Serum of Mothers Having Autistic Children Induces Cerebellar Purkinje cell Alterations in Experimental Model: A Possible Cause of Autism

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Rec date: May 29, 2014; Acc date: June 24, 2015; Pub date: July 02, 2015

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Abstract

Autism is a brain developmental disorder characterized by impaired social interaction. Our aim is to assess the morphology of plasma cells of mothers having autistic children and to evaluate the effect of administration of serum obtained from mothers having autistic children on the cerebellar Purkinje neurons. Two groups were used: control group, where pregnant rats remained intact while others were injected intraperitoneally with serum of mothers with no history of autism. In the experimental group pregnant rats were intraperitoneally injected serum of autistic children’s mothers.

The Transmission Electron Microscope showed altered plasma cells of autistic children’s mothers, where the heterochromatin material had inversed distribution in all nuclei examined in addition to other alterations. Also, modified Purkinje neurons were obtained in neonates of experimental group showing degeneration and atrophied dendrites. Recommended examination of peripheral blood is essential for early intervention.

Keywords: Autism; Purkinje neurons; Plasma cells; Rattus Norvegicus

Introduction

Autism is an ubiquitous heterogeneous neurodevelopmental disorder, associated with core deficits in social communication. To date, autism is diagnosed through similar behavioral symptomatology which comprises, abnormal speech patterns, inability to interact with others and the absence of social skills, as well as sustained, unusual repetitive behaviors with maladroit attachment to objects and aversion to changes in their surroundings [1,2].

Worth to note, that a wide range of factors are implicated to autism, some of which are environmental which include, exposures to toxins, chemicals like mercury, poisons and other substances Freitag [3] reported that exposure to infections especially to the rubella epidemic during pregnancy increases the risk of autism in newborns. Although scientists have focused their attentions to genetics, yet information about autism in general is relatively hypothetical and so far the genes related to autism are susceptible and no genes are yet proven to be surely involved in autism [4]. The hypothesis was derived from the concordance rate difference in autism between monozygotic (90%) and dizygotic (2-10%) [3]. Others added that although the consistent abnormality in autism is the decreased cerebellar Purkinje neurons, this decrease is due to genetic factors, thus suggesting that the genes involved in the growth and the differentiation of the cerebellar Purkinje cells would be an important step in the future studies as candidate genes for autism [5].

There are few studies suggesting that the cause of autism might be due to immature “Purkinje neurons”. Some authors believed that the reason for immature Purkinje is the maternal antibrain antibodies that recognize fetal brain antigens and therefore alter normal brain development [6]. They added that the immune system appeared to be deregulated in autistic patients and they believe that immune factors might be contributors to autism. Amaral et al., [7] reported that the number of antibody-producing B-cells had increased by 20 % in case of autism. On the other hand, Martin et al., [8] demonstrated reactivity of maternal serum against both human and monkey fetal brain antigens. On the other hand, Chauhan and Chauhan [11] reported that autistic patients had disturbance of energy metabolism in the brain and mild mitochondrial dysfunction and mentioned that damaged mitochondria not only produce more oxidants, but mitochondria are also vulnerable to oxidative stress which is associated with premature aging of cells provoking tissue inflammation, damaged cell membranes, autoimmunity, and cell death [12]. Furthermore, Rossini and Bradstreet [13] hypothesized that autism may be caused by mitochondrial dysfunction. They also added that individuals with autism show lower cell energy and deficiency in the capacity of mitochondrial energy reserve that could lead to cognitive impairment, language deficits, and abnormal energy metabolism. Abdelmeguid and Kourtian [14] reported that autistic candidates and their mothers possess altered peripheral blood cells. They also stated that erythrocytes were altered collectively, and related this to a decreased oxygen capacity of erythrocytes in blood circulation including, circulation in epitomes of fetal brain.
It is well known that plasma cells are the type of white blood cells rarely found in peripheral blood. They are specialized to yield a large amount of immunoglobulin and similar to other blood cells, they originate in the bone marrow, and depart in the form of B cells, before terminal differentiation. When an antigen enters the body, it must first bind to a B cell, which then proliferates to form plasma cells in lymph nodes. Those cells then secrete antibodies that inactivate the pathogen and mark it for destruction. To date, the possible mechanism that leads to autism is still unknown. Accordingly, the present study was undertaken for two objectives. First, to assess the ultrastructure of plasma cells in peripheral blood of mothers having autistic children. Second, to assess whether repeated administration of maternal serum containing antibodies of mothers of autistic children has any effect on the morphology and structure of the fetal Purkinje cerebellum cells in rats and then, correlate the results to the effect of maternal antibodies on the brain of autistic fetuses.

Materials and Methods

Healthy, sexually mature male and female rats (Rattus norvegicus), 3-4 months old weighing 180-190 grams each, obtained from the animal house of the pharmacology laboratory (of Beirut Arab University, Lebanon) were used in the present study. The rats were housed in Plexiglas cages and were maintained in the laboratory at room temperature with 14 dark and 10 light photoperiods. Rats were provided access to food (rat chow pellets) and tap water ad libitum. The experimental animals were bred in nine cages, where, in each cage, a male is placed with five females for mating.

Serum of blood was collected from mothers of ten families with no history of autism or other related diseases (for control group), as well as serum of blood obtained from 25 mothers having autistic children (for experimental group). The children were assessed as having autism according to the American Psychiatric Association criteria [1]. The Diagnostic and Statistical Manual of Mental Disorders (Table I) provides standard criteria for the classification of mental disorders.

### Table 1: According to the American Psychiatric Association (1996), a person could be labeled as having autism if he or she meets the above criteria.

<table>
<thead>
<tr>
<th>A total of six (or more) items from (A), (B), and (C), with at least two from (A), and one each from (B) and (C):</th>
<th>Qualitative impairment in social interaction, as manifested by at least two of the following:</th>
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<tr>
<td>Marked impairment in the use of multiple non-verbal behaviors such as eye-to-eye gaze, facial expression, body gestures, and gestures to regulate social interaction.</td>
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<td>Failure to develop peer relationships appropriate to developmental level.</td>
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<td>A lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest)</td>
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<td>Lack of social or emotional reciprocity</td>
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<td>Qualitative impairment in communication as manifested by at least one of the following:</td>
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<td>Delay in or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime).</td>
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<td>In individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others.</td>
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<td>Stereotyped and repetitive use of language or idiosyncratic language.</td>
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<td>Lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level</td>
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<td>Restricted repetitive and stereotyped patterns of behavior, interests, and activities, as manifested by at least one of the following:</td>
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<td>Encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus.</td>
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<td>Apparently inflexible adherence to specific, nonfunctional routines or rituals.</td>
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<td>Stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)</td>
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<td>Persistent preoccupation with parts of objects.</td>
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<td>Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years:</td>
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<td>Social interaction, Language as used in social communication, or Symbolic or imaginative play.</td>
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The disturbance is not better accounted for by Rett’s Disorder or Childhood Disintegrative Disorder.
Experimental Groups

The first group (GI)

This control group consists of two subgroups: the first (GIa) consists of 10 pregnant rats that were remained intact. The other group "GIb" in which 10 pregnant rats were i.p. injected three times during their gestation period, with 3 milliliters of serum withdrawn from mothers with no history of autism in their family.

The second group (GII)

In which pregnant rats were i.p. injected three times during their gestation period, with 3 milliliters of serum of blood obtained from twenty-five mothers having autistic children.

Rats were left in the cages undisturbed, except for weekly cage cleaning. Offsprings of both groups were housed in the same cage with their mother until the pups were slaughtered. Dissection of some neonates of each set of rats was undertaken at 2 weeks of age and others at almost two months. The head was directly immersed in 10% formalin for 10 days. The cerebellum was immersed in 10% formalin for one month. Then, the skull was removed and the cerebellum was immersed in 10% formalin for 10 days. The cerebellum was embedded in paraffin, sectioned and some fixed sections were stained with Hematoxylin and Eosin while others were stained with Masson [15].

Method of peripheral blood preparation to assess the ultrastructure of plasma cells

During this study, five milliliters of whole blood collected with 1% heparin withdrawn from mothers having autistic children, was centrifuged for 20 minutes using centrifugal separator at 2500 rpm. A thin white buffy coat was formed between erythrocytes below and plasma above. To avoid disturbance of the buffy coat, very gently 2cc buffered Formalin- Glutaraldehyde was dropped. The tube was covered and allowed to stand for 18 hours at 4ºC. 0.1M phosphate buffer (pH 7.4), was added into the test tube containing the precipitate free cells, then shaken to remove the fixative sticking to the free cells. After that, centrifugation was performed, and the supernatant liquid was discarded. L-shaped needle was inserted down the side of the tube to a position several millimeters below the buffy coat plug, and then moved around the wall of the tube to loosen the plug from the tube and from the red cells below. The plug was then pulled out of the tube by hooked needle, and placed in buffer solution. A thin l mm slice was cut through the middle of the plug, and then the slice was post fixed in 1% O2O2 for 2 hours, then trimmed into smaller pieces and processed as an ordinary tissue. The specimen of the buffy coat was dehydrated in a series of increasing concentrations of alcohol (60, 70, 80, 90 and 100%) for 10 minutes each. Dehydrated specimens were embedded in Epon-Araldite resin mixture. Ultrathin sections from the specimens were obtained using LKB ultratome, double stained with uranyl acetate for half hour, and lead citrate for 20-30 minutes then examined using Joel 100 CX electron microscope [16].

Results

Plasma cells of peripheral blood of mothers having autistic children

In the present study, "TEM" electron micrographs of peripheral blood of mothers having autistic children showed altered plasma cells. It was noticed that all plasma cells examined possess evidence of nuclear and cytoplasmic alterations (Figure 1a-c). A relatively altered nucleo-cytoplasmic ratio was observed since nuclei were large in some cells (Figure 1a, b), and exhibited small size in others (Figure1c). A major abnormality in the electron micrographs of plasma cells was the inverted distribution of heterochromatin clumps in all nuclei examined. Preparations also demonstrated that the perivesceral nuclear spaces were indistinct and their membranes appear electron dense (Figure 1a, b). Also, nuclear pocket like structure (Figure1b) and nuclear membrane blebbing (Figure 1c) were detected among plasma cells. In general, cytoplasmic organelles were abnormally distributed in more or less a narrow perinuclear basophilic area. Plasma cells free of organelles but with abundant free particles and hypertrophied Golgi apparatus in their cytoplasm were also observed. In addition, plasma cells characterized with different sizes and electron density cytoplasmic vacuoles were observed. Mitochondria of plasma cells were either absent (Figure1a) or mostly disrupted (Figure1b & c).

Structure and number of Purkinje neurons in different groups

Light microscopic preparations (fixed in 10% formalin and stained with either Hematoxylin and Eosin or Masson) of the cerebellar cortex

Figure 1: (a, b, c). TEM. Peripheral blood withdrawn from mothers having autistic children. a) Showing, plasma cell with centric large sized nucleus, radially arranged dense clumps of heterochromatin, distinct perinuclear area of the cytoplasm, dilated Golgi cisternae (bold arrow), large number of various sized vesicles; thin arrow: pointed at large vesicle containing granules. b) Showing, altered plasma cell nucleus, the heterochromatin shifted to inside instead of being arranged directly underneath the nuclear membrane, nuclear pocket type I (bold arrow). The cytoplasm houses large number of ribosomes, loss of mitochondrial ultrastructure (arrow head), multivesicular body (bent arrow) and autophagosome (double arrow), c) Showing, plasma cell with ruptured plasma membrane (bold arrow), with altered nucleus, highly dilated perivesceral nuclear space (thin arrow), the cytoplasm possesses numerous mitochondria and small sized dense granules (arrow head). [Specimen fixed in 4FG and double-stained with Uranyl acetate and Lead citrate.X, 10000, 10000, 7500].
of neonate rats (*Rattus norvegicus*) (Gia), in which their mothers were remained intact during their pregnancy showed that the histological architecture is comparable to those described in any textbook of histology. These preparations which were used as control revealed that the cerebellar cortex consists of three layers on a core of white matter in each folia: from outer to inner layer, these are the molecular, Purkinje, and granular layers (Figure 2a-d).

![Figure 2](image)

**Figure 2:** Light micrographs. Cerebellar cortex of 2 weeks R. norvegicus in which their mother remained intact (Gia). [Fig 3a, b, d. Specimen fixed in formalin and stained with Masson]. a) Showing, thousands of Purkinje neurons (arrow) aligned into rows between the molecular and the underlying granule cells [X 40]. b) Showing, the molecular layer (bold arrow), Purkinje cell layers (arrows), and granular layer (arrow head), Purkinjecells aligned into ranks between the molecular layer and the granule cells [X, 100]. [Specimen fixed in formalin and stained with Hematoxilin and Eosin] Illustrates, sequence of pear-shaped Purkinje neurons with normal aspect. Purkinje neurons exhibit large size, with large size nuclei, prominent nucleoli in almost all [X, 400]. d) Showing, a sequence of Purkinje neurons (arrow) forming a layer of grid-like construct between the molecular layer (bold arrow) and the granule cells (arrow head). Almost all the cells show the nucleus as well as the nucleolus [X, 250].

The molecular layer (the most superficial layer of the cerebellar cortex) is relatively devoid of neuronal cell bodies (Figure 2a, b), that is to say consists mainly of neuropil. Light micrographs showed that, immediately deep to the molecular layer is one layer composed of large Purkinje cell (Piriform) layer (Figure 2c). These are the most morphologically distinctive cells in the brain. They have flask-shaped somas. In a two-dimensional section they appear lined up neatly into a single row, forming a layer. Thousands of them were aligned into ranks and rows forming a grid-like construct between the molecular layer and the underlying granule cells (Figs, 2 c &d). Morphometric measurements based on light micrographs of the flask-shaped somas of Purkinje revealed that they possess an average of 5µm in diameter and the distance between every successive Purkinje neuron is around 2.5µm. In this investigation our attention was directed to the Purkinje cells only as it is well known they are central neurons. In addition to a large cell body, Purkinje cells possess an axon which forms the efferent pathway from the cerebellum, and sends collaterals in the granular layer. Our results showed that the Purkinje cells had a definite orientation in the folia. Purkinje cell nuclei appeared spherical and the nucleoli were prominent and clearly observed in almost all the nuclei. They were arranged in parallel arrays along the folia. Purkinje cells’ extensive dendritic arrays extended upwards into the molecular layer, oriented at right angles to the direction in which the fibers of the molecular layer run. They showed a great number of points of contact between the molecular layer axons and the dendrites of each Purkinje cell. It is worth to mention that Purkinje neurons in the neonates were less developed than in adults but were much closer to each other and more numerous within the same area (15 Purkinje neurons/100 µm area) and accordingly the distance between them was shorter (Figure 2d). Preparations showed also that the granular layer consisted of the Purkinje cell axons which passed through the granule cell layer and formed the fiber tracts of the cerebellar medulla.

Immediately deep to the Purkinje cell layer were the somata of the cells whose axons formed the bulk of the granular layer. These are small neurons called granule cells, forming a compact and easily distinguished region. Deep to the granule cell layer is the cerebellar medulla, made up of the axonal processes of the Purkinje cells. These pass through the granule cell layer and become bundled together into fiber tracts. Our preparations of this group showed also, that all of the three layers of the cortex, and the underlying layer of fibers, were present in each of the folia. In addition, the Purkinje cells which had a fixed orientation in the folia, were organized in parallel arrays along the folia as mentioned previously. Thus, we could trace the contours of a layer from one end of the cerebellum to the other without ever leaving that layer.

Profound observation of the light microscopic preparations of the cerebellar cortex of the neonates of group Gia, in which the mothers of the neonates were injected with the serum of mothers with no history of autism, showed that the cerebellar cortex had histological architecture comparable to the cerebellar cortex of the neonates in which their mothers were left intact; they revealed normal histological architecture (Figure 3a-d).
Sections of cerebellar cortex of a very young R. norvegicus showed a sequence of great number of Purkinje neurons forming a layer of grid-like construct between both the molecular and the granule cell layer. Each cell appeared rounded in shape and mononucleated. The nuclei showed nucleoli as in the case of group (Gia). Moreover, the cerebellar cortex of the older rats revealed also normal morphology. The granular layer as well as the molecular layer appeared comparable in structure to those of the older rats in (Gia). Similarly, the Purkinje neurons were of same morphology as those observed in the case of the older rats of Gia (their mothers remained intact). Their shape was piriform and their diameter was around 5µm. In addition, their number was around 11 Purkinje neurons/100µm and the distance between them was around 7.5µm. Measurements were all comparable to those in Gia. Their nuclei and the nucleoli were observed in almost all the cells. The Purkinje neurons in the older rat cerebellum were lined up precisely in a one row between the molecular and the granular layer. Worth to note that the Purkinje neurons did not appear as a network as in the case of the younger rats.

In contrast to those observed in the light microscopic observations of the control groups, the profound observation of the microscopic preparations of the cerebellar cortex of rats in which the mothers were injected with the serum of mothers having autistic children revealed degeneration of the layer of Purkinje neuron (Figure 4a-f). In addition, there was dislocation of the Purkinje neurons (Figure 4 e, f) and there were spaces in the layer void of Purkinje neurons. The micrographs of the cerebellar cortex of young (Rattus norvegicus), showed a decrease in the number of the Purkinje neurons where there was absence of the network layer which was observed in the case of the young rat cerebellum in the control rats. Although some of the cells revealed the nucleolus, others showed morphological alterations where the cell body lacked nucleolus and some were degenerated. In addition, some cells revealed the dendrites extended into the molecular layer but the cell lacked a cell body as well as nucleus and
nucleolus (Fig. 4b). The light micrographs also revealed a reduced diameter (3µm) in the Purkinje neurons where the nucleus was absent in almost all of them. There were great surfaces in the Purkinje layer void of Purkinje neurons (20µm) (Figure 4d) compared to the distance between the Purkinje neurons in control groups which varies between 2.5µm to 7.5µm much smaller than the distances calculated in the cerebellum of the treated rats. Accordingly, the average number of Purkinje cells within a certain area is very small (5 Purkinje cells / 100µm) since there are surfaces which do not contain any Purkinje (Figure 4c).

It is of interest to point out that pregnant rats injected with serum of the mothers who had the most severe cases of autism (all three of her children were autistic) died one day after delivery of neonates. Thus, these neonates were breastfed by another mother rat injected with serum of other autistic children.

Discussion

Profound examination of these preparations reveals few numbers of plasma cells. This may be due to the fact that these cells originate from B cells and stay within the bone marrow until an antigen appears in the body. Binding of antigen to B cells stimulates it to transform to plasma cells.

To highlight on either primary or secondary factors related to autism, a widespread investigation is in progress worldwide [17-20]. Maternal infection in prenatal life is a remarkable risk factor in the progress of neuropsychiatric disorders in later life, including autism. Patterson and Meyer et al. [21,22] reported that one established assumption proposes that infection-induced disorders of early prenatal brain development may predispose the organism for long-lasting structural and functional brain abnormalities, leading to the emergence of psychopathological behaviors in adulthood. It is worthy to note, that the feasibility of this causal link (between maternal infection during pregnancy and higher risk of brain and behavioral pathology in the offspring) has received extensive support from some experimental models established in both rats and mice [21].

Abdelmeguid and Kourtian [14] described for the first time, cellular and subcellular alterations in large number of erythrocytes, leukocytes, as well as, platelets in both autistic candidate and their mothers. The most important subcellular alteration was noticed among nuclear chromatin, nuclear membranes, as well as cellular organelles specifically mitochondria. Their observations indirectly pointed to a decline in oxygen carried by red blood cells.

It is well known that mitochondria are the energy-producing units of the cells. It is a source of production of adenosine triphosphate (ATP), the chemical energy in all living matter derived from a process known as oxidative phosphorylation [23]. According to Lombard [23], the function of the brain is critically dependent on ATP production, oxidative phosphorylation via the mitochondria which provides over 95% of total brain ATP. Furthermore, he related the possible cause of mitochondrial toxicity to the production of increased free-radicals which play a key role in immune-mediated neurotoxicity via mitochondrial inhibition. On the other hand, alterations in the mitochondria of the peripheral blood cells reflect the improper functioning of the bioenergetic metabolism in the central nervous system, since the blood cells are the source of oxygen for the cells which is necessary for the oxidative phosphorylation via the mitochondria. In addition, the plasma cells in the present study withdrawn from the peripheral blood of mothers having autistic children exhibited alterations also in their nuclei and cytoplasm. The observed plasma cells almost lacking normal mitochondria reflect their inability to produce antibodies. This is because alterations highlight other converging findings, pointed toward the mechanism of the production of maternal antibody with immune reactivity against epitopes of fetal brain and specify their neurons. Results obtained therefore suggest that the alterations revealed in the peripheral blood cells might be the cause for the alterations observed in the Purkinje neurons of the cerebellum.

Since the Purkinje neurons were mentioned in several studies as being modified in autistic children, it was worthy to test the effect of maternal serum on these neurons. As for the current study, it was impossible to study the Purkinje neurons of autistic candidates. Therefore, Rattus norvegicus, was chosen as a mammalian model in this investigation. The light microscope was the only tool used to detect alterations in the Purkinje neurons in the cerebellum of the rats in which their mothers, during gestation, were injected with the serum obtained from mothers having autistic children. In other words, the present investigation pointed to studying the Purkinje neurons of the newly born Rattus norvegicus, where their mothers were injected with the serum of the mothers of autistic children during gestation.

Examination of light micrographs of the cerebellar cortex of the postnatal and adult Rattus norvegicus off springs of female rats injected with serum obtained from mothers having autistic children as described before revealed morphological and quantitative changes in the Purkinje neurons. The numbers of Purkinje cells, as well as their size were reduced. Also, their nuclei were absent in most of them while other Purkinje neurons were degenerating. It is interesting to mention that the alterations were more severe in the adult rats than in the neonatal rats. In contrast, offspring of mothers that were not treated or were injected with the serum obtained from mothers with no history of autism, showed normal morphology of Purkinje cells. It is suggested that this might be due to the fact that the antibodies contained in the serum of the mothers of autistic children were pathogenic for the fetal purkinje cells of the neonate rats, i.e. the sera demonstrated autoreactivity to the purkinje neurons of the cerebellum of the neonate rats. The present results concerning the maternal antibodies to fetal brain in autism is in consistent with the findings of Zimmerman et al. [6], who reported that early embryonic brain may be a tissue target for maternal antibodies and may change the course of fetal brain development. They added that the identification of specific serum antibodies in mothers of children with autism that recognize prenatally expressed brain antigens suggests that these autoantibodies could cross the placenta and alter fetal brain development. Similarly, Dalton et al [24] reported that maternal antibodies might be pathogenic to the fetal brain in autism.

In conclusion, we can add that factors other than maternal antibodies might also be involved in fetus susceptible to autism since 22 out of 25 autistic children in our study have normal siblings. At the end of this study we hope that the present findings will be of use in future investigation concerning the diagnostic features of autism and/or the mechanism that leads to developing autism.

Ethical standards

All human and animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964...
Declaration of Helsinki and its later amendments. In addition, all persons included in the research have given their informed consent prior to their inclusion in the study.

References