

Review

Mitochondrial dysfunction in autism spectrum disorders: Cause or effect?

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ABSTRACT

Autism Spectrum Disorders encompass severe developmental disorders characterized by variable degrees of impairment in language, communication and social skills, as well as by repetitive and stereotypic patterns of behaviour. Substantial percentages of autistic patients display peripheral markers of mitochondrial energy metabolism dysfunction, such as (a) elevated lactate, pyruvate, and alanine levels in blood, urine and/or cerebrospinal fluid, (b) serum carnitine deficiency, and/or (c) enhanced oxidative stress. These biochemical abnormalities are accompanied by highly heterogeneous clinical presentations, which generally (but by no means always) encompass neurological and systemic symptoms relatively unusual in idiopathic autistic disorder. In some patients, these abnormalities have been successfully explained by the presence of specific mutations or rearrangements in their mitochondrial or nuclear DNA. However, in the majority of cases, abnormal energy metabolism cannot be immediately linked to specific genetic or genomic defects. Recent evidence from post-mortem studies of autistic brains points toward abnormalities in mitochondrial function as possible downstream consequences of dysreactive immunity and altered calcium (Ca²⁺) signalling.

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1. Introduction

Autism Spectrum Disorders (ASDs), encompassing Autistic Disorder, Asperger's Disorder, and Pervasive Developmental Disorder Not Otherwise Specified (PDDNOS), represent a group of severe neurodevelopmental disorders characterized by variable degrees of impairment in language, verbal and non-verbal communication, and social skills, as well as by repetitive behaviors and an excessive insistence on sameness, with onset prior to three years of age [1]. Collectively, the incidence of ASDs has dramatically risen during the last two decades from 2-5/10,000 to approximately 1-2/1000 children: broader diagnostic criteria and increased awareness in the medical community have certainly contributed to determine this trend, but a real increase in incidence possibly due to environmental factors cannot be excluded [2,3]. These disorders are often addressed as discrete clinical

entities, but they should instead be viewed as a continuum, ranging from minimal autistic traits to severe autism [4].

No neuropsychiatric disorder displays genetic underpinnings as prominent as those documented by family and twin studies for autism, with heritability estimates above 90% and sibling recurrence risk as high as 3–6% for strictly defined autism [3]. However, only in approximately 10% of cases the disease is "syndromic", i.e. secondary to a known genetic disorder [3,5], whereas in the vast majority of patients, the origin of "non-syndromic", "primary" or "idiopathic" autism remains unknown. Genome-scans unveiled the existence of approximately 15–20 loci contributing to non-syndromic autism, albeit in complex fashion due to genetic heterogeneity, incomplete penetrance, phenocopies, gene–gene and gene–environment interactions [3].

The cytoarchitectonic abnormalities present in autistic brains are most compatible with reduced programmed cell death and/or increased cell proliferation, abnormal cell migration, and altered cell differentiation with reduced neuronal size, all pointing toward the first/second trimester of pregnancy as the critical time for deranged neurodevelopment in autism [6,7]. The detection of fine motor symptoms already on the day of birth or very early on in neonates later diagnosed with an ASD converges with neuropathological findings in dating prenatally the origin of the disease, although behavioral symptoms typically appear at 6–24 months [8–10]. Finally, large subgroups of ASD patients also display systemic signs and symptoms, including macrosomy [11], non-specific enterocolitis [12], immune dysreactivity [12,13] and renal oligopeptiduria [14]. Autism thus involves primarily, but not exclusively, the central nervous system (CNS) and should be viewed as a multi-

Abbreviations: AGC, aspartate/glutamate carrier; ASD, autism spectrum disorders; CK, creatine kinase; CNS, central nervous system; CNV, copy number variant; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malonyldialdehyde; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; SAH, H-adenosylhomocysteine; SAM, S-adenosylmethionine

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organ systemic disorder encompassing several developmental components.

2. Primary mitochondrial disorders and autism

Interest into possible links between mitochondrial abnormalities and autism was initially spurred by the deleterious consequences of mitochondrial disorders on neurodevelopment. Indeed, mitochondrial disorders often result in CNS dysfunction, leading to developmental regression, learning disability, and various behavioural disturbances. Autism can indeed represent the main clinical presentation of a mitochondrial disease [15]. However, the clinical manifestations of mitochondrial disorders, even in the presence of well characterized mitochondrial DNA (mtDNA) mutations, are extremely heterogeneous, ranging from lesions affecting single tissues or organs, such as the optic nerve in Leber's hereditary optic neuropathy (LHON), or the cochlea in maternally inherited non-syndromic deafness, to myopathies, encephalomyopathies, cardiopathies, or complex multisystem syndromes with onset occurring anytime from neonatal to adult life [16]. Adult patients usually show signs of myopathy, associated with variable involvement of the CNS (ataxia, hearing loss, seizures, polyneuropathy, pigmentary retinopathy and, more rarely, movement disorders). Instead, children most frequently display severe psychomotor delay and generalized hypotonia, but symptoms can range from isolated myopathies, sometimes associated with cardiopathies, up to fatal multisystem syndromes [17,18]. The occurrence of muscle 'ragged red fibres' (RRFs), characterized by a segmental proliferation and accumulation of abnormal mitochondria under the sarcolemmal membrane, perhaps represents the best known morphological hallmark present in many, albeit not all, of these syndromes. Another common finding is the presence of muscle fibres that stain negative using cytochrome oxidase (COX, respiratory complex IV) histochemistry. However, these typical 'mitochondrial' clues may be absent in otherwise demonstrated mitochondrial disorders, such as LHON, and neuropathy ataxia and retinitis pigmentosa (NARP). The same also occurs in many paediatric cases: lactic acidosis and muscle tissue histology (even at the level of electron microscopy) will be negative in the majority of affected children, who rarely show "ragged red fibers" [15]. Finally, molecular investigations still fail to identify the responsible gene defect in 50% of adults affected by biochemically- and/or morphologically-defined mitochondrial disease. The percentage of undiagnosed cases increases to 80–90% for paediatric disorders [16].

Genetic mutations in mtDNA have been associated with myopathy, cardiomyopathy, neuropathy, seizures, optic atrophy, strokes, hearing loss, diabetes mellitus, and other clinical features [16]. In some cases, autism can directly stem from mutations in mtDNA, as documented in the following studies:

1) Pons et al. [19] reported two ASD patients carrying the 3243A>G mutation, located in the mtDNA tRNA^{Leu(UUR)} gene. The same mutation was also present in the two mothers of two other autistic children, whose peripheral tissues available for investigation did not unveil this mutation. The 3243A>G mutation typically causes mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), but has also been associated with maternally inherited progressive external ophthalmoplegia, and with developmental delay and seizures [19]. The autistic phenotype in these mutation carriers appears highly heterogeneous. The behavioral triad of autistic disorder is often (but not always) associated with different combinations of neurological signs or symptoms, including developmental delay, clumsiness, attention deficit, neurologic deterioration during intercurrent illnesses especially in the presence of fever, microcephaly or macrocephaly. A fifth child presented with mitochondrial DNA depletion, which is typically associated with muscle hypotonia, seizures, myoclonus, and developmental delay.

Notably, family history was generally positive for mitochondrial diseases in the maternal lineage, except in the presence of mitochondrial depletion.

2) Graf et al. [20] described one autistic patient carrying the 8363G>A mutation, affecting the mtDNA tRNA^{Lys} gene. This patient was characterized by behavioral regression at age 2, extreme hyperactivity, lack of attention, mild fine and gross motor dyspraxia. His sister was severely affected with partial complex seizures, unsteady gait, myoclonus, swallowing dysfunction, and moderate cognitive impairment. Importantly, muscle biopsies unveiled 86% and 61% of mitochondrial DNA mutated in this girl and in the autistic proband, respectively, highlighting a probable dosage effect. One of the patient's two maternal half-sisters suffered from seizures, learning disabilities, fine tremors and mild motor dyspraxia. Another maternal half-sister and his mother were neurologically and psychiatrically healthy.

3) Weissman and Colleagues [21] reviewed the medical records of 25 autistic children with evidence of a definite or probable ($N=21$ and 4, respectively) mitochondrial disorder. In addition to the three core diagnostic features of autism (impaired communication, social interactions, and repetitive behaviours or restricted patterns of interest), these patients presented additional signs and symptoms in various combinations, most commonly including:

- (a) excessive fatigability and/or exercise intolerance ($N=19$, 76%);
- (b) gastrointestinal dysfunction ($N=16$, 64%), in the form of gastroesophageal reflux ($N=9$, 36%) and/or constipation ($N=8$, 32%);
- (c) structural or functional cardiovascular abnormalities ($N=7$, 28%);
- (d) facial dysmorphisms ($N=6$, 24%);
- (e) microcephaly ($N=4$, 16%) or macrocephaly ($N=4$, 16%);
- (f) marked developmental gross motor delays ($N=8$, 32%); and
- (g) growth retardation ($N=5$, 20%).

Clinical heterogeneity was paralleled by heterogeneity at the biochemical and genetic levels. Lactate levels were elevated in 19 (76%) patients; defects in complexes I, II, III, and IV, assessed in muscle and/or fibroblasts, were observed in 64%, 8%, 20% and 4% of patients, respectively. Magnetic resonance imaging (MRI) was frequently positive for brain structure abnormalities, detected in 10/21 (47.6%) patients, but no single abnormality appeared specifically associated. At the genetic level, three mutations of probable pathogenetic meaning were found: (a) 3397A>G in the ND1 subunit of complex I; (b) 4295A>G in mtDNA tRNA^{Leu}, and (c) 11984 T>C in the ND4 subunit of complex I. These three missense mutations all cause aminoacid changes in highly conserved regions. Three other mutations (3394 T>C, 1039C>T, 11809 T>C) have unclear pathogenetic relevance.

In addition to mtDNA mutations, gene dosage abnormalities have been described by Fillano et al. [22] in five patients with autism carrying large deletions in their mtDNA. One recurrent deletion of 7.4 kb was present in three patients, whereas the remaining two patients unveiled at least three large deletions, including the 7.4 kb deletion. Approximately 5–15% of the mitochondrial genomes extracted from blood in four of these five patients carried these deletions. This leaves open the question of whether and to what extent mtDNA may undergo deletions more frequently in blood, as compared to muscle or to nervous tissue. Three of these patients, in addition to autism, also display ataxia and/or cardiomyopathy.

Several important conclusions can be drawn from these studies:

- 1) *At the clinical level*, autistic patients with an underlying mitochondrial disease can display highly heterogeneous clinical pictures. Some of their signs and symptoms are not unusual in idiopathic autism: for example, macrocephaly and macrosomy are present in approximately 20% of autistic patients [11]; gastrointestinal dysmotility is relatively frequent

both in mitochondrial diseases with neurological presentations and in ASDs [15], and by no means specific of ASDs among neurodevelopmental disorders. However, neurological signs and symptoms such as oculomotor abnormalities, hearing deficits, dysarthria, ptosis, hypertonia and movement disorders are indeed atypical for autism and were found in 15/25 (60%) children described by Weissman et al. [21] who also reports 24/25 (96%) of the children displaying at least 1 major clinical finding relatively uncommon in idiopathic ASD and 21/25 (84%) patients with an involvement of at least one organ/system outside the CNS. Regression occurs rather frequently, especially in the presence of fever [21,23].

- II) *At the laboratory level*, autistic patients with an underlying mitochondrial disease also display a high degree of heterogeneity, but almost all children display abnormalities in at least one biochemical parameter among several typically assessed to screen for mitochondrial disorders (i.e., increased blood levels of lactate, pyruvate, alanine; abnormal urinary levels of organic acids; increased lactate/pyruvate ratio in fibroblasts).
- III) *At the morphological level*, the incidence of microcephaly and microsomy is elevated, reaching approximately 20%, likely due to high incidence of developmental delay. At the neuroimaging level, neuroanatomical abnormalities are relatively frequent, although by no means consistent [15,21,23].
- IV) *At the epidemiological level*, these patients display an M:F sex ratio close to 1:1, whereas the M:F ratio in ASD is skewed much more toward males being affected with a 4:1 probability [24]. Furthermore, an excess of neurological or neurodevelopmental syndromes of suspect mitochondrial origin is often present in the maternal lineage.
- V) *At the genetic level*, mtDNA mutations or gene dosage abnormalities of pathogenetic significance are present only in a small minority of ASD patients with signs and symptoms of mitochondrial disorders, and collectively represent a rare cause of illness in unscreened samples of idiopathic autistic children. Furthermore, no compelling evidence of an association of any mitochondrial haplogroup to the risk of developing autism has been found in a large population study [25]. Since mitochondrial functioning has been estimated to require approximately 1500 nuclear genes [26] and oxidative phosphorylation (OXPHOS) involves at least 80 proteins, only 13 encoded by mtDNA [16], searches for mutations and chromosomal rearrangements in nuclear DNA are also warranted. The chromosomal rearrangements, or Copy Number Variants (CNVs), described in autistic patients and possibly involving mitochondrial function consist in deletions located in chromosomal regions 15q11–q13 (cytochrome C oxidase subunit 5A, COX5A), 13q13–q14.1 (mitochondrial ribosomal protein 31, MRPS31), 4q32–q34.68 (electron-transferring-flavoprotein dehydrogenase, ETFDH), 2q37.3 (NADH dehydrogenase ubiquinone 1 alpha subcomplex 10, NDUFA10), as recently reviewed by Smith et al. [27]. Null mutations in other genomic loci known to be involved in mtDNA metabolism have not been reported, but could lead, if present, to mitochondrial depletion [26] of the kind described by Pons et al. in their ASD patient n.5 (see above) [19]. However, collectively CNVs are only present in approximately 7–10% of ASD patients [27]. Considering that not all of them are autism-specific (1–2% of controls also carry CNVs) and that not all of them necessarily affect genes relevant to mitochondrial function, also the number of cases explained by CNVs and mutations present in the cell genome can hardly reach the 20–40% of cases consistently showing elevated lactate levels. In this majority of ASD patients, who are devoid of genetic mutations or genomic CNVs directly impacting mitochondrial function, biochemical signs and clinical symptoms of abnormal mitochondrial function are most likely

downstream of pathogenetic processes critically involved in the origin of ASDs.

3. Mitochondrial dysfunction in idiopathic autism spectrum disorders

Several studies have described an association between autism spectrum disorders and mitochondrial dysfunction [22,28,29], and an impairment of mitochondrial energy metabolism has been proposed to play a role in autism pathogenesis [30,31]. Mitochondrial dysfunction in the CNS of autistic individuals is supported by neuroimaging studies using positron emission tomography (PET) and nuclear magnetic resonance (NMR) spectroscopy, demonstrating reduced glucose utilization and diminished ATP levels especially in association areas of the cerebral cortex [32,33]. At the biochemical level, lactic acidosis has frequently been found in autism [34]. Three independent studies [35–37] reported moderate lactic acidosis (see Table 1) and elevated alanine levels in unselected samples of idiopathic ASD patients. In a larger cohort [38], a subset of ASD patients with increased lactate was also found to exhibit the biochemical features of mitochondrial disease, whose incidence in ASD was estimated at approximately 6%. Further case-control studies confirmed elevated plasma lactate in autism [39,40]. Also carnitine deficiency is commonly found in autistic patients [37]. Carnitine is essential for the utilization of fatty acids by the mitochondria, and carnitine deficiency results in impaired beta oxidation and decreased availability of high-energy phosphate compounds. Biomarkers of fatty acid elongation and desaturation, namely poly-unsaturated long-chain fatty acids (PUFA) and/or saturated very long chain fatty acids (VLCFA) containing ethanolamine phospholipids, were significantly elevated in autism, as a consequence of impaired mitochondrial beta oxidation [31]. Furthermore, in several studies of individuals presenting with autism increased ammonia has also been described [37].

Additional evidence pointing toward abnormal mitochondrial function in autism comes from investigations addressing oxidative stress. Under normal conditions, a dynamic equilibrium exists between the production of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, singlet oxygen, and hydrogen peroxide, and the antioxidant capacity of the cell, which involves both enzymatic and non-enzymatic processes. On one hand, the inner membrane of the mitochondria contains a large amount of free radical scavengers including glutathione, vitamin C, and vitamin E, as well as anti-oxidant enzymes such as superoxide dismutase (SOD). On the other hand, mitochondria represent the major cellular source of ROS. Excess production of free radicals or impaired antioxidant mechanisms may cause oxidative stress: impaired mitochondrial function then leads to further oxidative stress and a vicious negative cycle can ensue.

Converging evidence suggests that increased oxidative stress may be present in a sizable subgroup of autistic patients [41–44], where this harmful cycle is likely to be active and to persist. First, total glutathione (GSH + GSSG), and free reduced glutathione (GSH) were significantly decreased in plasma of children with autism when compared to normal children, while the oxidized disulfide form of glutathione (GSSG) was increased [45–47] (see Table 2). Similar results were observed in lymphoblastoid cells by James et al. [48]. The glutathione redox ratio (GSH/GSSG) and oxidized glutathione concentrations are dynamic indicators of cytosolic and mitochondrial

Table 1
Plasma lactate levels (mM) in autistic subjects.

Abnormal/tested (%)	Mean Abnormal (SD)	Ref value	References
9/34 (26.5%)	1.65 (1.07)	1.4	Filipek et al., 2004 [37]
14/69 (20.3%)	3.5 (1.3)	1.15	Oliveira et al., 2005 [36]
13/30 (43.3%)	2.17 (1.7)	<1.9	Laszlo et al., 1994 [35]

Table 2

Comparison of plasma biomarkers of mitochondrial dysfunction and oxidative stress between autistic children and control children.

	ASD/control	Mean ASD (SD)	Controls mean (SD)	Units	References
Lactate	15/15	15.3 (3.1)	10.9 (1.9)	mg/dL	Chugani et al., 1999 [39]
	15/15	1.398 (0.819)	0.872 (0.335)	mmol/L	Al-Mosalem et al., 2009 [40]
GSH	30/30	22.65 (8.1)	31.1 (11)	µg/ml	Al-Gadani et al., 2009 [43]
	28/120	3.1 (0.53)	4.2 (0.72)	µmol/L	Geier et al., 2009 [46]
	80/73	5.1 (1.2)	7.53 (1.7)	µmol/L	James et al., 2006 [45]
GSSG	10/10	21.72 (4.3)	26.48 (3.5)	(nmol/mg) ^a	James et al., 2009 [48]
	80/73	0.4 (0.2)	0.24 (0.1)	µmol/L	James et al., 2006 [45]
	10/10	0.356 (0.06)	0.287 (0.07)	(nmol/mg) ^a	James et al., 2009 [48]
MDA	28/120	0.46 (0.16)	0.35 (0.05)	µmol/L	Geier et al., 2009 [46]
	11/11	0.4969 (0.025)	0.396 (0.019)	nmol/ml	Chauhan et al., 2004 [42]
	27/26	0.032 (0.0077)	0.015 (0.0033)	(mmol/gHb) ^b	Zoroglu et al., 2004 [44]
Vitamin E	30/30	15.34 (4.8)	9.92 (4.1)	µmol/ml	Al-Gadani et al., 2009 [43]
	30/30	0.64 (0.39)	1.86 (0.47)	mg/dL	Al-Gadani et al., 2009 [43]
Methionine	80/73	20.6 (5.2)	28.0 (6.5)	µmol/L	James et al., 2006 [45]
SAM:SAH		4.0 (1.7)	5.5 (2.8)		
Adenosine		0.28 (0.13)	0.19 (0.13)	µmol/L	
Homocysteine		5.7 (1.2)	6.0 (1.3)	µmol/L	
Cystathione		0.24 (0.1)	0.19 (0.1)	µmol/L	
Cysteine		165 (14)	207 (22)	µmol/L	

^a Lymphoblasts.^b Erythrocytes.

redox status, as well as of the severity of oxidative stress. Secondly, under physiological conditions, glutathione reductase activity is sufficient to maintain an elevated reduced/oxidized glutathione ratio. However, excessive intracellular oxidative stress, exceeding the capacity of glutathione reductase, will result in oxidized glutathione export to the plasma in an attempt to regain intracellular redox homeostasis. Thus, an increase in plasma oxidized glutathione is a strong indication of intracellular oxidative stress. Moreover, oxidized glutathione export represents a net loss of glutathione to the cell and increases the requirement for cysteine, the rate-limiting amino acid for glutathione synthesis. The decrease in cysteine, methionine, and S-adenosylmethionine/S-adenosylhomocysteine ratio (i.e., an indicator of methylation capacity), recorded in the plasma of autistic patients by James et al. [45], paralleled by decreased glutathione levels, and reduced/oxidized glutathione ratios (Table 2), lends further support to the existence of an unbalanced redox homeostasis in autism, due to enhanced oxidative stress and reduced antioxidant capacity. Thirdly, some ASD children also present an increase in biomarkers of lipid peroxidation [42,44,49], such as malonyldialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids and related esters [36,37]. Furthermore, it has been claimed that α -tocopherol or vitamin E is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction: a marked decrease in vitamin E levels has been reported in autistic children compared to controls [43] (Table 2). Lastly, decreased activities of several antioxidant enzymes including SOD [50] glutathione peroxidase [50], catalase [44], and paraoxonase [51,52] have been detected in a significant subgroup of autistic patients.

4. Novel insights into the pathogenetic underpinnings of ASDs from post-mortem studies of autistic brains

As described above, many ASD patients display biochemical signs of abnormal mitochondrial function in the absence of genetic mutations or genomic CNVs directly impacting mitochondrial biochemistry. In order to shed light on the link between mitochondrial dysfunction and the pathogenetic underpinnings of autism, we have recently assessed possible roles for the *SLC25A12* gene and for its protein product in autism spectrum disorders [53]. We shall now briefly summarize our findings, which may serve as a useful paradigm to disclose possible mechanisms underlying secondary mitochondrial dysfunction in autism.

SLC25A12 is located on human chromosomes 2q24 and encodes for AGC1, the mitochondrial aspartate/glutamate carrier isoform predominantly expressed in the brain, heart and skeletal muscle [54,55]. AGC plays a pivotal role in energy metabolism by transporting glutamate into mitochondria across the inner mitochondrial membrane in exchange for matrix aspartate, a key step in the malate/aspartate NADH shuttle [54,55]. Importantly, AGC activity is regulated by intracellular Ca^{2+} through four “EF-hands” [56] located at its N-terminus, hanging into the intermembrane space [54,55]. Cytosolic Ca^{2+} , which is in equilibrium with the mitochondrial intermembrane space, can rapidly activate AGC transport, thereby increasing the NADH/NAD ratio in the mitochondrial matrix and consequently boosting electron flow through the respiratory chain and ATP generation by OXPHOS [54,55,57]. Through this mechanism, AGC1 plays an essential role in transducing small Ca^{2+} transients to neuronal mitochondria [55]. However, an excessive amplitude and/or duration of Ca^{2+} spikes leading to AGC activation can contribute to the formation of ROS and to oxidative stress [58]. Genetic and/or environmental factors could thus interfere with neuronal ATP production and with oxidative stress by affecting the AGC1 carrier, either directly or through Ca^{2+} homeostasis. Ramoz et al. (2004) raised interest in this molecule among autism investigators, reporting a significant association between autism and *SLC25A12* gene variants marked by the G allele at two intronic single nucleotide polymorphisms (SNPs), namely rs2056202 (13-21A>G) and rs2292813 (116+70A>G) [59].

In our study, we have assessed AGC function and *SLC25A12* genetics, using two parallel approaches: (a) examining AGC transport rates, AGC1 expression levels and *SLC25A12* genomic DNA and cDNA sequences in post-mortem temporocortical gray matter (Brodmann area 41/42 or 22) of six pairs of idiopathic ASD patients and sex-, age-, and post-mortem interval (PMI)-matched controls; (b) performing a family-based genetic association study using genomic DNA extracted from 309 simplex and 17 multiplex families with a non-syndromic autistic proband. The post-mortem study was performed on neocortical tissue from the superior temporal gyrus, because this region has been convincingly shown to host structural and functional abnormalities in autistic individuals [60]. Biochemical findings can be briefly summarized as follows: (a) an approximately three-fold increase in AGC transport rates was found in tissue homogenates from each of the six ASD patients compared to their matched controls. This increase was correlated neither with a clinical history of seizures and EEG abnormalities, nor with presence/absence or type of pharmacological treatment prior to death. Instead, excessive transport rates were

consistently blunted by the Ca^{2+} chelator EGTA, and no difference in AGC transport rates was found in isolated mitochondria from patients and controls following removal of the Ca^{2+} -containing post-mitochondrial supernatant; (b) when isolated mitochondria from each control were split into two aliquots, each exposed in parallel either to his/her own post-mitochondrial supernatant, or to the supernatant of his/her autistic match, AGC transport rates were activated 3.2- vs 1.7-fold by the patient and control supernatants, respectively ($P < 0.05$). Also this difference was blunted by EGTA; (c) finally, direct fluorimetric measurements of Ca^{2+} levels in the post-mitochondrial supernatant confirmed significantly higher Ca^{2+} levels in all six patients, compared to their matched controls; (d) AGC1 expression (RNA and protein levels) and cytochrome c oxidase activity were both increased in autistic patients, indicating an activation of mitochondrial metabolism; and (e) oxidized mitochondrial proteins were markedly increased in 5/6 patients vs 1/6 controls [53].

The extent and consistency of these biochemical findings strikingly contrasts with the paucity of genetic findings on the *SLC25A12* gene [53]. Sequencing the genomic DNA and cDNA extracted from the same brain tissues, unveiled only five known SNPs, none of them associated with the biochemical abnormalities described above. In addition, no evidence of genetic variants associated with ASDs was found in our genetic sample, although the unaffected brothers and sisters of autistic patients in our sample tend to inherit more frequently than expected by chance a “protective” *SLC25A12* genetic variant. We can thus confidently exclude that genetic variants, in the form of either non-synonymous coding mutations or common functional SNPs, represent the *primary* cause of the excessive AGC transport activity, mitochondrial metabolism and, to a more variable degree, oxidative stress documented in the autistic brain tissue samples assessed in our study. Instead, abnormal functioning appears *secondary* to excessive Ca^{2+} levels. Importantly, an excess of AGC1 transport activity due to overexpression of *SLC25A12* has been shown to yield enhanced neurite growth *in vitro* [61]. Moreover, AGC1 activity significantly modulates myelination rates through the synthesis of N-acetylaspartate, a critical intermediate in myelin formation [62]. In fact, humans and animals entirely devoid of AGC1 display profound cerebral hypomyelination [63,64]. Hence, the abnormal hyperactivation of AGC1 we recorded in post-mortem tissue, if present *in vivo*, could yield neurodevelopmental consequences at the structural level, perhaps contributing to explain the overgrowth of neocortical brain regions recorded during the first few years of postnatal life in many autistic children [65,66].

The existence of altered Ca^{2+} signalling in autism is supported by several converging lines of research [67]. Timothy syndrome, a multisystem disorder including autism and mental retardation, is caused by gain-of-function mutations in the L-type voltage-gated Ca^{2+} channel $\text{Ca}_v1.2$ (*CACNA1C*) [68]. Similarly, gain-of-function mutations in the L-type voltage-gated Ca^{2+} channel $\text{Ca}_v1.4$ (*CACNA1F*) cause an incomplete form of X-linked congenital stationary night blindness (*CSNB2*) frequently accompanied by cognitive impairment and either autism or epilepsy, whereas *CSNB2* due to loss-of-function *CACNA1F* mutations is not accompanied by these neurodevelopmental components [69]. In general, these gain-of-function mutations prevent voltage-dependent channel inactivation, yielding excessive Ca^{2+} influx. Also mutations indirectly boosting cytosolic Ca^{2+} levels or amplifying intracellular Ca^{2+} signalling by hampering Ca^{2+} -activated negative feedback mechanisms have been found associated with autism [67,70]. Post-mortem studies do not allow to unravel the mechanisms responsible for enhanced Ca^{2+} levels *in vivo*. Nonetheless, two other recent studies using brain tissue specimens largely overlapping with those assessed by Palmieri et al. [53], can significantly contribute to place the chain of events centred around AGC1 into its proper framework:

- (a) Garbett et al. [71] performed a genome-wide expression study on some of the same brain tissue specimens assessed by

Palmieri et al. [53]. They found 130 genes showing increased expression, whereas 22 genes were down-regulated in the brains of autistics subjects compared to controls. Upregulated genes fall into 31 gene sets, and as many as 19 of them are involved in targeting of the immune response to specific cells (NKT pathway), inflammation (NFkB, IL1R, INFLAM, GSK3, P38MAPK, IL6, NTH1, and TH1TH2 pathway), cell death (NFkB, TNFR2, P38MAPK, TID, 41BB, CASPASE, and FAS pathway), autoimmune diseases (NFkB, TOB1 and FAS pathway), migration (MCALPAIN pathway) and antigen-specific immune response (TOLL, TNFR2, HIVNEF, DC and IL2R pathway). Altogether these patterns of expression were most consistent with an inability to attenuate a cytokine activation signal, as occurs in the late recovery phase of autoimmune brain disorders. Instead, several downregulated genes were involved in neuronal differentiation and outgrowth, which is suggestive of altered neurodevelopment as a consequence of dysreactive autoimmunity.

- (b) Lintas et al. [72] assessed the involvement of the *PRKCB1* gene in autism both at the genetic and post-mortem level. *PRKCB1* generates two mRNA isoforms named *PRKCB1-1* and *PRKCB1-2*, yielding the two PKC β isoenzymes β I and β II, expressed in CNS, immune system, digestive tract and kidney. Downregulation of PKC β either by homologous recombination or by selective pharmacological inhibition is associated with immunosuppression, reduced oxidative stress, and blunted gene expression for chemokines such as ICAM-1, MCP-1, and TGF β [73–76]. Instead, activation of PKC β boosts oxidative stress [77,78]. At the genetic level, Lintas et al. [72] assessed a sample of 229 simplex and 5 multiplex families with an autistic proband, detecting a significant association between ASD and a “risk” allele marked by a specific haplotype located in intron 2. At the post-mortem level, temporocortical tissue specimens of ASD patients unveiled a profound decrease in *PRKCB1* gene expression for both isoforms, at the RNA and protein levels. Importantly, decreased gene expression characterizes patients carrying the “normal” *PRKCB1* allele, whereas patients homozygous for the “risk” conferring allele identified in our genetic study display mRNA levels comparable to those of controls. Whole genome expression analysis [71] unveils a partial disruption in the coordinated expression of PKC β -driven genes, including several cytokines: the positive correlation generally present in controls, becomes a strong negative correlation in ASD patients. These results argue against *PRKCB1* driving the ongoing immune activation documented by Garbett et al. [71] and the enhanced oxidative stress shown by Palmieri et al. [53] in patient samples. On the contrary, a compensatory downregulation in *PRKCB1* gene expression occurs in ASD patients, most likely in an attempt to buffer an inappropriate activation of the immune system. Results from these two studies are in agreement with previous reports demonstrating an ongoing immune reaction in a set of post-mortem brain tissue samples of autistic patients largely non-overlapping with ours, and in the CSF of autistic children collected *in vivo* [79].

Altogether, these studies from ours and other groups, point toward an abnormal neuroimmune response as a relevant player in elevating intracellular Ca^{2+} levels, deranging neurodevelopment, driving oxidative stress, and ultimately affecting synaptic function and neural connectivity especially in long-range neuronal pathways physiologically responsible for integrated information processing (Fig. 1) [80]. Cytokines, such as TNF- α and IL6, and cytokine receptors, like CD38, are all known to mobilize intracellular calcium stores and/or to facilitate calcium entry from the extracellular compartment [71,79,81]. Increased cytoplasmic Ca^{2+} levels producing mitochondri-

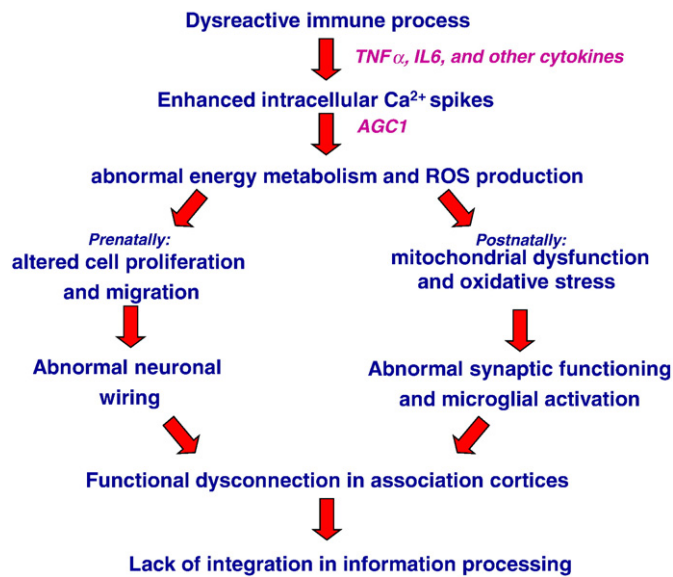


Fig. 1. Pathogenetic model proposed to underlie immune-driven forms of autism spectrum disorders. Molecules discussed in the present article are highlighted in italics. Genetic forms of the disease can be due to mutations or CNVs affecting genes critical to each step of the cascade.

al dysfunction with the generation of oxidative stress and impaired OXPHOS have been described during the course of HCV infection, a condition characterized by a persistent activation of the immune system [82]. Interestingly, an 8-year-old female control who died of a heart transplant rejection, displayed an EGTA-sensitive AGC activation reaching levels intermediate between those recorded in autistic patients and in other controls [53]. This immune-mediated mechanism could thus characterize a large subgroup of ASD patients, while others could well be explained either by rare genetic mutations, or by the coexistence in the same individual of several unfavourable common gene variants. In these genetically-based cases, mutations, CNVs or polymorphic variants could affect, among others, mtDNA or nuclear loci directly involved in mitochondrial function, genes involved in Ca^{2+} signalling, or genes critical to down-stream synaptic function (Fig. 1). In this context, it is worth noting that mitochondria are key players in cellular Ca^{2+} handling [58,83] and an increase in intracellular Ca^{2+} , possibly through NO and/or oxidative stress, would in turn be able to trigger an immune response similar to the one observed in autistic brains.[84–87].

5. Conclusions

Autism Spectrum Disorders are often associated with clinical, biochemical, or neuropathological evidence of altered mitochondrial function. In some patients, these signs stem from rare mutations in their mtDNA, or from mutations or chromosomal rearrangements affecting nuclear loci involved in mitochondrial function and/or energy metabolism. Autistic behaviors can here be viewed as part of a broader mitochondrial disease, typically, though not always, displaying relatively unusual neurological or systemic signs and symptoms. At least some biochemical parameters of diagnostic value in mitochondrial diseases will also be abnormal in these cases. On the other hand, the majority of autistic patients displays functional abnormalities in mitochondrial metabolism seemingly secondary to pathophysiological triggers, such as genetic mutations affecting loci involved in Ca^{2+} signalling, or a dysreactive immune system leading to elevated cytosolic Ca^{2+} levels. Within this framework, mitochondrial function may play a critical role not just in rarely causing the disease, but also in frequently determining to what extent different prenatal triggers will derange neurodevelopment and yield abnormal

postnatal behavior. The understanding of mitochondrial roles in autism progressively achieved in recent years will soon allow investigators and clinicians to define whether and to what degree targeted therapeutic and perhaps even preventive approaches can be designed on the basis of a strong pathophysiological rationale.

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