

Decreased Plasma Myeloperoxidase Associated with Probiotic Therapy in Autistic Children

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ABSTRACT

AIM: To assess plasma myeloperoxidase (MPO) levels in autistic children and to test the hypothesis that there is an association between decreased MPO concentration and probiotic therapy.

SUBJECTS AND METHODS: Plasma from 49 autistic children (39 males; mean age 11.4 years) (17 with diagnosed gastrointestinal (GI) disease – chronic diarrhea and/or constipation (10 of these GI patients were taking probiotics) and 26 receiving probiotic therapy) and 36 neurotypical controls (29 males; mean age 10.2 years; controls were not assessed for GI disease) were tested for MPO plasma concentration using Enzyme Linked Immunosorbent Assays (ELISAs). Plasma concentration of MPO in autistic individuals was compared to plasma concentration of copper and zinc.

RESULTS: We found that individuals with autism, receiving no therapy, did not have significantly lower plasma MPO levels when compared to controls. In the autistic group, MPO levels were significantly lower in individuals taking probiotic therapy. In addition, plasma copper levels were significantly lower in autistic individuals taking probiotics compared to those not taking probiotics, but plasma zinc levels were not different in the probiotic group.

DISCUSSION: These results suggest a relationship between low MPO levels found in a group of autistic individuals and probiotic therapy. By possibly changing gut bacterial flora and thereby changing absorption properties in the gut, probiotic therapy was also associated with lower copper levels.

KEYWORDS: ASD, autism, myeloperoxidase, MPO, copper, probiotics, oxidative stress

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Introduction

Myeloperoxidase (MPO) is an enzyme found in the azurophilic granules of neutrophilic polymorphonuclear leukocytes (PMNs) and in the lysosomes of monocytes in humans. Its major role is to aid in microbial killing. MPO is a dimeric molecule, consisting of a pair of heavy- and light-chain promoters and two iron atoms. MPO has only one gene; thus, the mature enzyme is synthesized from a single polypeptide product. The synthesis of MPO primarily occurs during the promyelocytic stage of myeloid development, concurrent with the development of the azurophilic granules. The MPO gene, located on chromosome 17 (17q23.1), encodes a single-stranded

primary translational product, which is glycosylated to yield an enzymatically inactive precursor, apopro-MPO. Heme is then inserted, yielding the enzymatically active precursor pro-MPO.^{1,2}

When neutrophils become activated, in conjunction with phagocytosis, they undergo respiratory burst. This causes production of superoxide, hydrogen peroxide, and other reactive oxygen derivatives, which are all toxic to microbes. During respiratory bursts, granule contents are released into the phagolysosomes and outside the cell, allowing released contents to come into contact with any microbes present. MPO catalyzes the conversion of hydrogen peroxide and chloride ions (Cl)



into hypochlorous acid. Hypochlorous acid is 50 times more potent in microbial killing than hydrogen peroxide.²

MPO may have a role in atherosclerosis, carcinogenesis, and degenerative neurological diseases. The role of MPO in atherosclerosis is supported by the fact that MPO deficiency may protect against cardiovascular disease.^{3,4}

In addition to killing bacteria, the products of the MPO–hydrogen peroxide–Cl system are believed to play a role in killing fungi, parasites, protozoa, viruses, tumor cells, natural killer (NK) cells, red cells, and platelets, and they may be involved in terminating the respiratory burst, because individuals with MPO deficiency have a prolonged reaction.^{3–8}

Other functions of MPO include tyrosyl radical production, generation of tyrosine peroxide, mediation of the adhesion of myeloid cells via b2-integrins, and oxidation of serum lipoproteins.^{3,5} Because of this, MPO is considered a marker for inflammation and oxidation.

Autistic spectrum disorder (ASD) is a neurodevelopmental syndrome with onset prior to age of 36 months. Diagnostic criteria consist of impairments in socialization and communication plus repetitive and stereotypic behaviors.⁹ Traits strongly associated with autism include movement disorders and sensory dysfunctions.¹⁰ Although autism may be apparent soon after birth, many autistic children experience at least several months, up to a year or more in some cases, of normal development, followed by regression, defined as loss of function or failure to progress.^{10–12}

Children with ASDs frequently have accompanying gastrointestinal (GI) symptoms^{13–15} and pathology, which includes inflammation of the GI tract.^{16,17} Many autistic children, particularly those with GI disease, may have increased incidence of fungal infection,^{18,19} but one study found no difference in levels of intestinal yeast between children with autism and controls using both culture methods and microscopic evaluation.²⁰

We recently reported that there was a relationship between low MPO levels and GI disease seen in autism spectrum disorder individuals.²¹ In this study, we measured MPO levels in a general population of autistic children and analyzed the relationship between these levels and probiotic therapy.

Materials and Methods

ELISAs were used to measure plasma MPO (ELISA Kits, R&D Systems, Minneapolis, MN). The ELISA and plasma protocols have been previously reported and are explained below.²²

All reagents and specimens were equilibrated at room temperature before each assay was performed. A 1:51 dilution of the patient samples was prepared by mixing 10 μ L of the patient's plasma with 0.5 mL of plasma diluent. In all, 100 μ L of calibrators (20–200 EU/mL antibodies), positive and negative control plasma, plasma diluent alone, and diluted patient samples were added to the appropriate microwells of a microculture plate (each well contained affinity purified polyclonal IgG to the appropriate marker). Wells were incubated for 60 minutes

(\pm 5 minutes) at room temperature, and then were washed four times with wash buffer. In all, 100 μ L of pre-diluter anti-human IgG conjugated with Horse Radish Peroxidase (HRP) was added to all microwells, incubated for 30 minutes (\pm 5 minutes) at room temperature, and then washed for four times with wash buffer. A total of 100 μ L of enzyme substrate was added to each microwell. After approximately 30 minutes at room temperature, the reaction was stopped by adding 50 μ L of 1 M sulfuric acid, and then the wells were read at 405 nm with an ELISA reader (BioRad Laboratories, Inc.).

Copper and zinc measurements. Copper and zinc plasma concentrations were measured by LabCorp, Inc. using inductively coupled plasma–mass spectrometry, as previously described.²³

Plasma. All plasma, experimental and control, were treated in an identical fashion – frozen at -70°C immediately after collection and cell/serum separation, and then stored at -70°C until thawed for use in ELISAs. Plasmas were collected on a non-fasting basis, under conditions which were the same for both individuals with autism and controls.

Subjects. Plasma and diagnostic criteria (below) has been previously reported.²² Plasma from 49 autistic children (39 males; mean age 11.4 years) (17 with diagnosed GI disease – chronic diarrhea and/or constipation (10 of these GI patients were taking probiotics) and 26 receiving probiotic therapy) and 36 neurotypical controls (29 males; mean age 10.2 years; controls were not assessed for GI disease) were tested for MPO plasma concentration using ELISAs. Plasma concentration of MPO in autistic individuals was compared to plasma concentration of copper and zinc.

The diagnostic criteria used in this study were defined by the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) criteria. In 2012, the separate diagnostic labels of autistic disorder, Asperger's disorder, and pervasive developmental disorder – not otherwise specified (PDD-NOS) were replaced by one umbrella termed *autism spectrum disorder*.

Plasma was obtained from patients presenting to the Health Research Institute (HRI) (The HRI is a comprehensive treatment and research center, specializing in the care of neurological disorders, including autism.) over a two-year period. All autistic individuals who presented to HRI were asked to participate. Plasmas from individuals who participated in this study were randomly chosen from all patients who volunteered. Neurotypical control plasma was obtained from HRI (wellness patients) and the Autism Genetic Resource Exchange (AGRE) (The AGRE is a repository of biomaterials and phenotypic and genotypic data to aid research on autism spectrum disorders.), and randomly chosen from a selection of about 200 samples. The autistic individuals in this study met the DSM-IV criteria, and many were diagnosed using the autism diagnostic interview – revised (ADI-R) before presenting to the HRI.

Patient consent was obtained from all patients involved in this study, and this study was approved by the institutional review board (IRB) of the HRI, and this research complied with the principles of the Declaration of Helsinki.



Statistics. Inferential statistics were derived from unpaired t -test and odds ratios with 95% confidence intervals. Pearson moment correlation test was used to establish degree of correlation between groups.

Results

We found that individuals with autism, receiving no probiotic therapy, had lower, but did not have significantly lower plasma MPO levels ($2463 \text{ pg/mL} \pm 420$) when compared to controls ($3161 \text{ pg/mL} \pm 150$) ($P = 0.06$). In the autistic group, however, MPO levels were significantly lower ($1054 \text{ pg/mL} \pm 116$) in individuals taking probiotic therapy, compared to those not taking probiotics ($3161 \text{ pg/mL} \pm 336$) ($P = 0.0009$) (Fig. 1). In addition, plasma copper levels were significantly lower in autistic individuals taking probiotics ($88.8 \text{ mg/dL} \pm 4.7$) compared to those not taking probiotics ($103.1 \text{ mg/dL} \pm 3.6$) ($P = 0.02$) (Fig. 2), but plasma zinc levels were not different in the probiotic group ($89.30 \text{ mg/dL} \pm 25.9$) compared to the non-probiotic group (86.40 ± 24.7) ($P = 0.7$). MPO levels did not correlate by gender ($P = 0.4$) or age ($P = 0.7$). GI disease ($N = 17$) had no effect on MPO levels ($P = 0.46$). Autistic patients receiving zinc therapy ($N = 25$) did not have different plasma concentrations of MPO when compared to individuals with autism who had not received zinc therapy ($P = 0.46$). Table 1 summarizes the above data.

Discussion

Our results show that probiotic therapy may serve to significantly decrease MPO concentration in individuals with autism. We also found that probiotic therapy is associated with significantly lower copper levels.

A large population of autistic children suffers from GI disorders, implying a link between autism and abnormalities in gut microbial functions. Increasing evidence associated with sequencing analyses indicates disturbances in composition and diversity of gut microbiome, and that these changes may be associated with various disease conditions.^{20,24,25}

Recent research indicates that autism and accompanying GI symptoms are characterized by distinct and less diverse gut microbial compositions than controls, with lower levels of *Prevotella*, *Coprococcus*, and unclassified *Veillonellaceae*.²⁵

Probiotics are live microorganisms, such as *Lactobacilli* sp., *Bifidobacteria* sp., and *Streptococcus thermophilus*, that provide various health benefits upon ingestion and serve to often normalize gut flora populations.²⁶ These probiotics are commercially available as spores in lyophilized forms or in the form of probiotic-fortified fermented dairy products. Probiotics have shown potential as a component of treatment for eczema,²⁷ pediatric antibiotic-associated diarrhea,²⁸ acute upper respiratory tract infections,²⁹ chronic and acute enteric infections, and their associated diarrheal complexes.³⁰

Probiotics have been shown to modulate the intestinal immune system by production of secreted factors and metabolites that affect the growth and function of intestinal epithelial and immune cells.³¹

Probiotics may manipulate intestinal microbial communities and suppress growth of pathogens by inducing the host's production of β -defensin and IgA. Probiotics may be able to fortify the intestinal barrier by maintaining tight junctions and inducing mucin production. Probiotic-mediated immunomodulation may occur through mediation of cytokine secretion through signaling pathways such as NF κ B (nuclear

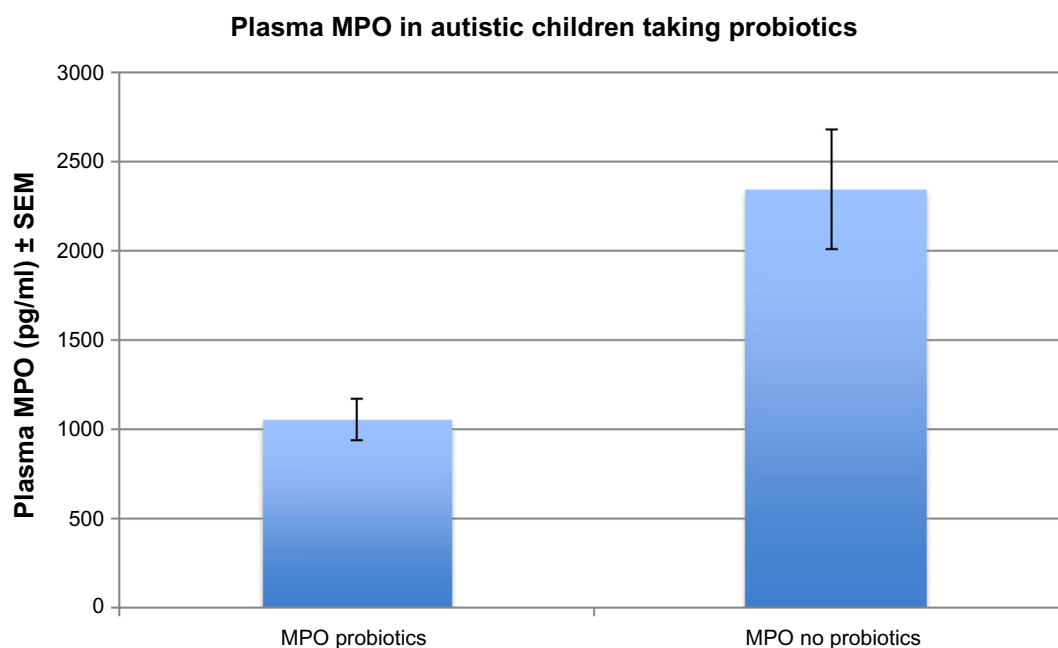


Figure 1. MPO levels were significantly lower ($1054 \text{ pg/ml} \pm 116$) in individuals taking probiotic therapy, compared to those not taking probiotics ($3161 \text{ pg/ml} \pm 336$) ($P = 0.0009$).



Effect of probiotics on plasma copper levels in autistic children

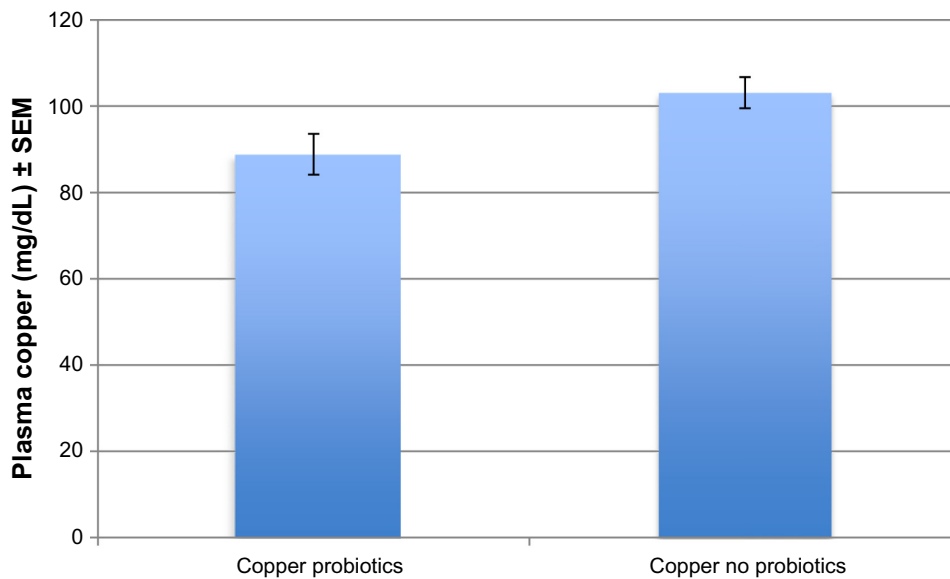


Figure 2. Plasma copper levels were significantly lower in autistic individuals taking probiotics (88.8 mg/dL ± 4.7) compared to those not taking probiotics (103.1 mg/dL ± 3.6) ($P = 0.02$).

factor kappa-light-chain-enhancer of activated B cells) and MAPK (mitogen activated protein kinase), which can also affect proliferation and differentiation of immune cells (such as T cells) or epithelial cells. Gut motility may be modulated

through regulation of pain receptor expression and secretion of neurotransmitters.³²

The gut microbiota comes into contact with metals and other contaminants as they are ingested through diet.³³ It is likely that microbes are presented with metals in water and food and may play a role in protecting the host from metal adsorption. Microbial sequestering of heavy metals by the intestinal microbiota is strongly supported by studies that show that when these contaminants are consumed at much higher concentrations, there is a lower detection in clinical samples, excluding absorption and dilution factors.^{34,35} Mechanisms for the binding of metals to bacterial cell walls include ion exchange reactions with peptidoglycan and teichoic acid, precipitation through nucleation reactions, and complexation with nitrogen and oxygen ligands.³⁶⁻³⁸ Since we found that copper levels, but not zinc levels, are lowered when associated with probiotic therapy, changes in microbiota may selectively affect the absorption of copper. The dynamics of this will need to be elucidated with future studies.

There were limitations to this study. We were unable to differentiate effect of different types of probiotics. This was based on varied therapy and the number of individuals in our study. Also, we did not follow individual patients, but the data represent a comparison of individual MPO levels, patients who had not taken probiotics with those who received the therapy. We did not find a relationship between length of probiotic use and MPO levels (unpublished data).

These data strongly suggest that autistic children taking probiotics have significantly lower levels of MPO and copper. It is possible that altered gut flora, because of probiotic therapy, results in less inflammation, which, in turn, results in lower MPO levels. Lower copper levels may also be the result

Table 1. Effects of probiotics on MPO concentration (pg/ml ± SEM).

1. Plasma MPO concentration significantly lower in autistic children compared to controls	
MPO autistic	MPO controls
Mean = 1692.03 ± 181.1	Mean = 3161.28 ± 150.5
2. Plasma MPO concentration significantly lower in Autistic individuals taking probiotics compared to autistic individuals not taking probiotics	
MPO probiotics	MPO no probiotics
Mean = 1054.21 ± 116.8	Mean = 2344.56 ± 336.8
3. Plasma MPO levels are not significantly lowered by zinc therapy	
MPO pre zinc therapy	MPO post zinc therapy
Mean = 2100.54 ± 411.3	Mean = 1557.36 ± 228.7
4. Plasma MPO is not affected by GI disease.	
MPO GI disease	MPO without GI disease
Mean = 1929.87 ± 442.4	Mean = 1607.18 ± 212.9
5. Plasma copper levels (mg/dl ± SEM) are significantly lower on autistic children taking probiotics compared to those not taking probiotics	
Copper probiotics	Copper no probiotics
Mean = 88.84 ± 4.7	Mean = 103.14 ± 6.3
6. Plasma zinc levels (mg/dl ± SEM) are not affected by probiotics	
Zinc probiotics	Zinc no probiotics
Mean = 89.30 ± 7.1	Mean = 86.40 ± 6.3



of changes in gut flora, resulting in bacteria that more readily bind heavy metals and prevent high absorption rates.

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Author Contributions

Conceived and designed the experiments: AJR. Analyzed the data: AJR. Wrote the first draft of the manuscript: AJR. Contributed to the writing of the manuscript: AJR. Agree with manuscript results and conclusions: AJR. Jointly developed the structure and arguments for the paper: AJR. Made critical revisions and approved final version: AJR. The author reviewed and approved of the final manuscript.

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