Effects of the probiotic k11-tmax on symptoms and inflammatory markers in children with autism spectrum disorder

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EFFECTS OF THE PROBIOTIC K11-TMAX ON SYMPTOMS AND INFLAMMATORY MARKERS IN CHILDREN WITH AUTISM SPECTRUM DISORDER

SUMMARY

Disorder Autism is a neurological disorder that affects social, communication, and behavioral development. Since the initial description of the disorder by Kanner (1943), there has been a wide range of research on autism, including genetic, neurobiological, behavioral, and intervention studies. The exact cause of autism is not fully understood, but studies indicate that genetic and environmental factors may be involved, while some studies suggest that metabolic indices of serotonin, dopamine, amino acids, among others, may be affected, while others suggest that genetic predisposition is an important factor in the origin of autism. Ding et al. (2021) discuss evidence of imbalances in intestinal microbiota with autism development. Conducting a literature review, various pieces of evidence suggest that inflammatory processes may contribute to the development and symptoms of autism. In this context, metabolic deficiencies, inflammatory processes, and intestinal dysbiosis may be directly related to autism and its symptoms. The connection between gut microbiota and the nervous system occurs through the gut-brain axis, a well-established association in the scientific community. Numerous studies have investigated the effects of probiotics on intestinal and mental health, including the production of neurotransmitters such as dopamine and serotonin. This has been documented in research conducted by Marco et al. (2015), Xie et al. (2020), Dinan et al. (2013), among others. These findings underscore the importance of understanding the role of probiotics in modulating gastrointestinal health and mental well-being. Similarly, Martins et al. (2022) concluded that there is a significant association between inflammatory bowel disease and autism. We propose a clinical trial to evaluate the effects of a probiotic called nanoencapsulated K11-Tmax on improving symptoms and inflammatory biomarkers in children with ASD.

Keywords: Autism; Biomarkers; Inflammation; Probiotics; Gut Microbiota

1. THEORETICAL FRAMEWORK

The autism spectrum disorder (ASD) is a complex and multifaceted condition, with many different definitions provided by scientists and researchers. According to Kanner (1943), autism is a separate and distinct condition from other psychiatric conditions. Kanner described autism as a condition characterized by "an inability to establish emotional contact with other people, a lack of desire to establish social relationships, an inability to use language to communicate experiences, and ritualistic and stereotyped behavior." These characteristics are still widely recognized as part of the definition of autism, although the understanding of the condition has evolved significantly since Kanner's time. [15]

Another important definition of autism was provided by Hans Asperger (1944),[22] an Austrian pediatrician who first described what is now known as Asperger's syndrome. In 1944, Asperger published a paper titled "Autistic Psychopathy in Childhood," in which he described a group of children who shared characteristics like those described by Kanner. However, Asperger emphasized the importance of recognizing the exceptional abilities and talents of these children, rather than just focusing on their difficulties. He described the children as having "special intelligence," with skills in areas such as memory, visual perception, and mathematics. He also noted that these children often had intense and restricted interests, which could become a source of creativity and achievement. [22]

Asperger's definition became significant as Asperger's syndrome was recognized as a subtype of autism. Although Asperger's syndrome is no longer a separate diagnostic category in the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders, 5th edition), many people still use the term to refer to a specific type of autism that includes exceptional skills and talents in some areas.[23] Various studies have been conducted to better understand autism, from its etiology to clinical intervention. One prominent area of research is neuroscience, which seeks to understand the neurological changes that may be associated with autism. Brain imaging studies, for instance, have demonstrated differences in connectivity and brain activity, as well as in neuroendocrine and metabolic aspects, in individuals with autism compared to typical individuals. [2,3, 5,8,10,24]

Although there is no formal classification of "autism levels," many autism specialists use terms like "high-functioning" or "low-functioning" to describe different degrees of autism's impact on a person's daily life.[11,15,7,18,22,25] Tony Attwood (1998) discusses the different levels of functioning within the autistic spectrum, stating that autism is a continuous spectrum, with some people experiencing severe difficulties in areas such as communication and social skills, while others have fewer difficulties in these areas but may still exhibit repetitive and restricted interests and behaviors. He also emphasizes the importance of understanding that each person with autism is unique and may present a wide variety of symptoms and needs.[24]

Kanner (1943)[15] was the author who defined the symptoms of autism as being divided into three main areas:

a) Communication: difficulty initiating and maintaining conversations, stereotyped use of language, difficulty understanding context, and interpreting nonverbal language.

b) Social interaction: difficulty in developing and maintaining relationships, lack of interest in shared play, lack of empathy, and understanding of others' emotions.

c) Restricted and repetitive behaviors: repetitive or ritualized behaviors, intense interest in specific topics, difficulty coping with changes in routine, hypersensitivity to sensory stimuli (such as light, sound, and textures).

It is important to note that not all people with autism exhibit the same symptoms and that they can vary widely in intensity and frequency. Some individuals with autism may have milder

symptoms and be able to live independently, while others may require constant support in their daily activities. [15,18,24,25]

Several hypotheses have been proposed to explain inflammation related to ASD.[1] The dysregulation of the maternal immune system during pregnancy has been implicated, and the transfer of maternal fetal brain-reactive antibodies is associated with an increased risk of ASD.[2] In maternal autoimmunity, IgG antibodies derived from the mother enter the fetal compartment freely crossing the placenta; these antibodies recognize proteins and can interfere with fetal development.[4,7]

The gastrointestinal system has a direct connection with the immune system. Gastrointestinal symptoms associated with ASD may be a manifestation of an underlying inflammatory process.[3,12,26,27] Increased intestinal permeability has been associated with ASD (leaky gut hypothesis.[4,28,29] A landmark study detected increased intestinal permeability in 37% of autistic patients and in 21% of their relatives through the lactulose/mannitol test. Interestingly, autistic individuals on a gluten-casein-free diet had significantly lower intestinal permeability than those on an unrestricted diet and control individuals. Calprotectin was used as a non-invasive marker to assess intestinal inflammation.[5,30,31,32,33] Intestinal barrier defects predispose individuals with ASD to sensitization by environmental antigens. Notably, the abnormal immune system in autistic individuals may be due to gluten/casein-derived molecules that, once moved to a damaged intestinal barrier, trigger pro-inflammatory processes (increased pro-inflammatory cytokines and pro-inflammatory monocytes).[6,13,34,35] These pro-inflammatory mediators or immune-activated complexes reach higher brain centers through the bloodstream, and a permissive blood-brain barrier further contributes to neuroinflammation events.[7,37,38,39]

Growing evidence supports dysbiosis of the intestinal microbiota in ASD. Through cultivation methods, aggressive forms of Candida spp. were identified in the stools of 57% of children with ASD and not in healthy controls of the same age.[9] Decreases in Lactobacillus spp. and Clostridium spp. could sustain dysbiosis. Different fecal flora was observed in regressive autism compared to normal controls.[10] Harmful intestinal colonizing microorganisms produce various chemicals from their cell cycles that affect behaviors, as these metabolites have molecular structures similar to brain neurotransmitters.[11,40,41,42] In summary, inflammatory state, cellular viability, and genetic factors are intrinsically related to ASD, and the increasing understanding of these mechanisms underscores the need to explore new directions for future treatments and therapies for ASD.

2. GENERAL OBJECTIVE

The objective of this study is to evaluate the effectiveness of probiotic compounds K11-T and K11-Tmax (enriched with amino acids, minerals and vitamins), in their solid and nanoencapsulated formulations, in improving neuropsychological, psychiatric and psychopedagogical outcomes, as well as reducing inflammatory parameters in children with ASD.

3. SPECIFIC OBJECTIVES

- Characterize the sample of individuals with ASD who will participate in the study through a questionnaire with sociodemographic and pedagogical data such as: Age in years; gender in percentage terms of the sample according to female or male; Weight in kg; Height in centimeters; Time since diagnosis of ASD in years and presence of psychiatric or neurological comorbidities in percentage terms of the sample.

- Investigate the association between probiotic consumption and neuropsychological results using the scale unit validated in the literature Vineland Adaptive Behavior Scales - Third Edition (Vineland-3) in the questionnaire format answered by parents/guardians of the child with ASD;
- Investigate the association between probiotic consumption and the change of psychiatric parameters through the application of the validated Autism Diagnostic Observation Schedule (ADOS) scale with children with ASD,
- Investigate the association between probiotic consumption and the change of psychopedagogical parameters using the Childhood Autism Rating Scale (CARS) validated in the literature, which will be answered by the teachers responsible for each child participating in the study;
- Analyze the possible effect of probiotics on inflammatory aspects in individuals with ASD, through serum (Insulin, C-Reactive Protein, Prolactin and Cortisol) and fecal (fecal calprotectin) biomarkers;
- Analyze the difference of using the K11-Tmax version, enriched with supplements, on the neuropsychological, psychiatric and psychopedagogical outcomes described above in comparison to the group that used K11-Tmax alone with the presence of a control group that will receive placebo.

4. MATERIALS AND METHODS

The present study will be characterized as a randomized, double-blind, placebo-controlled clinical trial. The sample calculation was based on the current prevalence of ASD in children according to the latest release from the Center for Disease Control, with a target of 270 volunteer participants with ASD, maintaining a confidence interval of 5%.
Pre-screening will have immediate action, and volunteers can register by filling out a virtual recruitment questionnaire available on the website *www.gon1.com.br*. This phase is crucial in raising sponsorship funds to ensure the feasibility of the research, considering the minimum sample safety requirements. It is emphasized that the analysis of inclusion criteria and contact with families will only be conducted after the project approval by this Research Ethics Committee.
The safety of using the probiotic mix will be ensured by the results obtained from toxicity and pathogenicity tests and by defining the supplement ceiling (zinc 20% 0.148g, selenium 1% 0.032g + magnesium 65mg + vitamin A 0.300g) contained in the K11-Tmax version according to the positive list from ANVISA stratified at the age of 3 years.

4.1. SAMPLE SELECTION

Inclusion Criteria:

- Volunteers must have a confirmed diagnosis of ASD conducted by qualified professionals, according to the criteria established in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) or another recognized diagnostic classification.
- The study will include children and adolescents in the specific age range of 3 to 11 years.
- Must be regularly enrolled in a public or private school, or in a special education center.
- Absence of uncompensated clinical or psychiatric comorbidities.
- Informed consent: Legal guardians must provide informed consent, understanding the study objectives, procedures involved, risks and benefits, as well as the freedom to withdraw from the study at any time.
- Informed assent: Children aged 7 to 11 years must provide informed assent, understanding the study objectives, procedures involved, risks and benefits, as well as the freedom to withdraw from the study at any time.

Exclusion Criteria:

- Concomitant restrictive medical conditions that may interfere with study results, such as severe gastrointestinal diseases, significant metabolic diseases, or immunodeficiencies.
- Use of specific medications such as broad-spectrum antibiotics or immunosuppressive drugs during the follow-up period.
- Allergies or intolerances to components of the probiotics that will be administered.
- Previous or current participation in recent clinical studies with distinct therapeutic interventions.
- Uncontrolled cardiac conditions or severe unstable medical illnesses.
- Inability to be present on the predetermined dates of clinical assessments.

4.2. EXPERIMENTAL PROTOCOL

4.2.1. Study Design

This study will be conducted in a medical clinical specifically adapted for testing and with a permissive playful space to mitigate the stress of the involved children and avoid social conflicts during evaluation sessions. The space will comprise at least 17 individual analysis rooms, and the evaluation will involve 20 neuropsychologists, 2 pediatricians and 1 psychiatrist with prior and ensured training.

Children will be evaluated on DAY 0, DAY 45 and DAY 90 to quantify inflammatory markers through blood collection; for measuring fecal calprotectin, by collecting fecal samples; and finally, for the primary outcomes mentioned in this project: neuropsychological (Vineland-3); psychiatric (ADOS) and psychopedagogical (CARS). After the first data collection, individuals will be randomized to receive the K11-T probiotic mixture or the K11-Tmax probiotic mixture or placebo, in a 1:1:1 ratio. After the initial 90-day follow-up, up to 30% of the sample using K11-Tmax will continue to be monitored, being re-evaluated after 365 days in all aspects mentioned. Children will be stratified by age, gender and level of support (see Appendix B) and then randomized to intervention according to the groups below.

Condition or Disease	Intervention/Treatment	Phase
Children with ASD	Drug 1: Probiotic K11-T Drug 2: Probiotc K11-Tmax Drug 3: Placebo	Phase 3

4.2.2. Research Arms

Groups	Intervention/Treatment
Group 1	Probiotic K11-T 1 dose/day Orally
Group 2	Probiotc K11-Tmax 1 dose/day Orally
Group 3	Placebo 1 dose/day Orally

4.2.3. Quantification of C-reactive Protein (CRP)

For the quantification of PCR, individuals were instructed to fast for 8 hours. Polymerase Chain Reaction (PCR) is a widely used technique for amplifying specific DNA sequences in vitro. It allows selective and rapid amplification of a target DNA region, enabling its detection and analysis. The PCR methodology involves the following steps:

- Reaction preparation: Reaction components are prepared in a reaction tube, including the target DNA, primers (specific oligonucleotides that bind to sequences of interest), nucleotides (DNA building blocks), a DNA polymerase enzyme (which catalyzes DNA synthesis), and a reaction buffer providing optimal conditions for enzyme activity.
- Denaturation: The reaction mixture is heated to a high temperature (usually between 94-98°C) to denature the target DNA, separating the two complementary strands.
- Primer Annealing: The temperature is reduced (usually between 50-65°C) to allow the primers to anneal (bind) to the complementary DNA sequences at the ends of the target.
- Extension: The temperature is then raised again (usually around 72°C) to enable the activity of the DNA polymerase enzyme. The enzyme synthesizes a new complementary DNA strand to each of the original strands.
- Amplification cycles: The denaturation, annealing, and extension steps are repeated in successive cycles. Each cycle doubles the number of copies of the target DNA. The total number of cycles may vary depending on the desired amount of amplification.
- At the end of PCR, multiple copies of the target DNA sequence are obtained, allowing for its subsequent detection and analysis. Amplification can be visualized using various methods such as agarose gel or capillary electrophoresis, and detection can be performed using fluorescent probes, specific dyes, or DNA sequencing.

4.2.4. Serum Cortisol Quantification

For serum cortisol quantification, participants were instructed to fast for 8 hours and ensure that sample collection is done between 7 and 10 am. To measure serum cortisol levels, serum samples will be centrifuged at 2,200 g for 10 minutes at 18°C and stored in gel tubes at a temperature of 2 to 8°C. The analysis will utilize the chemiluminescence method, and the results will be expressed in mcg/dL.

4.2.5. Insulin Quantification

For insulin quantification, individuals were instructed to fast for 8 hours. To measure serum insulin levels, serum samples will be centrifuged at 2,200 g for 10 minutes at 18°C and stored in gel tubes at a temperature of 2 to 8°C. The analysis will utilize the chemiluminescence method, and the results will be expressed in mcUI/ml.

4.2.6. Prolactin Quantification

For prolactin quantification, individuals were also instructed to fast for 8 hours. To measure serum prolactin levels, serum samples will be centrifuged at 2,200 g for 10 minutes at 18°C and stored in gel tubes at a temperature of 2 to 8°C. The analysis will utilize the chemiluminescence method, and the results will be expressed in ng/dL.

4.2.7. Fecal Calprotectin Measurement

The sample for fecal calprotectin measurement will be collected by convenience sampling, considering the inherent behavioral difficulties of the disorder, while respecting a minimum sample value of 30% of the total sample of children, totaling at least 81 children. Families will be instructed to collect the first stool of the day and keep it refrigerated at a temperature ranging from 4 to 5 degrees Celsius (in a regular refrigerator) until the evaluation time. Children undergoing colonoscopy, using laxatives or suppositories, or undergoing examinations using radiological contrasts within 72 hours before collection will have their samples discarded. The fecal material will be stored in a refrigerated environment, processed using a specific kit for measurement, and expressed in µg/g.

4.2.8. Neuropsychological Assessment

Neuropsychological assessment in patients with ASD involves a multidisciplinary approach, considering different aspects of neuropsychological functioning. Standardized analysis will be

conducted using the Vineland Adaptive Behavior Scales - Third Edition (Vineland-3) [46]. The forms will be completed in their self-administered version by parents/caregivers and teachers, under the supervision of a qualified and trained health professional. Vineland-3 assesses adaptive behavior by dividing it into five domains: communication skills, daily living skills, socialization skills, motor skills and maladaptive/behavioral skills of the child. Those responsible will respond to the items in each domain on a scale ranging from 0 to 2, with 0 being never; 1 sometimes and 2 often. To calculate the final score, the scores are transformed into a population average of 100 based on age and a standard deviation of 15 according to the specific and standardized manual for the scale. A higher score indicates greater adaptive functioning.
The Vineland-3 is clinically validated for use in tracking change over time [47] and is used as an outcome measure in several randomized controlled trials for children with ASD. [48]
It should be noted that the adaptive scale in question is a specific neuropsychological battery, and its content must be acquired for individual application. Therefore, its independent disclosure or copying for any purpose is prohibited. Therefore, it is not possible to include it in its entirety in this project.

4.2.9. Psychiatric Assessment

The ADOS-2 (Autism Diagnostic Observation Schedule, Second Edition) observational assessment will be applied. This is a standardized assessment that involves direct observation of the child in different situations and social interactions. [45] The evaluated parameters are divided into 5 items according to the psychiatric criteria for ASD diagnoses by the Diagnostic and Statistical Manual of Mental Disorders (DSM): language and communication; reciprocal social interaction; play and imagination; stereotypical behaviors and restricted interests and, finally, atypical behaviors. The observation lasts 60 minutes and will be carried out by trained and qualified professionals supervised by a reference neuropsychologist. This assessment has the possibility of covering children according to their language level and chronological age. Modules 1 and 2 will be aimed at children with a language level under 48 months, adapting activities such as playing with soap bubbles, using a remote-control car and pretending to have a birthday party. While module 3 will be aimed at children who can use complex sentences, as they have questions about emotions and relationships, as well as retelling a story from a book; as demonstrated in Annex B.[45,49]
After applying the ADOS, the domains are coded on a scale that varies from 0 to 3 points, where 0 indicates that the child has a certain ability and 3 indicates abnormal or dysfunctional behavior. Higher scores indicate worse results in terms of the skills described. A standardized severity score based on codes within these domains can be calculated to compare autism symptoms between modules, according to the Standardized Manual. [50]

4.2.10. Psychopedagogical Assessment

The psychopedagogical assessment will be carried out using a questionnaire validated in literature in the version translated into Portuguese, called the Childhood Autism Rating Scale (CARS).[51] It consists of a scale with fifteen parameters on the symptoms of autism spectrum disorder in children, which include: interpersonal relationships, imitation, emotional response, body use, use of objects, response to changes, visual response, auditory response, response and use of taste, smell and touch, fear or nervousness, verbal communication, non-verbal communication, level of activity, level and consistency of intellectual response and finally a general parameter of that individual's autism. Because it is brief and complete, it will be answered by the teachers of each child participating in this study, in order to evaluate the possible improvement in the child's behaviors and skills also in the school context. Variations from 1 to 4, with number 1 corresponding to no difficulty in performing that skill, 2 being mild difficulty, 3 being moderate difficulty and 4 being severe difficulty. Thus, the global score varies from 15 to 60 points, and the interpretation is based on the severity of the autism; If you are

between 15 and 30 years old, you do not have autism; 30 to 36 mild to moderate autism and 36 to 60 correspond to severe autism.[52]

4.2.11. Biostatistics

Mean and SD (standard deviation) will be used to describe quantitative data, while risk factors, frequency and percentage will be used to describe qualitative data. Shapiro–Wilk test will confirm or not the normality of data distribution in the groups, therefore, parametric, or non-parametric tests, respectively, will be used to assess the research variables. To compare quantitative variables in the two or more groups, independent t-test (between groups) or paired samples t-test (within groups), ANOVA (between groups) or Repeated Measures (within groups), with post hoc testing to assess individual differences among the categories, will be used as parametric options. The relatives nonparametric options will be used otherwise and chi square test, if qualitative variables. All statistical calculations will be performed at the significance level of less than 0.05 ($P < 0.05$). Data will be analyzed using SPSS version 26.0.

5. JUSTIFICATION

Autism Spectrum Disorder (ASD) is a complex neurobiological disorder characterized by deficits in social communication, restricted and repetitive patterns of behavior, narrow interests, and atypical sensory sensitivities. Although the exact cause of ASD remains unknown, growing evidence suggests the contribution of genetic, environmental, and immunological factors in its development. [1,2,3,4,6,7,12,14,17,19]

An emerging area of research related to ASD focuses on the role of the intestinal microbiome and its interaction with the immune system and the brain. Studies by Finegold et al. (2002)[10], Kang et al. (2017)[40], Navarro et al. (2019)[4], and Adams et al. (2011)[39] have demonstrated alterations in the composition and diversity of the intestinal microbiota in children with ASD, as well as a higher prevalence of gastrointestinal disorders compared to neurotypical individuals. These alterations in the intestinal microbiome may be associated with chronic inflammation and immune dysfunction.

In this context, probiotics emerge as a potentially promising intervention for managing ASD symptoms. Probiotics are beneficial live microorganisms that, when administered in adequate quantities, can confer health benefits to the host. They have the potential to modulate the composition and function of the intestinal microbiota, reduce inflammation, and improve gastrointestinal health. [41,42,43]

Preclinical investigations, exemplified by Hsiao et al. (2013)[41] and initial clinical studies, such as Tomova et al. (2015)[42], have demonstrated that probiotic supplementation could potentially ameliorate symptoms associated with ASD, encompassing repetitive behaviors, social aptitude, communication, and alleviation of gastrointestinal issues. Nevertheless, it is imperative to emphasize the necessity for additional research endeavors aimed at thoroughly and rigorously assessing the effectiveness of probiotics as a therapeutic modality for children diagnosed with ASD.

Therefore, a study investigating the action of probiotics in improving symptoms manifested due to the disorder in children with ASD is justified. This study would contribute to understanding the underlying mechanisms of probiotic effects on ASD and provide robust scientific data to support probiotic-based therapeutic interventions, potentially positively impacting the quality of life of these children and their families.

6. PROPOSED SCHEDULE

The literature review and volunteer registration (pre-recruitment) commenced prior to the review of this project by the Research Ethics Committee (REC), owing to the imperative to review existing literature for document preparation and ensure the minimum sample size feasibility for the investor, thereby facilitating project execution.

Scheduled Activity	**Month (2024)**										
	Jan	Feb	March	April	May	Jun	Jul	Aug	Sept	Oct	Nov
Literature review	X	X	X	X	X	X	X	X			
Pre-recruitment of volunteers and posting of the project on the CEP		X	X								
Conducting neuropsychological evaluations (Vineland-3)				X	X		X				
Conducting psychiatric evaluations (ADOS)				X	X		X				
Conducting psychopedagogical evaluations (CARS)				X	X		X				
Blood collection and analysis of fecal material				X	X		X				
Questionnaire completion				X	X		X				
Data tabulation of demographic and anthropometric data							X	X			
Statistical analysis of the data								X	X		
Presentation of the data at scientific events										X	X
Preparation of the scientific article for publication in indexed journal										X	X

7. ESTIMATED COSTS

It is emphasized that the research will be sponsored by Gon-1 Project Management Ltd. - 12.410.656/0001-61, with financial support from an associated sponsor to be defined after the first round of investments from institutions that have already shown interest during the pre-recruitment phase of volunteers, which is carried out after approval by the Research Ethics Committee (CEP).

Expense Type	**Expense Amount (R$)**	**Public Funding (R$)**	**Private Funding (R$)**
Inflammatory Tests	56.487,13		56.487,13
Biochemical Tests	65.196,29		65.196,29
Clinical Assessments (DAY 0 / DAY 45 / DAY 90) and Acquisition of Vineland-3 Test	57.846,00		57.846,00
Clinical Assessments (T180/T365) and Acquisition of Vineland-3 Test	53.923,00		53.923,00
Researchers and Team	280.000,00		280.000,00

Probiotic K11-Tmax	260.000,00		260.000,00
Probiotic K11-T	151.890,00		151.890,00
Placebo	3.559,10		3.559,10
Technical Support for Volunteers	65.800,00		65.800,00
Research Management	360.000,00		360.000,00
Fees and Taxes	144.367,77		144.367,77
General Expenses	38.079,77		38.079,77
Publication	50.000,00		50.000,00
TOTAL	**R$ 1.587.148,88**		

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Annex A

Interview Instrument

Name: _____
Guardian's Name: _____
Gender: _____ Date of Birth: _____
Support Level: ( ) 1 ( ) 2 ( ) 3 Randomization Code: _____
Clinical Comorbidities: _____

Annex B
Autism Diagnostic Observation Schedule (ADOS)

MODULE 1 - 31 MONTHS - PRE-VERBAL AND SINGLE WORDS

1. Free play
2. Name response
3. Response to joint attention
4. Bubble play
5. Anticipation of a routine with objects
6. Response to social smile
7. Anticipation of a social routine
8. Functional and symbolic imitation
9. Birthday party
10. Snack time

Note: Joint attention onset requires spontaneous shifting of gaze between three points - object and person - for the sole purpose of sharing interest or pleasure (may or may not involve pointing gesture).

A three-point gaze shift requires the child to look at the object, then at the person (to catch their gaze and direct it to the object), and then back to the object, or the child looks at the person first, then at the object, and then back to the person.

PARENT GUIDANCE

- Observe how these parents interact with the child.
- Observe the child's reaction to parental stimuli.
- Communicate to parents that from activity 2 onwards (toy block), parental stimulation is not advised unless requested.
- Parental stimulation does alter the score.
- Be cautious with reinforcements, we need to observe whether the child genuinely possesses the skill, as well as the level of autonomy and what she can actually do independently.

1- FREE PLAY

Aspects to observe:

- Does the child attempt to engage the caregiver spontaneously? Requests help looks at parents or therapist.
- If yes, how does she do it?
- Does this involve any type of joint referencing of objects, such as giving and showing to them, or just seeking affection or help?
- How does she communicate if she does?
- Does the child direct her emotions towards others?
- How does she express them?
- Does she explore materials symbolically and functionally?
- Does she remain engaged in activities, quickly shifting from one object to another, or getting stuck in repetitive actions.

2- NAME RESPONSE

Aspects to observe:

- Observe and evaluate the consistency of the child's response to the hierarchy of stimuli.

- What sounds and actions do you or the family member or caregiver need to make to get the child's attention?
- How does the child respond?
- Does she establish eye contact?
- Does she look at your face or in your direction, or look at the family member or caregiver, or in your direction?
- Does she vocalize?

Stimulus Sequence:
1. Call the child by name.
2. Ask the family member or caregiver to call the child by name up to two times.
3. Ask the family member or caregiver to say other words or make a familiar noise or sound.
4. Ask the family member or caregiver to do whatever is necessary, including touching the child, to get her to look at the relative (gestural help).

3- RESPONSE TO JOINT ATTENTION

- Does the child follow a simple gaze shift or follow a gaze shift accompanied by a pointing gesture?
- Pay attention to the child's behaviors when playing with the remote-control toy, including eye contact, vocalizations, requests, shared pleasure, initiations of joint attention, and pretend play (e.g., hugging or kissing the teddy bear).

4- BUBBLE PLAY

Aspects to observe:
- Observe emotions, shared attention, if she points.
- Does the child display stereotypies, atypical motor movements, how is her behavior (participates, ignores, aggressive, irritating...)
- If she has hypersensitivity.

5- ANTICIPATION OF OBJECT ROUTINES

Aspects to observe:
- Observe emotions and the onset of joint attention by the child, shared pleasure, how the child makes requests, motor responses during the activity, especially those repetitive mannerisms, stereotypies.

6- RESPONSE TO SOCIAL SMILE

Aspects to observe:
- Evaluate the child's smile appearance in response to:
  (a) the smile of the family member or caregiver,
  (b) the smile of the family member or caregiver combined with the performance of a familiar sound or with you calling in a way that suggests physical contact, but without touching the child (e.g., "I'm going to get you"), Or being touched.

7- ANTICIPATION OF A SOCIAL ROUTINE

Aspects to observe:
- Evaluate the child's emotional reaction and attempt to initiate a routine repetition.
- Pay special attention to the social intent of the child's behaviors and the extent to which she integrates gaze, facial expression, vocalization, and gestures into actions directed toward the examiner or the family member or caregiver, especially those behaviors that are indicative of shared pleasure.

8 - JOINT ATTENTION RESPONSE

- Does the child follow a simple gaze shift or follow a gaze shift accompanied by a pointing gesture?
- Pay attention to the child's behaviors when she's playing with the remote-control toy, including eye contact, vocalizations, requests, shared pleasure, initiations of joint attention, and pretend play (e.g., hugging or kissing the teddy bear).

Guidance:

- Orient the child's body towards your face. Yes, it's possible.
  Up to FIVE attempts should be made to draw the child's attention to his (the examiner's) face.
- Proceed with the activity. If you fail to get the child's attention, move on to the stimulus hierarchy, including activating the toy.
- Say: "(child's name), look!" (with exaggerated eye gesture).
- Say or name the child and say "look at" (do this) 2 times *
- Use the toy.

9- FUNCTIONAL AND SYMBOLIC IMITATION

Aspects to observe:

- How does the child use miniature objects and substitute to imitate known actions?
- Are these acts performed with social awareness and shared pleasure?

10- BIRTHDAY PARTY

Aspects to observe:

- Evaluate the child's interest and ability to integrate into the "script" of the doll's birthday party.
- Does the child treat the doll as a representation of a living being?
- Does the child contribute spontaneously to the party representation?
- If not, does the child spontaneously imitate the examiner's actions or participate when asked to do so or when directed?
- Pay attention to shared pleasure, initiations, and reciprocity.

11- SNACK TIME

Aspects to observe:

- Does the child indicate a preference for a snack and ask for it?
- If yes, how does she do it?
- How does she use visual cues, gestures, facial expressions, and vocalizations to communicate a request to you and engage in social initiations?
- Does the child show her snack to her family member or caregiver or try to feed adults in the room or share food with them?

MODULE 2 - VERBAL EXPRESSION WITH PHRASES

1. Building task
2. Pretend play
3. Joint interaction game
4. Demonstration task
5. Describing an image
6. Telling a story from a book
7. Cartoons
8. Narration and conversation
9. Emotions

10. Social difficulties
11. Break
12. Friends and marriage
13. Loneliness
14. Creating a story

1- BUILDING TASK
Observation Focus: The observation focus is on determining if the child indicates the need for more pieces and how she does it (e.g., reaching over the examiner's arm, vocalizing, or making eye contact).
Communication sample recording:

2- PRETEND PLAY
Observation Focus: The observation focus is on determining to what extent the child produces sequences of actions involving the use of materials beyond their most obvious intention. Special attention should be given to how the child assigns an animated role to dolls and how she pretends that dolls interact with each other.

3- JOINT INTERACTION GAME
Observation Focus: The observation focus is on the reciprocity demonstrated by the child in interactive play. The goal is for the child (not the examiner) to develop interaction and demonstrate a new initiative that goes beyond a direct response to the examiner's proposals.

4- DEMONSTRATION TASK
Observation Focus: The objective of this task is to determine if and how the child represents familiar actions through gestures, especially using her body to represent an object (e.g., using a finger to represent a toothbrush) or mimicking the use of an imaginary object. Additionally, this task provides an opportunity to assess the narration of a familiar event.

5- DESCRIPTION OF AN IMAGE
Observation Focus: The objective of this task is to obtain an example of the child's spontaneous discourse and communication, as well as to understand what captures her interest.

6- TELLING A STORY FROM A BOOK
The objective of this task, like the previous one, is to obtain an example of the child's spontaneous discourse and communication and to understand what captures her interest. This task also provides an opportunity to assess the extent to which the child can provide a sense of continuity to a story.

7- CARTOONS
Observation Focus: The objectives of this task include (a) observing the use of gestures and their coordination with speech by the subject, (b) observing their response to humor, (c) recording a sample of language, (d) gaining an idea of their degree of insight and flexibility in adapting a narrative to the listener audience, and (e) noting any comments the subject may make about affect and relationships.

8- CONVERSATION NARRATION
Observation Focus: The observation focus is on the subject's ability to move from the examiner's sentences and fully engage in dialogue, particularly about a topic outside the immediate context. Special attention should be paid to how the subject recounts routine and non-routine events and describes relationships and emotions.

9- EMOTIONS

Observation Focus: It has a dual objective: (a) to identify which events or objects elicit different emotions in the participant, especially if they are social or not, and (b) to observe how the subject describes their emotions. Questions:

- What do you do to feel happy or content?
- What kind of things make you feel this way? How do you feel when you are happy? Can you describe (tell me about) it?
- What scares you?
- What makes you feel scared or anxious? How do you feel? What do you do?
- And when you're angry?
- What makes you feel this way? How do you feel "inside" when you're angry?
- Everyone feels sad sometimes. What makes you feel this way?
- How do you feel when you're sad? What happens when you're sad? Can you describe it?

10- SOCIAL DIFFICULTIES

Observation Focus: The observation focus is on the subject's perception of their social difficulties, their insight into the nature of these problems, and whether the subject has made any attempt to change their behavior to better adapt to others. Attention should be paid to the subject's understanding of the appropriateness and implications of their feelings. Questions:

- Have you ever had problems with people at school (or work)?
- Is there anything others do that irritates or annoys you? What?
- Have you ever been teased or threatened? Why do you think that happened?
- And do you do anything that annoys others?
- Have you ever tried to change those things? Have you ever done anything so others wouldn't bother you? Did it work?

• Break:

Observation Focus: (a) how the subject occupies themselves during free time, (b) how they react to the examiner's withdrawal of interaction, and (c) if and how the subject initiates and participates in an unstructured conversation or interaction with the examiner.

• Friends and Marriage:

Observation Focus: It focuses on how the subject perceives the concept of friendship and/or marriage and the nature of these relationships, as well as how they perceive their role in these relationships. Questions about marriage and long-term relationships also focus on the subject's understanding of why a person would want to be in a long-term relationship and their perception of their possible role in such a relationship. Questions:

- Do you have any friends? Can you tell me about them? (Note down their ages, names, occupation.)
- What do you like to do together? How did you meet? Do you spend a lot of time together?
- What does being a friend mean to you?
- What is the difference between a friend and someone you work with or go to school with?
- Do you have a boyfriend/girlfriend? What is their name? How old are they?
- When was the last time you were together?
- What are they like? What do you like to do together?
- How do you know they are your boyfriend/girlfriend?
- Have you ever thought about having a long-term relationship or getting married (when you're older)?
- Why do you think people get married when they get older?
- What do you think about marriage? What are the good parts? What do you think might be difficult?

• Loneliness
Observation Focus: These questions refer to the subject's perception of the concept of loneliness and how they feel about it. Questions:

- Have you ever felt lonely?
- Do you think other kids in your situation also feel lonely?
- Is there anything you do to feel better?

14- CREATING A STORY
Observation Focus: Creative use of objects by the subject to tell a new story or invent the transmission of a news bulletin or advertisement.

MODULE 3 - VERBAL FLUENCY

1. Building Task *
2. Tell a story from a book
3. Description of an image *
4. Conversation and reports
5. Current employment or school *
6. Social difficulties and inconveniences
7. Emotions
8. Demonstration task
9. Video
10. Break
11. Daily life *
12. Friends and marriage
13. Loneliness
14. Plans and aspirations
15. Invent a story

(* OPTIONAL ACTIVITIES)

1- BUILDING TASK (OPTIONAL)
Aspects to observe: The objective of this activity is to observe if the individual indicates the need for more pieces and, if so, how they do it (e.g., reaching over the examiner's arm to grab the pieces, do they vocalize or gesture, or make eye contact?)
Communication sample recording

2- TELL A STORY FROM A BOOK
Aspects to observe: The objective of this activity is to obtain a sample of the participant's language and spontaneous communication and get an idea of what interests them. This task also provides an opportunity to assess their response to conventional humor and their understanding of visual cues in the social context (e.g., what the characters are doing in the story and how they are feeling).

3- DESCRIPTION OF AN IMAGE (SEE PREVIOUS MODULES) - OPTIONAL ACTIVITY

4- DIALOGUE

Aspects to observe: The objective is to observe the extent to which the participant can build a conversation around the examiner's claims and take an active role in the conversation, especially about an issue without immediate context. Special attention should be paid to how the participant reports routine and non-routine events and how they describe relationships and emotions. This task also offers an opportunity to observe the characteristics of their communication including the use of gaze, facial expression, intonation, and gestures.

5- WORK OR SCHOOL

If the individual is in full-time school, has no job, and has never worked (including volunteering outside of school), skip the questions about work and move on to the questions about school.
Questions about school:

- Aspects to observe: The objective of the initial questions is to obtain general information about the individual's employment to be able to ask follow-up questions. It is especially interesting to know if the individual has had to leave previous jobs and what the reasons were. These questions also allow assessing if the participant has a realistic view of their future employment possibilities. In addition to expectations about work.

Questions about work:

- Do you have a job?
- If yes, how is it: What kind of work is it? How did you find it? Have you had other jobs before?
- If you haven't: What do you do during the day? Have you had a job before? Why did you leave your previous job, was it planned? Are you happy where you are now or would you eventually like to change to do something else? What else would you do? What should you do to find this type of work?

Questions about school:

- Did you attend school? Where?
- What courses are you taking?
- What grade are you in? How are you doing?
- If the participant is no longer in school and not working, ask: Until which grade did you reach in school? How was it? What do you plan to do next? What experience or training would you need to be able to do that?

6- SOCIAL DIFFICULTIES

Aspects to observe: The focus here is on the participant's perception of their social difficulties, their understanding regarding the nature of these problems, and if they have made any attempt to change their own behavior to better fit in with the people around them. You should pay attention to the degree of understanding the participant has about the implications of their feelings and whether they are appropriate or not. Interview questions:

- Have you ever had difficulty relating to people in school or at work?
- Are there things that others do that annoy or bother you? What are they?
- Have they ever teased or bullied you in any way?
- Why do you think they did that?
- And the things you do that bother others?
- Have you ever tried to change these things? Ever done anything so that others don't make fun of you? Did it work?

7- EMOTIONS

Observation Aspects: The aim of these questions is twofold: (a) to identify which events or objects elicit different emotions in the participant, especially whether they are of a social nature or not, and (b) to observe how they describe their emotions. Interview Questions:

- What do you enjoy doing that makes you feel happy and joyful?

- What kind of things make you feel this way? How do you feel when you're happy? Can you describe it?
- What things scare you?
- What makes you feel afraid or anxious? How do you feel?
- What do you do in those situations?
- And what about feeling angry (upset, irritated)?
- What kind of things make you feel this way? How do you feel "inside" when you're angry?
- Most people have moments when they feel sad.
- What kind of things make you feel this way?
- How do you feel when you're sad?

8- DEMONSTRATION TASK

Observation Aspects: The objective of this task is to ascertain if the participant can perform familiar actions (and if so, how) using gestures, especially if they use their body to represent an object (for example, using a finger as a toothbrush) or simulate the use of an imaginary object.

Additionally, the examiner should also consider to what extent the participant can (a) appropriately adjust the level of detail for the current context and (b) narrate an action that is familiar to them.

9 - VIDEO (OPTIONAL)

Observation Aspects: The objectives of this task include: (a) observing the use of gestures and their coordination with speech; (b) another opportunity to observe their response to humor; (c) an opportunity to gather another language sample; (d) forming an opinion on the participant's "insight" and flexibility in adapting a story to their audience; and (e) noting any comments they may make about affect and relationships.

10 - BREAK

Observation Aspects: There are several factors to observe: (a) how the participant spends their free time; (b) how they respond to the examiner's withdrawal from interaction; and (c) if they initiate and engage in an unstructured conversation or interaction with the examiner and how they do so.

11 - DAILY LIFE

Observation Aspects: The questions: (a) provide information about the individual's level of financial responsibility; (b) establish where they are living and how they came to be in this situation; and (c) indicate the extent to which the participant is realistic about their plans for independence and the complexities involved in each form of accommodation. These questions are optional; if sufficient information has already been obtained about the participant's "vision" and responsibility, these questions on generally positive topics (for example, saving money for something, favorite activities, etc.) are not necessary.

Money Questions:

- How about money? Do you handle money by yourself?
- Where does it come from? Who pays your bills?
- Have you ever saved money to buy something or do something special? What was it?

Housing Questions:

- Where are you living now?
- If you are living at home with your parents:
    - Have you ever lived away from your parents?
    - What would be different if you lived on your own?
    - Do you want more?
    - What would be difficult?

- If you live on your own:
    - How did you find the place where you are living now?
    - Can you tell me a bit about it?

Leisure Activities Questions:

- What do you like to do in your free time at home?
- How about going out?

12- FRIENDSHIP - MARRIAGE

Observation Aspects: This set of questions is not intended to determine if the participant has friends or not, but to understand how they comprehend the concepts of friendship and marriage, the nature of these relationships, and how they perceive their own role in them. Questions about marriage and long-term relationships are also intended to discover their understanding of why someone would want to be in such a relationship and their perception of their possible role in them. Interview Questions:

- Do you have friends? Can you tell me something about them? (Write down the ages of your friends. It may be helpful to ask you to specify their names if the participant speaks generally about who their friends are.)
- What do you like to do together? How did you get to know them? How often do you get together?
- What does being a friend mean to you?
- What is the difference between a friend and someone you simply know or go to school with?
- Do you have a boyfriend or girlfriend? What's their name? How old are they?
- When was the last time you saw each other?
- What are they like? What do they like to do together?
- How do you know they are your boyfriend or girlfriend?
- Have you ever thought about having a long-term relationship or getting married (when you are older)?
- Why do you think some people get married when they are older?
- What's good about getting married? What can be difficult about getting married?

Loneliness

Observation Aspects: These questions aim to discover if the participant understands the concept of loneliness and how they feel about it. Interview Questions:

- Do you sometimes feel lonely?
- Do you think other people (young people) in your same circumstances have felt lonely?
- Have you ever felt lonely?
- Is there anything you do to feel better?

Future

Observation Aspects: The question aims to provide a positive conclusion to the interview and gather information about what the individual anticipates for their future. Interview Questions:

- Do you have plans or dreams about things you would like to have or do or see happening to you in the future?
- Tell me something about them.

Telling a Story

Observation Aspects: This activity focuses on the creative use the participant makes of objects, telling an original story, or creating a news report or a commercial.

Annex C

CHILDHOOD AUTISM RATING SCALE

Date:
Child's Name:
School Name:
Teacher:

What level of difficulty does the child have in the following aspects?
Consider
1) No Difficulty
2) Mild difficulty
3) Moderate
4) Serious

DOMAIN	CLASSIFICATION						
INTERPERSONAL RELATIONSHIP	1	1,5	2	2,5	3	3,5	4
IMITATION	1	1,5	2	2,5	3	3,5	4
EMOTIONAL RESPONSE	1	1,5	2	2,5	3	3,5	4
BODY USE	1	1,5	2	2,5	3	3,5	4
USE OF OBJECTS	1	1,5	2	2,5	3	3,5	4
ADAPTATION TO CHANGES	1	1,5	2	2,5	3	3,5	4
VISUAL RESPONSE	1	1,5	2	2,5	3	3,5	4
AUDITORY RESPONSE	1	1,5	2	2,5	3	3,5	4
RESPONSE TO TASTE, SMELL AND TOUCH	1	1,5	2	2,5	3	3,5	4
FEAR OS NERVOUSNESS	1	1,5	2	2,5	3	3,5	4
VERBAL COMUNICATION	1	1,5	2	2,5	3	3,5	4
NON VERBAL COMUNICATION	1	1,5	2	2,5	3	3,5	4
ACTIVITY LEVEL	1	1,5	2	2,5	3	3,5	4
LEVEL OF COHERENCE OF THE INTELLECTUAL RESPONSE	1	1,5	2	2,5	3	3,5	4
GENERAL IMPRESSIONS ABOUT AUTISM	1	1,5	2	2,5	3	3,5	4
ESCORE TOTAL							