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Communication

Biomedical approach in autism spectrum disorders—the importance of assessing inflammation

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Abstract: Autism spectrum disorders (ASD) are severe heterogeneous neurodevelopmental disorders characterized by dysfunctions in social interaction and communication skills, repetitive and stereotypic verbal and nonverbal behaviors. Published findings have identified widespread changes in the immune systems of children with autism, at the systemic and cellular levels, suggesting that autism may, in fact, be a systemic disorder with connections to abnormal immune responses. Evaluating autism is hindered by a lack of specific biomarkers, making these pathologies difficult to diagnose. A critical priority for the future of ASD management is the identification of potential targets for the development of diagnostic and therapeutic strategies. The purpose of this brief report is to raise awareness regarding the involvement of different inflammatory processes in ASD and the need to assess them as a part of a biomedical evaluation. An extensive analysis of biomarkers relating to inflammation, immune dysfunctions, intestinal dysfunctions and infections will assist in the management of the autistic patient through a more personalized therapy.

Keywords: autism; inflammation; immune dysfunction; intestinal dysfunctions; biomedical evaluation

1. Introduction

Autism spectrum disorders (ASD) are complex, and severe heterogeneous neurodevelopmental pathologies with accepted, but complex immune system abnormalities. Previously, ASD were strictly considered to be a result of interactions between epigenetic and environmental factors, however, they are now generally recognized as multifactorial diseases. ASD pathologies are increasing in incidence and the prevalence in children may vary from 1 in 100 to 1 in 50 [1–3]. Unfortunately, too often ASD are underreported and, therefore, the healthcare needs of the impacted children are inadequately addressed. Furthermore, in spite of significant efforts on the part of many researchers, currently there are no ASD-specific biomarkers making an exact diagnosis of these pathologies difficult. For this reason, the current diagnostic criteria for autism are mainly based on the evaluation of behavior and social communication skills [4].

Indeed, a critical priority for the future of ASD management is the identification of potential targets for the development of diagnostic and therapeutic strategies. To this end, several dysregulated biochemical pathways and physiological observations have been associated with ASD including oxidative stress; endoplasmic reticulum stress; decreased methylation capacity; limited production of glutathione; mitochondrial dysfunction; intestinal permeability and dysbiosis; increased toxic metal burden; immune dysregulation; cytokine overproduction; endocannabinoid system dysregulation; and neuron-glia cross talk imbalance (reviewed in [5–7]). In light of this, an increasing number of practitioners are adopting a biomedical approach for managing their patients with ASD. Diagnostic testing for immune parameters such as cytokine expression, metagenomic analysis of intestinal microbiota, and screening for different chronic infections, such as mycoplasmas and tick-borne pathogens are becoming routine. However, at this time, few publications exist that articulate the utility of these diagnostics. The primary source of information available to the healthcare practitioner is primarily conferences-related communications. The purpose of the present brief report is to raise awareness about the involvement of different inflammation processes in ASD and the need to assess them as a part of biomedical evaluation.

2. Material and methods

2.1. Specimen collection

As part of the respective patient's biomedical assessment, peripheral EDTA-anticoagulated blood, RNA-stabilized blood (collected using Tempus™ Blood RNA Tubes (Applied Biosystems, Inc., Foster City, CA)) and serum were collected by venipuncture, along with fresh stool samples from ASD-diagnosed patients at the Himmunitas clinic (Neder-over-Heembeek, Belgium). Each specimen was sent the same day to R.E.D. Laboratories (Zellik, Belgium) by courier for analyses. The present study represents a retrospective investigation of existing clinical data. Accordingly, all patients did not undergo the same clinical testing. A total of 42 available files were surveyed. The patients ranged from 3–17 (mean 11 $\pm$ 3.7 SD) years old and were primarily from Europe (Belgium, Holland, Italy, Spain, UK, and Ukraine).

Written informed consent to use the patient's clinical data was obtained from the respective patient's parent or legal guardian(s), in accordance with the Code of Ethical Principles for Medical

Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki). All haematological testing were conducted as part of the respective patient's clinical work-up.

In order to determine the range of normal values, samples from healthy controls (referred to the laboratory for a general check-ups) were tested. The impact of age and gender were evaluated when determining the normal control ranges. These analyses demonstrated no differences linked to the age but differences linked to gender were observed in the case of Prostaglandin E_2 quantification (see below) and gender-related ranges were established.

2.2. Prostaglandin E_2 quantitative determination in human serum

The DetectX® Prostaglandin E_2 (PGE2) Immunoassay kit (Arbor Assay, Michigan, USA) was used to quantitatively measure PGE2 present in serum according to the manufacturer's instructions. Serum PGE2 concentrations were determined using a standard curve generated with PGE2 standards provided in the assay kit and calculated using 4PLC fitting routine provided with the microplate reader (BioRad, Nazareth, Belgium). To obtain the sample concentrations, the values were multiplied by the dilution factor to yield neat sample values. In order to determine the range of normal values, 79 samples from healthy controls (referred to the laboratory for a general check-up) were tested. These 79 healthy controls (42 female and 37 male) were used as the reference panel to establish the median value for the healthy group. This group was analyzed to assess any potential age- and gender-related impact on the results. The analyses demonstrated no differences linked to the age but a noticeable difference was linked to gender. Accordingly, gender-specific median values of the healthy group were established. This median value is used to normalize all other values in the format of a ratio. The normal range is established using percentile 10 (P10) and 90 (P90) of the healthy group as limits for normality i.e. P10 ratio to normal as lower limit and the P90 ratio to normal as upper limit.

2.3. Quantification of serum cytokine levels

Serum levels of nine proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70, MCP-1, MIP-1β, TNF-α, activated TGFβ), were evaluated by flow cytometry multiplex assessment using the BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (Becton Dickinson (BD) Biosciences, San Jose, CA, USA), on a BD FACS Canto II™ platform and analyzed using FCAP Array™ software. All flow cytometry—related reagents were obtained from BD. Briefly, the BD CBA assays is a bead-based multiplex assay that utilizes beads of known size and fluorescence intensity, making it possible to detect and distinguish each bead by flow cytometry. Each respective "capture" bead in the kit has been conjugated with an antibody specific to one analyte. The detection reagent is a mixture of phycoerythrin (PE)—conjugated antibodies, specific to a different epitope on the analyte of interest. The respective bead to which it binds identifies each specific analyte and the quantitative value for each analyte is proportional to the fluorescent signal produced by the detection antibody and determined by fitting to a standard curve generated using know concentrations of standards. A full standard curve was run at each assay, along with positive controls and interassay controls.

2.4. Quantification of soluble CD14 (sCD14) levels in serum

Serum levels of sCD14 were also assessed by flow cytometry using BD CBA Human Soluble CD14 Flex Set on a BD FACS Canto II^{TM} platform according to the instructions provided by manufacturer. The assay principle is similar to that provided above for multiplex cytokine detection. A standard curve was run at each assay, along with positive controls and interassay controls.

2.5. Quantification of elastase mRNA expression levels

Total RNA was extracted from stabilized whole blood, collected using Tempus^{TM} blood RNA collection tubes, using the Tempus^{TM} RNA extraction kit, according to the manufacturer's instructions (Applied Biosystems) and converted into cDNA using RNA to cDNA kit (Applied Biosystems). The cDNA was quantitatively analyzed on a CFX96 Touch^{TM} Real-Time PCR Detection System (Biorad, Inc. Nazareth, Belgium). All primers and PCR reagents were obtained from Life Technologies (Palo Alto, CA, USA).

2.6. Quantification of soluble IgA in stool

The sIgA ImmuChrom Immunoassay kit (ImmuChrom GmbH, Heppenheim, Germany) was used to quantitatively measure sIgA present in stool according to the manufacturer's instructions.

3. Results and discussion

In order to articulate the importance of different potential biomarkers we have presented here the clinical laboratory results from 42 patient files of children diagnosed with ASD. Notably, the obtained results are not the consequence of a planned prospective study, rather a retrospective biomedical survey conducted in the framework of a medical consultation. Many inflammation-related parameters have been evaluated (although not always the same ones in all children). Inflammation-related markers were tested both in blood (cytokines, PGE2 and elastase expressions) and in stool (sIgA). As qualitatively depicted in Figure 1 and Table 1, the vast majority of patients tested show a notable increase in inflammation-related markers: 86.5% presented with elevated IL-8, 71% with elevated PGE2, 57% with elevated MCP-1, 47% with elevated IL-1β, and 41% with elevated elastase while 68% displayed an abnormal expression of sIgA in the stool (Figure 2).

Abnormalities in cytokine expression have been previously reported for ASD by several groups and are consistent with the observations presented here. Ashwood et al. reported a significant increase in plasma levels of a number of cytokines in the ASD cases when compared to healthy controls, including IL-1β, IL-6, IL-8 and IL-12p40 [8]. Also, Suzuki et al. reported that the plasma concentrations of IL-1β, IL-1RA, IL-5, IL-8, IL-12p70, IL-13, IL-17 and GRO-α were significantly higher in subjects with ASD when compared with matched controls [9]. Additionally, Okada et al. and Ashwood et al. independently reported decreased serum levels of transforming growth factor beta1 (TGFβ1) in patients with autism, with lower TGFβ1 levels associated with lower adaptive behaviors and worse behavioral symptoms, suggesting that immune responses in autism may be inappropriately regulated due to reductions in TGFβ1 [10,11]. Al-Ayadhi and Mostafa reported that children with autism had significantly higher serum IL-17A levels in comparison to healthy

controls ($p < 0.001$), with increased serum levels of IL-17A found in 48.9% of the autism group [12]. Moreover, based on their study, which evaluated cytokine levels in 17 ASD children and matched controls, Inga Jacome et al. suggested that peripheral inflammatory markers might be useful as potential biomarkers to predict comorbidities in autism [13]. Finally, a very recent publication by Siniscalco et al. [14] nicely review the role of cytokines and inflammatory condition in ASD patients.

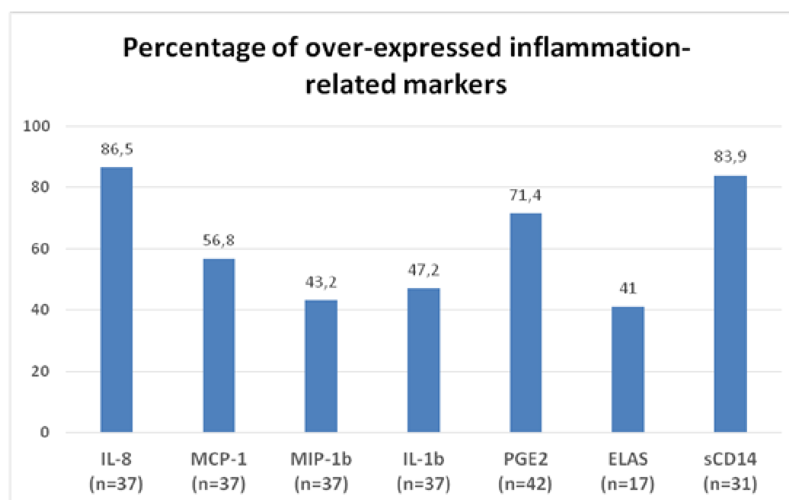

Figure 1. Percentage of over-expressed inflammation-related markers. The values of the graph represent the percentage of ASD patients with abnormal laboratory results for serum interleukin 8 (IL-8), MCP-1, MIP-1β, IL-1β, PGE2 and sCD14 and mRNA levels for elastase (ELAS). The numbers in brackets indicate the total number of patient records used to derive each respective value.

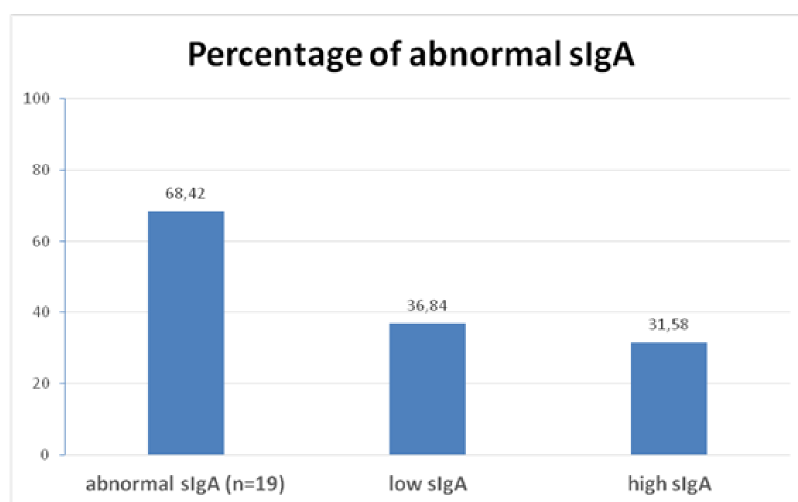

Figure 2. Percentage of abnormal sIgA results. The values of the graph represent the percentage of ASD patients with abnormal sIgA as measured in the stool. The numbers in brackets indicate the total number of patient records used to derive each respective value. The abnormal results may represent values that are either higher or lower than the established healthy range.

Table 1. Mean of over-expressed levels of interleukin 8 (IL-8), MCP-1, MIP-1β, IL-1β, PGE2, Elastase, sCD14 and sIgA as measured in serum, blood or stool of ASD patients. Over-expression indicates patient values above the normal range, as established on healthy subjects.

Analyte	Mean	Normal Range
sCD14	4167.78	1430–2800 ng/mL
sIgA	1981.73	510–2040 µg/mL
Elastase	356.47	0–150*
Il-1b	24.9	0–3 pg/mL
IL-8	2541.14	0–15 pg/mL
MCP-1	734.25	0–165 pg/mL
MIP-1	467.92	0–155 pg/mL
PGE2f	13.53	0.10–2.81**
PGE2-f	0.84	0.37–1.63**
PGE2m	20.42	0.17–6.45**
PGE2-m	3.8	0.30–2.06**

*Ratio normalized to the B2M gene; **Expressed as a ration to the normal median; f and m for PGE2 indicate female or male values, respectively.

In line with our findings on PGE2, El-Ansary & Al-Ayadhi also observed PGE2 to be significantly higher in autistic patients, recording an average increase of 91.15% [15]. It is noteworthy that this particular marker is also emphasized in a recent book by Drs. Skorupka and Amet (*Autisme: causes et solutions*, Ed Essai 2017 [16]). As a reminder, PGE2 is a compound derived from membrane phospholipids and is a key mediator of immunopathology in chronic infections and cancer. Elevated prostaglandins could be related to the recent report of Rossignol and Frye that immune dysregulation and inflammation are the first etiological factor of autism [17]. It is widely believed that autism may be associated with viral infections that cause an upregulation in prostaglandins, which then mediate fever production. This suggestion could support the observation of elevated inflammatory cytokines in the plasma and brains of autistic patients.

Elastase is an inflammatory protease expressed by immune cells (monocytes, neutrophils) that contributes to immune defense by inactivating foreign bacteria but at the same time can cause damage to connective tissue, and break down cytokines, immunoglobulins and immune cells receptors. Excess, chronic production of elastase is therefore detrimental. To the best of our knowledge, the present study is the first to suggest its increase in ASD patients.

Several reports have revealed a high prevalence of gastrointestinal symptoms, including gut inflammation and dysfunction in children with autism (reviewed by Horvath and Perman, [18]). Indeed, the number of reports on ASD-related intestinal dysbiosis is increasing, suggesting that gut flora and gastrointestinal status in children with autism correlate with autism severity. For instance, Adams et al. reported that gastrointestinal symptoms were strongly correlated with the severity of autism [19]. Of the four types of beneficial bacteria that were investigated, children with autism presented with significantly lower levels of Bifidobacterium (45% lower), lower levels of Enterococcus (16% lower), and significantly higher levels of Lactobacillus (100% higher). Additionally, Finegold et al. reported that in the control children's stool, Firmicutes accounted for 63.6% of the total flora but only 38–39% of the flora of autistic children's stool [20].

Bacteroidetes accounted for 30% of the stool flora in controls and for 51% in the flora of stool of autistic children. Whereas, Actinobacteria made up 1.8% of stool flora of control children and between 0.4 and 0.7% of the flora of autistic children, Proteobacteria made up 0.5% of the flora of control children and between 2.3 and 3.1% of the flora of autistic children. Interestingly, Iovene et al. reported an aggressive form (pseudo-hyphae presenting) of *Candida spp*., together with lowered *Lactobacillus spp.* and decreased number of *Clostridium spp*. in the stool samples of autistic children. Also, in this case, a significant linear correlation was found between autism severity and the presence of *Clostridium spp.* [21]. Overall, the fecal flora of autistic children tends to be perturbed when compared to the fecal flora of healthy children. In line with this, our data suggest that of the 33 ASD children presented with evaluated microbiome composition; the diversity index was low in 85% of cases, 100% showed low Asaccharobacter, 82% low Ruminococcus, and 39% presented with high Bacteroides (data not shown).

Upon medical evaluation, mild to moderate degrees of inflammation were found in both the upper and lower intestinal tract. In children with ASD, the presence of gastrointestinal dysfunction is often associated with increased irritability, tantrums, aggressive behavior, and sleep disturbances (reviewed by Critchfield et al., [22]). In our analysis, 68% of assessed patients showed an abnormal expression of sIgA. A key function of sIgA in the stool is to bind to invading microorganisms and toxins and entrap them in the mucus layer or within the epithelial cells, thereby inhibiting microbial motility, agglutinating the organisms and neutralizing their exotoxins and then assist in their harmless elimination from the body in the fecal flow. A lack of sIgA indicates a diminished activity of the intestinal immune system while an increased level of sIgA shows intestinal inflammation. Interestingly, half of patients who present with abnormal sIgA have increased levels while the other half have sIgA below the normal level.

The very high prevalence of increased inflammation-related markers is most probably the end result of several dysfunctions in ASD patients, as already reported in previous studies. Among them, a large number point towards intestinal dysfunctions (including gut dysbiosis and leaky gut) and chronic infections (including intracellular pathogens and tick-borne infections). This hypothesis is further supported by the very high percentage (84%) of patients with increased serum soluble CD14 (sCD14) (Figure 1). The membrane bound form of CD14 is expressed by macrophages, and to a lesser extent, by dendritic cells and neutrophils. It plays a critical role in the recognition of the lipopolysaccharides (LPS), which are found in the outer membrane of Gram-negative bacteria. The extracellular part of CD14 can be cleaved and released in the plasma, where it will inactivate circulating LPS; accordingly, sCD14 is also a putative surrogate marker of serum LPS and bacterial translocation. Serum sCD14 levels are significantly elevated in patients with inflammatory bowel disease, and Crohn's disease, but also in patients suffering from Brucellosis or Lyme disease.

The increased concentration of LPS can originate (i) from the intestinal bacteria that translocate across the intestinal epithelium, as seen in leaky gut (as already emphasized by Siniscalco and Antonucci [23], and/or from (ii) infections (as emphasized by Bransfield et al. [24] and Kuhn et al. [25]). Indeed, when we analyze the data for markers associated with leaky gut on the same patient population, we observed that 73% of patients tested displayed a notable increase of zonulin in stool (data not shown). Of note, zonulin regulates tight junctions of intestinal epithelial cells and is considered a biomarker of increased intestinal permeability [26]. Specifically, decreased cellular zonulin leads to increased spaces between the cells of the intestinal lining. In subjects with leaky gut, the spaces between the cells increases allowing larger protein molecules and bacterial products, such

as LPS, to translocate into the bloodstream, resulting in inflammatory immune responses. As the cellular zonulin level decreases, the seal between the intestinal cells diminishes. Zonulin is the only physiological modulator of intercellular tight junctions described, thus far, that is involved in trafficking of macromolecules and, therefore, in tolerance/immune response balance. As a consequence of bacterial overgrowth and leaky gut, toxic bacterial metabolites are also substantially increased. For instance, we have observed elevated ammonia in 79% and D-lactate in 61% of ASD patients analyzed (data not shown). D-lactate is a product of bacterial metabolism; it is neither produced nor metabolized by mammalian cells. Typically, elevated D-lactate levels are due to bacterial infection or short bowel syndrome in humans. As a result of its slow metabolism and excretion, elevated D-lactate can cause acidosis and encephalopathy [27]. Ammonia is derived from the actions of bacterial enzymes on ingested amino acids. It is absorbed from the gastrointestinal tract and delivered through the portal vein to the liver, which converts most of it into urea. Abnormally high levels of ammonia can result from colic or "enteric hyperammonemia" (combination of increased bacterial production and increased gut permeability) that occurs despite normal hepatic function. Hyperammonemia is a metabolic condition characterized by elevated levels of ammonia in the blood. Increased entry of ammonia to the brain is a leading cause of neurologic disorders, metabolic disorders and some toxic encephalopathies.

Finally, we observed that 77% of patients tested positive for one or more zoonotic infections (tested by regular serology, Elispot LTT and/or by PCR). These findings are further supported by data obtained by Nicolson et al. indicating that a large subset of ASD patients show evidence of bacterial and/or viral infections [28]. Furthermore, Bransfield et al. indicated that their preliminary data suggested borreliosis may be a contributor in 20–30% of ASD cases, and pathogenic Mycoplasma may be a contributor in 58% [24]. Many other infections continue to be investigated and these results will further address the hypothesis of the origin of multiple inflammatory processes evidenced in ASD patients.

4. Conclusion

As emphasized by Careaga et al., "Autism is a complex and clinically heterogeneous disorder with a spectrum of symptoms" [29]. They state "Published findings have identified widespread changes in the immune systems of children with autism, at both systemic and cellular levels, suggesting that autism may, in fact, be a systemic disorder with connections to abnormal immune responses". When taken together, the small sampling of qualitative data discussed in this report, in conjunction with previous reports, clearly point towards the importance of assessing the inflammation-related biomarkers in ASD patients. A larger, well controlled, and quantitative investigation is needed in order to further define the most useful biomarkers. Moreover, these data emphasize the need for a more general assessment of immune-related dysfunctions, intestinal dysfunctions and infections in the framework of biomedical approaches to ASD patient management. In order to define a subset of biomarkers with real clinical utility, it will be necessary to assess a wide range of parameters on a large ASD population. Ultimately, the knowledge gained will allow for efficient and efficacious biomedical-based treatment and management strategies for the ASD patient.

Conflicts of interest

TM and EB are employees of R.E.D. Laboratories, which conducted the clinical diagnostic testing presented in this report. All other authors report no conflicts of interest.

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