Research Proposal

I. Research Background

Research Significance of the Project

Autism spectrum disorder (ASD) is a pervasive developmental disorder that has become the leading cause of mental disability among children in China [1], characterized by early social dysfunction, repetitive behaviors, and impaired interests, often coexisting with epilepsy, depression, anxiety, attention deficit hyperactivity disorder, as well as sleep and self-injurious behaviors [2]. According to the Centers for Disease Control and Prevention in the United States, approximately 1 in 44 children is diagnosed with ASD, with boys being about 4 times more likely to be affected than girls. The onset of autism is closely linked to genetic factors and environmental factors during early development. Recent studies have found that changes in the gut microbiome are associated with the pathogenesis of ASD [3].

cavities are a chronic, progressive disease that causes destruction of hard tooth tissue under the influence of multiple factors, primarily bacteria. Dental plaque in the mouth is closely related to cavity disease, and the dental community has continuously focused on studying the bacterial composition and characteristics in plaque. cavities have three main traditional etiological hypotheses: the specific plaque hypothesis, the non-specific plaque hypothesis, and the ecological plaque hypothesis. The specific plaque hypothesis suggests that only certain specific bacterial species are associated with the disease, such as Streptococcus mutans and Streptococcus sobrinus. The non-specific plaque hypothesis indicates that cavities are the result of the collective action of plaque microorganisms. The ecological plaque hypothesis suggests that cavities are the result of an imbalance caused by changes in the resident microbial flora.

Saliva Immunoglobulin A（IgA）is the main protective antibody in oral mucosal immunity, working with the innate immune system to inhibit the adhesion of microorganisms to the mucosa and tooth surface, and promoting the elimination of cariogenic microorganisms such as Streptococcus mutans[4].IgA deficiency makes patients more susceptible to oral mucosal infections and cavities[5]. Compared to individuals without ASD, previous studies have observed poorer oral health conditions in the ASD cohort[6,7]. Given the changes in oral health conditions in ASD patients, as well as the correlation between oral microbiota dysbiosis and mucosal immunity, saliva IgA levels may change in ASD subjects.

Microorganisms that cause oral diseases may enter brain tissue through various pathways, directly damaging the central nervous system, affecting neuroimmune activity and inflammation, thereby influencing the occurrence of ASD. On one hand, the brain sends 5 cranial nerves that participate in the sensory and motor innervation of the oropharynx. These can serve as direct channels for oral bacteria to reach the central nervous system, or as transmitters of neural signals from the oral cavity to the brain. On the other hand, oral microorganisms can enter the bloodstream through damaged oral mucosa or tooth roots due to conditions like gingivitis or periodontitis, and then breach the blood-brain barrier to enter the brain. It has been reported that mice infected with Pseudomonas aeruginosa exhibit reduced blood-brain barrier integrity [8].

The indirect effects of oral microorganisms on the brain may be mediated by systemic inflammation and metabolism. ASD patients exhibit central nervous system inflammation in

their brains, and elevated levels of cytokines and chemokines in cerebrospinal fluid [9], which may be due to the pro-inflammatory activity of lipopolysaccharides abundant in Gram-negative bacteria and periodontal-related pathogenic bacteria, leading to synaptic dysfunction [10]. Hicks et al. [11] found that children with ASD exhibit upregulation of microbial RNA related to lysine degradation in their oral cavity. Lysine degradation produces glutamate, which is a key neurotransmitter involved in learning and memory. Therefore, the oral microorganisms of ASD children may be connected to the brain, suggesting the existence of a "microbiome-brain-mouth axis" -.

Analysis of the current situation domestically and internationally:

Currently ASD research on the imbalance of pediatric microecology primarily focuses on gut microbiota, with the "microbiota-gut-brain axis" being considered by many scholars to be related to ASD [12–14].Chaidez et al.[15]reported that46%–84%ofASD patients exhibit gastrointestinal symptoms, which are associated with the severity of their core symptoms and can exacerbate social withdrawal, stereotyped behaviors, and other behaviors in ASD children. Iglesias et al.[16]found that the abundance of Bacteroides, Parabacterium, Clostridium, Faecalibacterium, and Coprococcus genera in the gut microbiota of ASD patients is significantly higher, while the abundance of Faecalibacterium and Bifidobacterium genera is lower. Desbonnet et al.[17]experimentally demonstrated that when the fecal bacteria of neurobehaviorally normal mice are used to colonize autistic male mice raised in a sterile environment, many social avoidance and repetitive behavior defects, as well as a lack of interest in social novelty and social motivation, were reversed.

②The mouth is the only entrance to the gut. In the Human Microbiome Project, oral bacteria and fecal bacteria overlap in nearly half of the participants. Olsenet al.[18]found that Porphyromonas gingivalis, which enters the gut through swallowing, causes gut microbiota dysbiosis, further increases intestinal epithelial permeability, and induces immune activation, leading to systemic inflammation. Narengaowa et al.[19]believe that Porphyromonas gingivalis surviving in the gut and the gut microbiota or its metabolites may enter the bloodstream and ultimately reach the brain via the enteric nervous system. Hicks et al.[11]studied2-6year-oldASDchildren, non-autistic developmental delayed children, and normally developing children's saliva microbiota. Through metagenomic shotgun sequencing, they found5types of oral microbiota that could distinguish ASD children from normal children, and3types of microbiota that could distinguish ASD children from globally developmental delayed children, with an accuracy close to80%. Qiao et al.[6]compared the bacterial diversity between normally developing children and ASD children using high-throughput sequencing. They found that the abundance of pathogens such as Haemophilus and Streptococcus in the saliva and plaque of ASD children was higher, while Prevotella, Sebacibacterium, Actinomyces, Porphyromonas, and Fusobacterium were less abundant.

③Gonget al.[20] demonstrated that Streptococcus mutans can regulate the downregulation of polymeric immunoglobulin receptor (PIGR) in human salivary gland (HSG) cells. The impact of other bacteria with altered abundance on PIGR. Meanwhile, they also found that the saliva IgA levels in mice models of autism spectrum disorder (ASD) established by sodium valproate (VPA) were reduced and correlated with autistic-like behaviors in mice. Guo et al.[21] studied the blood IgA levels of 75 children with ASD and 75 normally developing children. They found that the blood IgA levels of children with ASD were significantly lower than those of the

control group and showed a significant negative correlation with the severity of ASD in children.

This project aims to address the scientific questions and the main research approach

Since oral health is influenced by multiple factors, previous studies have observed ASD patients exhibiting poorer oral health conditions. We propose the main scientific question of this project: What are the changes in the oral microbiome of autistic children? This study will collect saliva and plaque samples from both ASD children and healthy children, while also distributing oral hygiene questionnaires. Subsequently, we will utilize RNA-seq technology to analyze the distribution of oral microbiota and compare the dominant microbial species in ASD children to those in typically developing children. Using the Elisa method, we will measure saliva IgA levels to validate the host's immune response under changes in oral microbiota. Additionally, we will analyze the correlation between the severity of autism and the oral microbiome. This research aims to provide experimental evidence for the screening of cariogenic microorganisms, the assessment of immunoglobulin A levels, the prediction of the relationship between oral microbiota and brain development, and the establishment of active disease indicators for caries, saliva immunity, and autism.

II. Research Objectives

① To clarify the changes in ASD children's IgA levels and oral microbiota abundance across different severity groups;

② To determine the differences in ASD children's and healthy children's IgA levels and oral microbiota abundance between groups;

③ To determine the host immune response under oral microbial changes.

III. Research Design and Methods

[Research Type]

This study is a multicenter retrospective cohort study. The main center is the Affiliated Hospital of Zhejiang University School of Medicine(Department responsible: Department of Conservative Dentistry; Investigator: Tu Yan). Other participating centers include the Seventh People's Hospital of Hangzhou (Department responsible: Department of Child Psychology; Investigator: Zhou Guoling).

[Research Period]

Research Duration:3 years

Start Date: From the date of approval

End Date:2025 year9 month

【Research Methods】

An oral health status assessment questionnaire was released to patients with ASD aged 6-18 and their families to collect information on the oral hygiene habits, dietary habits, bad habits, and oral hygiene maintenance of children with ASD. Additionally, it investigated the degree of concern parents have regarding the oral hygiene of children with ASD and their level of knowledge about oral hygiene. Simultaneously, children with ASD aged 6-18 and their corresponding control normally developing children were recruited, and questionnaires were issued to their parents.

（1）Inclusion Criteria: This project is supported by Hangzhou Seventh People's Hospital

and the Stomatological Hospital Affiliated to Zhejiang University School of Medicine; all patients' ages must be clearly recorded6-18 years old, gender unrestricted; Participants or/ their families must understand the trial objectives, be willing to cooperate, and voluntarily or/ with their families' consent to participate in the trial and sign an informed consent form.

（2）Exclusion Criteria: Obvious brain structural abnormalities detected by MRI; Severe sensory organ damage (blindness, hearing loss); organic gastrointestinal problems;1month of antibiotic or immunosuppressive medication use; confirmed autism associated with genetics; obvious oral mucosal abnormalities; wearing various orthodontic appliances and accessories.

（3）Exit Criteria：The patient, after being informed, does not consent to the use of their information for research; Unexpected circumstances arise during the study, causing the patient to no longer meet the inclusion/exclusion criteria.

Children had not brushed or rinsed their mouths the night before and on the morning of sample collection; they needed to rinse gently with sterile distilled water half an hour before sampling. Saliva collection: Keep saliva in the mouth1min, gently spit into the collection funnel, and collect5mLwith a sterile tube3mLnon-irritating saliva sample, mark it, and immediately store in a dry ice bucket, then transfer to a-80℃ freezer for storage. Plaque collection: Isolate the sampling area from saliva with sterile gauze, use sterilized swabs to scrape supragingival plaque from the subject's11、46labialpalatal/buccal lingual supragingival plaque, place the sample in1mL Tris-EDTA(TE)buffer solution, mark it, and store in a dry ice bucket, then transfer to a-80℃ freezer for storage.

Under natural light, conduct an oral health survey using visual and tactile examination, employing a specialized examination record form to record simplified oral hygiene indices such as the OHI-S and DMFS/dmefs for oral hygiene status. OHI-S: Record the indices of soft plaque (DI-S) and calculus (CI-S) on the buccal side of 16, 26, 36, the lingual side of 46, the labial side of 11, and 31, as well as the soft plaque (DI-S) and calculus (CI-S). DI-S: 0 = no soft plaque on the tooth surface; 1 = soft plaque covering an area of less than 1/3 of the tooth surface; 2 = soft plaque covering an area of 1/3 to 2/3 of the tooth surface; 3 = soft plaque covering an area of more than 2/3 of the tooth surface. CI-S: 0 = No dental calculus on or below the gum; 1 = Dental calculus on the gum covers less than 1/3 of the tooth surface; 2 = Dental calculus on the gum covers between 1/3 and 2/3 of the tooth surface, or scattered subgingival dental calculus on the tooth neck; 3 = Dental calculus on the gum covers more than 2/3 of the tooth surface, or continuous and thick subgingival dental calculus on the tooth neck.

DMFS/dmefs： D refers to teeth in the oral cavity that exist due to caries that have not been repaired. M refers to teeth lost due to caries. Some statistics classify M into two categories, using Mi—teeth that need to be extracted due to severe caries; Me—teeth already lost due to caries. F represents teeth that have been filled due to caries (excluding fillings done for non-caries conditions).At age 9 or younger, if a child loses deciduous teeth that should not fall out, such as molars or cuspids, it is considered lost due to caries. For those over 9 years old, there is no longer a distinction between teeth lost due to caries or physiological shedding; they can be treated as unerupted teeth (corresponding permanent teeth).d: Carious teeth requiring treatment; f:Already treated carious teeth; e:Deciduous teeth that should be extracted due to caries; m:Teeth lost due to caries.

The Childhood Autism Rating Scale (CARS) is used by professionals to assess children with autism. This scale consists of 15 items, each scored on a seven-level scale from 1 to 4, with increments of 0.5 points. A score of 1 indicates normality, while 4 indicates the most severe level, and the total score is calculated by summing the individual item scores. For CARS total scores, ages 6-12 with ⩽29.5 points are considered normal, 30-36.5 points indicate mild-to-moderate autism, and ⩾37 points indicate severe autism; for ages 13 and older, ⩽27.5 points are normal, 28-34.5 points indicate mild-to-moderate autism, and ⩾35 points indicate severe autism.

DNA extraction and PacBio SMRT high-throughput sequencing: Refer to the instructions of the genomic DNA extraction kit to perform genomic DNA extraction on the samples. Bacterial16S universal primers27F (5-AGAGTTTGATCCTGGCTCAG-3′),1492R (5-GGT-TACCTTGTTACGACTT-3′) are used. To distinguish between different samples, each pair of primers is separately added with a16 base pair length barcode sequence, and PCR amplification is performed on the products, followed by purification, quantification, and normalization to form a sequencing library.

Sequencing and bioinformatics analysis: Quality control of the constructed libraries was performed, and qualified libraries were sequenced using the PacBio SMRT high-throughput sequencing technology. After quality control of the offline data, raw data were subjected to quality control and subsequent bioinformatics analysis using the Mothur andQIIME2 platforms. Qualified sequences were split into different samples based on barcode sequences, and barcode and primer sequences were removed for subsequent analysis. Bioinformatics analysis was performed on high-quality sequences, including: (1) clustering to obtain operational taxonomic units (Operational taxonomic units, OTU)) using the Usearch software at97% similarity; (2) calculating α diversity indices for each sample using the Mothur(v.1.30） software, including Shannon index, Simpson index,Chao1 index, and abundance-based coverage estimate (ACE） index, and plotting sample dilution curves and rank abundance curves; (3) β diversity analysis to assess differences in microbial community structure between the two groups, including principal coordinate analysis based on the binary jaccard matrix; (4) classifying feature sequences using a naive Bayes classifier combined with alignment based on the SILVA reference database to obtain taxonomic classification information for each feature, generating community composition statistics for each sample at the phylum, class, order, family, genus, and species levels using the QIIME software, and using the R language tool to plot community structure diagrams at each taxonomic level for each sample, comparing species differences between the two groups.

According to the manufacturer's instructions, use an enzyme-linked immunosorbent assay (ELISA) kit (Bayerin, Wuhan, China) to measure the IgA levels in human saliva samples.

Data processing: First, determine normality through the Shapiro-Wilk test and check for homogeneity of variances using the Brown-Forsythe test. If the two continuous variables are normally distributed, use the Student t test; otherwise, use the Mann-Whitney U test. If the three continuous variables are normally distributed and meet the homogeneity of variance conditions, use one-way analysis of variance (ANOVA); otherwise, use the Kruskal-Wallis test. Use the chi-square test to analyze categorical data. Use the Student t test (for age) and the chi-square test (for gender) to compare the demographic information of ASD children and normal control children. Use the Student t test to analyze human saliva IgA content and

evaluate the diagnostic performance of human saliva IgA through receiver operating characteristic (ROC) analysis.

IV Sample size

This study is a multicenter retrospective cohort study, with sample size determined primarily based on saliva IgA content as the main observation index. According to the average concentration of the control group (161.61 ± 193.37 μg/mL) and the average IgA concentration in saliva of children with ASD (116.55 ± 90.97 μg/mL) [22], with a significance level of bilateral 0.05, power of 90%, the formula was used for calculation. Considering the dropout rate, approximately 50 participants (total of 150 people) are needed in each group to detect statistically significant differences between the groups.

V: Data Management and Confidentiality

The original data involved in the study were obtained from the database of the Stomatological Hospital Affiliated to Zhejiang University School of Medicine and the database of the Seventh People's Hospital of Hangzhou. The data were entered and stored only by the research personnel. Missing data, unused data, and illogical data will be excluded. All eligible participants will be included in the participant dataset for statistical analysis. All records related to participant identities will be kept confidential, and the data will not be disclosed outside the scope permitted by relevant laws and regulations.

VI: Informed Consent

This study will be conducted by Tu Yan team to obtain informed consent from participants through signature before the evaluation. The informed consent process adheres to the principles of full disclosure, full understanding, and autonomous choice; the wording of the informed consent is easy to understand and aligns with the comprehension level of the participant group. This study involves vulnerable populations including children, children with autism spectrum disorder. Before the study begins, child psychiatry doctors assess whether children with ASD children have self-awareness based on self-perception ability evaluation and provide conclusions and signatures; other relevant clinical department physicians (such as neurology, neurosurgery, critical care neurology, etc.) evaluate the consciousness status of participants with other disorders of consciousness ;if the ASD child has self-awareness, the participant signs and dates it; if they have partial self-awareness, both the participant and the legal guardian sign and date it; if they do not have self-awareness, the legal guardian of the participant signs and dates it

Before the routine oral examination and collection of saliva and plaque must inform the patient and their family of relevant precautions, potential risks, etc., and sign an informed consent form, informing that clinical examination data may be used for non-commercial medical research, and the content of the informed consent form includes permission for the non-commercial use of clinical data in scientific research, sign the informed consent form, complete the informed consent. When obtaining informed consent, follow the principles of full disclosure, full understanding, and autonomous choice, ensuring that the personal information and medical information of the data source are not disclosed, and do everything possible within the law to protect the personal medical records and privacy of the data source.

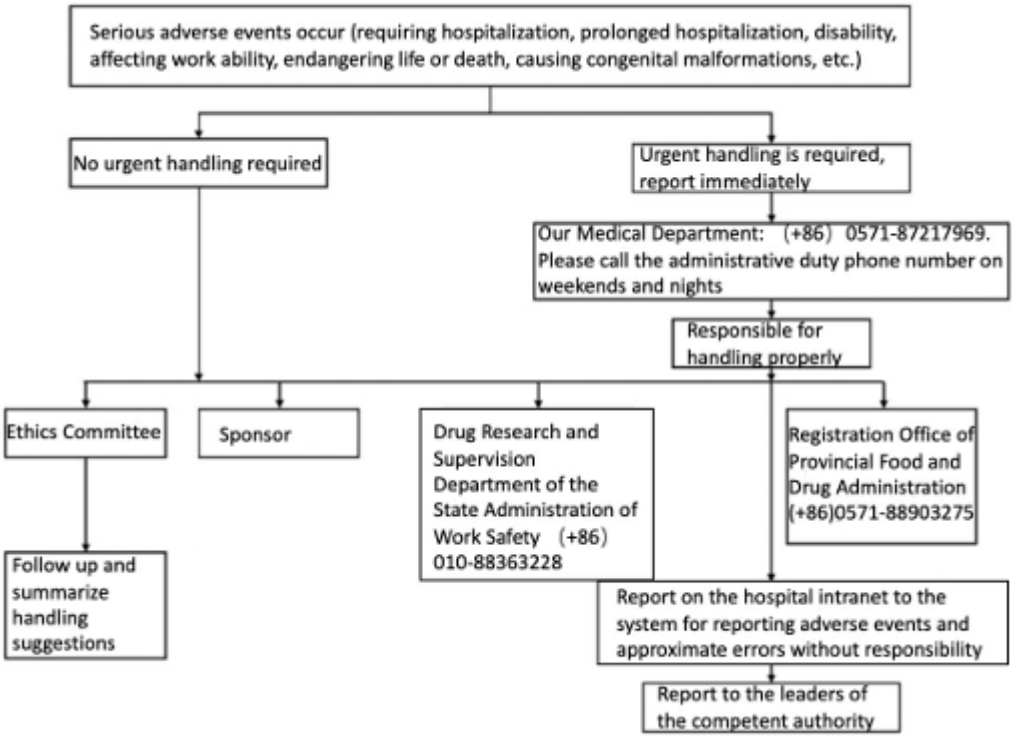
VII, Adverse event reporting

All patients participating in this study need to undergo routine oral examinations and collection of saliva and plaque‹ which is one of the contents for understanding the specific condition before treatment，not an invasive treatment，and almost no risk。Due to poor cooperation from children and patients with autism spectrum disorders，in clinical procedures and follow-upshouldstrictly adhere to the diagnosis and treatment specifications，standardized operations。

If an adverse event occurs，we will report it according to the following procedures：

Various adverse events：Take timely measures to handle them，record them in the case report form.

Severe adverse events（SAE）：Take timely measures to handle them，record them in the case report form，decide to discontinue or reduce medication by the investigator，immediately report to the ethics committee、drug clinical trial institutions and sponsor，24 hours to report to the national and provincial food and drug administration。



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