura in one. Three other children with non-specific headaches before head injury had more frequent and migrainous headaches after head injury.

Recurrent severe headache is common after head injury in children. It usually has the clinical features of tension headache or migraine and clears up within 2 years.