



## **STUDY PROTOCOL**

**Official Title:**

### **Human Milk Oligosaccharides in Breast Milk and Its Relation to Gut Bifidobacterium, vitamin D and Immune Modulation in Infants**

**NCT Number : (not yet assigned)**

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## **Summary:**

An observational study aims to analyse maternal Human Milk Oligosaccharides (HMO) secretor status and Breast Milk (BM) Bifidobacterium and their correlation to gut Bifidobacterium, serum vitamin D and immune regulatory status (sCD14, TGF beta, IgA, CD4 T cells, and CD8 T cells) in infants. This study will be done on 100 subjects (each mothers and infants), who fulfil the eligibility (inclusion and exclusion) criterias. The samples include breast milk from mother, faecal and blood samples from infants, would be collected from the subjects and will be processed and analysed in the laboratory.

## **Introduction:**

Human Milk oligosaccharides (HMOs) are oligosaccharides derived from lactose and are the third major component of breast milk(BM).<sup>1,2</sup> To date, more than 200 individual HMO structures have been isolated and identified. This diversity depends on genetic (maternal secretory status) and non-genetic factors (duration of lactation, gestational age, geographic location).<sup>2</sup> HMOs are thought to play an important role in infant growth and development. Recent studies show that HMOs stimulate the growth of beneficial bacteria (such as Bifidobacteria) in the infant's intestine by providing a carbon source, supporting an optimal gut microbiome, and promoting immune development.<sup>2</sup> In parallel, the effects of vitamin D on health is mediated in part by the microbiome including bifidobacterium. On the other hand, some studies show that vitamin D supplementation induces the changes in the composition of gut microbiome.<sup>3</sup> However, there are less evidences of the studies which are focused on the relationship among HMO status of the mother, vitamin D and the abundances of gut microbiome especially Bifidobacterium in infants.

The purpose of this study is to analyse maternal HMO secretor status and BM Bifidobacterium and their relation to vitamin D and gut Bifidobacterium in infants. Additionally, we will also analyse the relationship between maternal HMO secretor status and infant immune regulatory status.

## **Objectives of Trial:**

### **1. Primary Objective**

To analyse maternal Human Milk Oligosaccharides (HMO) level, secretor status and Breast Milk (BM) Bifidobacterium and their correlation to gut Bifidobacterium, serum vitamin D and immune regulatory status in infants.

### **2. Secondary Objective:**

To analyze the correlation between :

- a. HMO and infant vitamin D levels.
- b. HMO and infant gut Bifidobacterium.
- c. HMO and immune regulatory markers in breast milk, including sCD14, TGF-beta, and IgA.
- d. Breast milk Bifidobacterium and infant vitamin D levels.
- e. Breast milk Bifidobacterium and infant gut Bifidobacterium.



- f. Breast milk Bifidobacterium and immune regulatory markers in breast milk, including sCD14, TGF-beta, and IgA.
- g. Infant vitamin D levels and infant gut Bifidobacterium.
- h. Infant vitamin D levels and immune regulatory markers in breast milk, including sCD14, TGF-beta, and IgA.

### Hypothesis:

**H0** : There is no significant correlation between maternal Human Milk Oligosaccharide level and secretor status, breast milk *Bifidobacterium*, infant gut *Bifidobacterium*, infant serum vitamin D levels, and immune regulatory status in infants.

**H1** : There is a significant correlation between maternal Human Milk Oligosaccharide level secretor status, breast milk *Bifidobacterium*, infant gut *Bifidobacterium*, infant serum vitamin D levels, and immune regulatory status in infants.

### Trial Design:

Observational study (non-randomized cross-sectional study)

### Study Population:

#### Number of samples:

100 subjects (each of mother and infant samples)

#### Eligibility Criteria

##### *Inclusion criteria:*

##### **a. Mother Criteria :**

- Healthy woman at least 18 years of age (at the time of subject recruitment and sampling procedures);
- Having a healthy 1 month-old infant with full term gestational age (37 – 42 weeks) and normal birth weight (2500 – 4000 grams);
- Exclusively breastfeeding;
- Agree to participate in the study by signing the informed consent

##### **b. Infant Criteria :**

- All infants from mother who fulfil the inclusion criterias

##### *Exclusion Criteria:*

##### **a. Mother Criteria :**

- Currently participating in another trial;
- Not from an Indonesian ethnicity
- Presenting conditions such as mammary abscess or any other mammary pathology
- Currently taking medications for conditions that may affect breast milk such as antibiotic



- Subjects not able to comply the study protocol

**b. Infant Criteria :**

- Currently participating in another trial
- Not from an Indonesian ethnicity
- History of antimicroba medications (oral or parenteral antibiotic)
- Subjects not able to comply the study protocol

**Material and Method Description:**

This study is a non-randomized cross-sectional study. Subject recruitment was conducted during immunization activities at community health centers (Puskesmas) and integrated health posts (Posyandu) in Kota Makassar, South Sulawesi, Indonesia. The subject collection technique used the quota sampling method, a non-probability subject selection technique carried out by determining the desired sample size (quota) from a population with predetermined criteria, and researchers collected sample units until the target quota was reached.

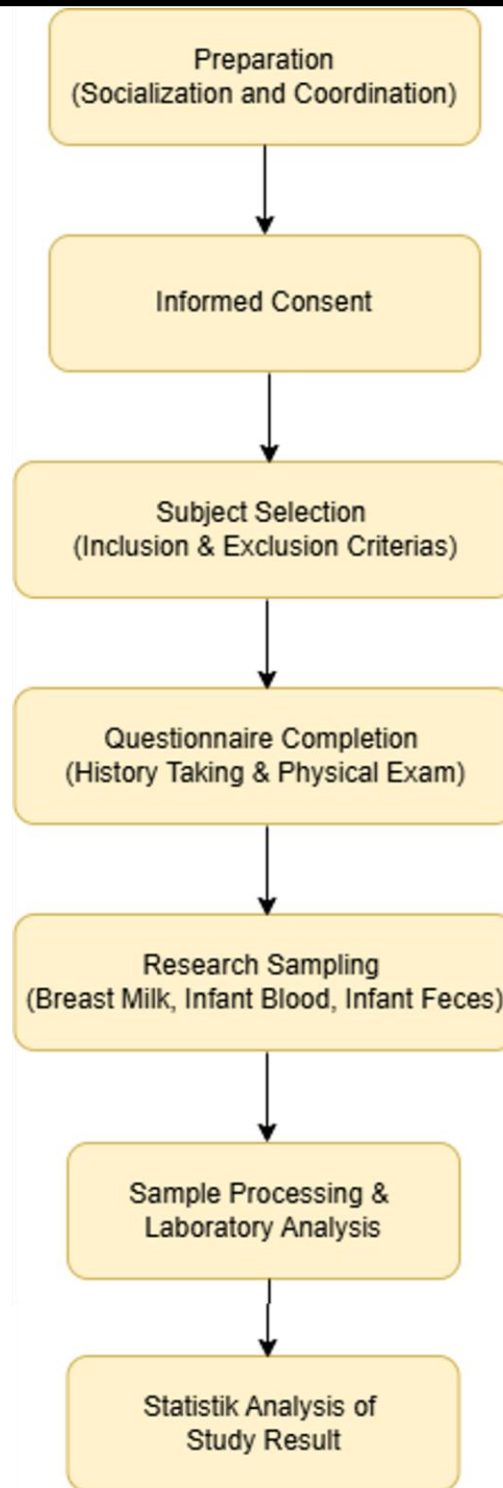
Mature breast milk samples will be analyzed to determine maternal secretion status, HMO status, Bifidobacterium in breast milk, and the status of immune markers such as sCD14, TGF-beta, and IgA in breast milk. Stool samples will be analyzed to determine the infant's intestinal Bifidobacterium. Blood samples will also be analyzed to determine vitamin D levels.

**Conduct of Trial:**

A pilot study was conducted to test the research protocol. Once the research protocol was confirmed, subject recruitment began. Sampling is preceded by providing an explanation by researchers of the participants' questionnaire and obtained informed consent from participants regarding their willingness to participate in the study. Participants who met the eligibility criteria (inclusion and exclusion criteria) were declared as research subjects. Next, a research questionnaire was completed, including recording the subject's identity (mother and baby), the mother's health and nutritional history, childbirth and breastfeeding history, observation of the baby's stool, history and risk factors for allergies, and a general physical examination.

**Outcomes:**

Practically, the results of this study will give us the new insight of the relationship among HMO status of the mother, the BM and gut Bifidobacterium abundances and their impacts on immune development in infants. Besides, the results will give the direction for the future researches on the better formula milk containing HMO and Probiotics design for infants.



*Figure 1. Research Flow*



### Statistical Aspects and Considerations:

A statistical analysis will be performed using statistic program (SPSS/JASP). The Normality of data distribution will be tested using the Shapiro–Wilk test. Statistical correlations between these parameters will be performed to determine the research objectives. The collected data is grouped based on the purpose and type of data, then the appropriate statistical method is selected, namely:

1. *Univariate analysis*

Used to describe basic data characteristics, in the form of frequency, mean value, standard deviation, and range.

2. *Bivariate analysis*

a) Comparison Test → To see differences between groups.

- Chi-Square Test → If the variable is categorical (e.g., the relationship between maternal secretory status (Secretory vs. Non-Secretory) and delivery method (Vaginal vs. Cesarean section). An alternative test, the Fisher's Exact test, is performed if the basic assumptions of the Chi-Square are not met.
- T-Test (Independent T-Test) → If you want to compare the means of two groups (e.g., the difference in vitamin D levels between male and female infants). An alternative test, the Mann-Whitney test, is used if the data is not normally distributed.
- ANOVA Test → If you want to compare the means of more than two groups (e.g., the difference in vitamin D levels based on the timing of early breastfeeding initiation: <1 hour, 1-6 hours, >6 hours). An alternative test, the Kruskal-Wallis test, is used if the data is not normally distributed.

b) Correlation Analysis → To see the relationship between two numeric variables)

- Pearson Correlation → If the data is normally distributed.
- Spearman Correlation → If the data is not normally distributed or is ordinal

A p-value <0.05 will be considered significant.

### Ethical Aspects:

The trial followed the research protocol and must be ethically reviewed and approved by the Ethics Committee of each participating institution.



**References:**

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4. Sjödin KS, Vidman L, Rydén P, et al. Emerging evidence of the role of gut microbiota in the development of allergic diseases. *Cur Opin Allergy Clin Immunol* 2016;16:390-395.