LMU KLINIKUM

Dr. von Haunersches Kinderspital

Minimally Processed Cow's Milk as Prevention for Asthma, Allergies and Respiratory Infections in Infancy and Childhood - MARTHA -

Statistical Analysis Plan for an Interim Analysis on Intestinal Microbiota - Interim Analysis 1 -

Version 1.0 of 2021-10-01

Investigational medicinal product:	Minimally processed full cream cow's milk
Comparator	UHT-treated semi-skimmed cow's milk
Indication:	Prevention of asthma, allergies, and respiratory infections
Sponsor:	Investigator initiated trial of LMU Klinikum
Financial support:	Dutch Longfonds
Protocol registry identification:	DRKS00014781
Development phase:	Feasibility study, interim analysis

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1. BACKGROUND

1.1 TRIAL OBJECTIVE

Primary Objective: To demonstrate the superiority of minimally processed, microbiologically safe full cream cow's milk over ultra-heat treated (UHT) semi-skimmed milk for the prevention of atopic sensitization

Secondary Objective: To demonstrate the superiority of minimally processed, microbiologically safe cow's milk over ultra-heat treated (UHT) semi-skimmed milk for the prevention of childhood-onset asthma, respiratory tract infections, otitis media, and low-grade inflammation

1.2 TRIAL DESIGN

The design is a national, 2-center, randomized, double-blind, placebo-controlled, two-arm parallel-group prevention trial with a nutritional intervention.

The recruitment phase lasts until age 6 months. Infants are checked for in- and exclusion criteria and randomized during Visit 1. The duration of the intervention period is 2.5 years, during which Visit 2 will take place and which ends with Visit 3, the final visit. The secondary outcome asthma diagnosis is determined after a subsequent follow-up period of 2 years.

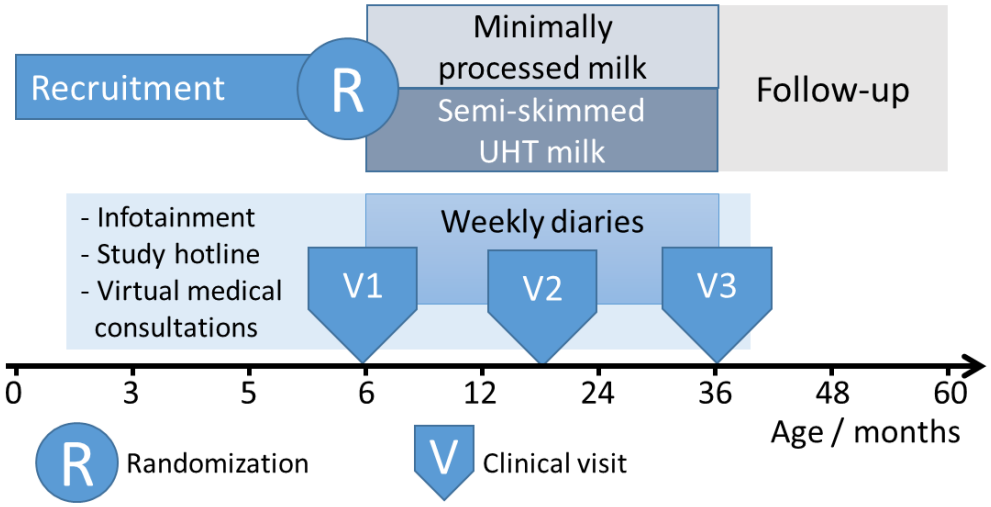


Figure 1: Study design of the MARTHA trial

1.3 INTERIM ANALYSIS

A grouped interim analysis was originally scheduled after one year from the start of the intervention (FPFV), where about a fourth of the study population was expected to have reached their first birthday. This interim analysis was aimed at comparing the effect size of the intervention on infections with the effect size assumed based on previous observations (Loss et al 2015). However, the prolonged and recurrent lockdown measures to fight the COVID-19 pandemic led to delay in recruitment and a substantial decrease in risk of respiratory tract infections, which rendered the scheduled interim analysis inutile.

Therefore, we intend to perform a modified interim analysis assessing the richness and the maturation status of the intestinal microbiome between the intervention and comparator group.

1.3.1 PRELIMINARY DATA

The rational for this approach is the observation that consumption of milk obtained directly from a farm (farm milk) leads to a higher richness (Table 1) and Shannon diversity of the gut microbiome in the PASTURE birth cohort as compared to shop milk consumption (Pechlivanis, von Mutius, Ege, 2021, unpublished data). Furthermore, we have previously shown that farm milk consumption influences the gut microbiome towards an asthma-protective composition, as illustrated for the first dimension of a principle components analysis of a maturation score and three scores reflecting the bacterial capability of producing short-chain fatty acids including butyrate (Depner et al, Nat Med 2020).

Table 1: Effect of farm milk consumption on bacterial richness in the PASTURE birth cohort

Model	N*	Estimate
richness in farm milk drinkers (mean ± SD)	140	47.5 ± 12.4
richness in shop milk drinkers (mean ± SD)	92	42.6 ± 10.4
difference in richness (±weighted average of SD)	232	4.9 ± 11.6
Welch Two Sample t-test (p-value)	232	0.0014
linear regression (β [95%-CI], p-value)**	232	4.65 [0.93 – 8.34], p=0.0154

* From the analysis set of Depner et al, Nat Med 2021, only children with at least 2 months of farm or shop milk consumption were included in the current analysis.

** Adjustment for center, farming, exposure to cowsheds, breastfeeding, diversity of food introduced in the first years did not change the association to a relevant extent.

2. ANALYSIS SETS

2.1 INTERIM ANALYSIS SET

The interim analysis set includes all subjects who, by 2021-10-01, have provided fecal samples after at least 2 months of intervention. The latter condition may exclude individuals with a Clinical Visit 1 performed after 10 months of age. An intervention period shorter than 2 months is anticipated to exert no substantial effect on the interim outcome microbial richness as suggested by an adverse effect of prolonged breastfeeding and possibly delayed introduction of cow's milk (Depner et al, Nat Med 2020)

2.2 INTERIM SAFETY SET

The interim safety set includes all individuals randomized until 2021-10-01 including those who prematurely terminated the study.

3. TRIAL CENTERS

The interim analysis will only involve Clinical Center Munich, which all enrolled individuals are currently assigned to.

4. UNBLINDING

To maintain blinding, all analyses will be performed on a trusted server (von Bomhard et al, 2018) run by the Data Trustee of the Medical Faculty (DMF). Blinded statisticians will prepare the data sets and write the R code to be run on the trusted server.

5. ANALYSIS VARIABLES

5.1 DEMOGRAPHY AND BASELINE CHARACTERISTICS

The study population will be described with respect to demography, diversity, and self-selection and will include variables such as sex, age at randomization, parental age, history of atopy (asthma, hay fever, atopic eczema, and atopic sensitization), educational level, and country of birth.

5.2 ALLOCATION TO STUDY ARMS

Allocation to study arms is defined by randomization and coded by 8 different letters, half of them representing intervention and comparator group, respectively. Analyses including information on allocation to study arms will only be run on the trusted server after sealing (von Bomhard et al, 2018).

5.3 PRIMARY VARIABLE

The primary variable will be microbial richness at the first time point following 2 months of intervention as defined by number of individual amplicon sequence variants (ASVs) after rarefication as detailed below.

5.4 SECONDARY VARIABLES

As secondary variables, we will use Shannon diversity and bacterial composition as represented by relative abundance of ASVs. Moreover we will use the maturation and short chain fatty acid scores as introduced by Depner et al, NatMed 2020.

5.4.1 EFFICACY

Efficacy will not be assessed during this interim analysis.

5.4.2 SAFETY

Safety outcomes will be assessed descriptively and include confirmed adverse events according to the following definitions:

Adverse events:

- milk allergy demonstrated by specific IgE against milk allerges ≥ 0.7 kU/L and a positive provocation test

- milk intolerance as demonstrated by improvement of symptoms during discontinuation of milk consumption over 14 days followed by a rebound effect

- lactose intolerance as clinically suspected and supported by molecular testing

Serious adverse events include failure to thrive as documented by persistent drop in weight percentiles, unscheduled hospitalization for more than 3 days, COVID-19 infection with hospitalization, and death of any reason.

6. HANDLING OF MISSING VALUES AND OUTLIERS

6.1 MISSING VALUES

The percentage of missing values will be described per variable. Bacterial genera with missing values will be considered censored data due to insufficient sequencing depth. Zero values in relative abundance measures for bacterial genera will be replaced by a pseudocount of 0.5 for subsequent logarithmic transformation. All other variables will not be imputed.

6.2 OUTLIERS

Samples with less than 5% reads of the average number of reads will be considered technical failures and removed from the analysis set.

7. STATISTICAL ANALYSES / METHODS

7.1 INCLUDED INDIVIDUALS AND INTERVENTION TIME

Absolute numbers will be given for individuals randomized and those who terminated the study prematurely. The numbers will also be broken down to study arms. Number of weeks in total and per study arm will be given for time under intervention and, more specifically, weeks with at least 4 days of intake of interventional milk products.

7.2 DEMOGRAPHY AND BASELINE CHARACTERISTICS

Demography and baseline characteristics will be summarized by frequencies and percentages in total and stratified by study arms.

7.3 ADHERENCE AND EXPOSURE TO TREATMENT

Adherence to electronic diaries will be quantified as proportion of weekly diaries completed of those sent out, exposure to milk intake will be quantified as proportion of weeks with at least 4 days of consumption of those reported and with milk available. Both measures will be broken down to study arms.

7.4 PRIMARY ANALYSIS

Reads per sample will be rarefied at the minimum sequence numbers of all biosamples. Rarefaction and calculation of species richness will be iterated 1,000 times, and the resulting richness values will be averaged. Richness will be compared between study arms by a t-test. If the effect of the minimally processed milk is similar to that of farm milk in the PASTURE study (Table 1), it can be assessed with reasonable power in 140 children receiving the intervention or the comparator milk for at least 2 months (Table 2).

Table 2: Sample size calculation for a higher richness in the intervention group

Parameter	Estimate
Significance level	0.05
Power	0.8
Sides of test	1 (superiority)
difference in richness	4.9
weighted average of SD	11.6
Required sample size per group	70
Overall sample size required	140

7.5 SECONDARY ANALYSES

7.5.1 MICROBIAL DIVERSITY

Calculation of Shannon diversity index in rarefied samples will be iterated 1,000 times, and the resulting index will be averaged. Shannon diversity will be compared between study arms by a Mann-Whitney-Wilcoxon test.

7.5.2 COMPOSITION OF THE GUT MICROBIOME

Microbial composition will be assessed on a genus level. Rare taxa will be defined as having a mean relative abundance below 0.5% in the analysis population and will be considered an individual category termed 'rare'. The maturation and short chain fatty acid scores will be predicted from the prediction models established in the PASTURE study (Depner et al, Nat Med 2020).

7.5.3 RESPIRATORY TRACT INFECTIONS

Since respiratory tract infections substantially decreased during hygiene measures and lockdown, these secondary outcomes will currently not be assessed.

7.5.4 SAFETY / ADVERSE EVENTS

Safety / adverse events will be assessed descriptively.

7.6 PLANNED SUBGROUP ANALYSES

This interim analysis does not involve subgroup analyses.

7.7 INTERIM ANALYSES

This document describes the first interim analysis and does not include further analyses.

8. DEVIATIONS FROM THE PROTOCOL

Deviations from the protocol will be assessed descriptively. The following situations are considered major deviations from the protocol: non-compliance with investigational milk product, frequent consumption of raw milk or raw milk products, duration of the intervention of less than 1 year. Tolerable deviations from inclusion criteria are slightly premature infants and randomization before a positive result for sIgE against milk was received.

9. INTERPRETATION OF RESULTS

The results will be interpreted with respect to effect size and may lead to adjustment of the sample size calculation. Furthermore, the results from the feasibility phase may inform the choice of adequate surrogate outcomes in case respiratory tract infections cannot be evaluated in the future.

10. DATA PROBLEMS

We will list major changes in eCRF forms with impact on data quality. This listing will be complemented by a list of measures undertaken to limit the impact of the changes.

11. SOFTWARE

Statistical analysis will be performed with R version 4.x.x (https://www.r-project.org/), particularly with the R package *phyloseq*. Relative abundance values will be transformed by centered log-transformation using the R package *composition*.

12. REFERNCES

Depner M, Taft DH, Kirjavainen PV, Kalanetra KM, Karvonen AM, Peschel S, et al. Maturation of the gut microbiome during the first year of life contributes to the protective farm effect on childhood asthma. Nature medicine. 2020;26(11):1766-75.

von Bomhard N, Ahlborn B, Mason C, Mansmann U (2018) The Trusted Server: A secure computational environment for privacy compliant evaluations on plain personal data. PLoS ONE 13(9): e0202752. https://doi.org/10.1371/journal.pone.0202752

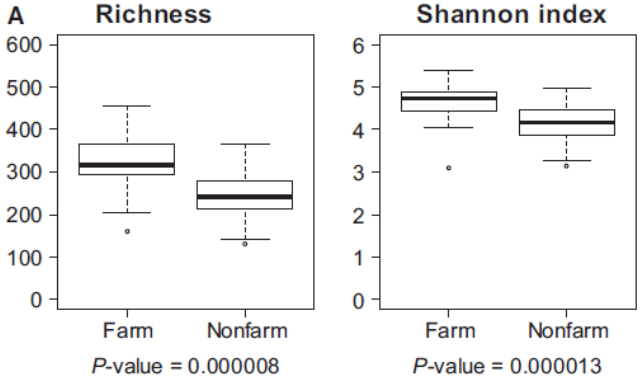
13. APPENDICES

13.1 PLANNED TABLES SAMPLE

	All	included in the analysis population		
		no	**yes**	
	N=	N=	**N=**	
	%	%	**%**	p-value
Male gender				
Cesarean section				
Birthweight > 3500g				
Gestational age > 40 weeks				
Maternal asthma				
Parental asthma				
Parental atopy				
Any breastfeeding at month 2				
Any breastfeeding beyond 3 months				
Consumption of intervention milk more than 50% of observation time				

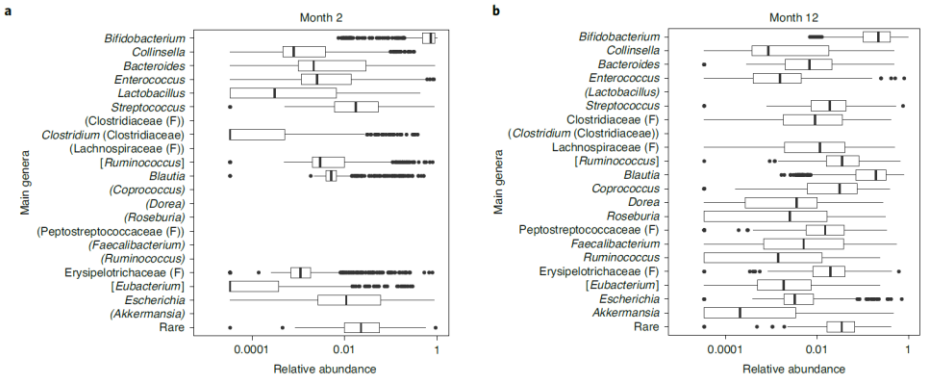
13.2 PLANNED GRAPHICS SAMPLE

13.2.1 DISTRIBUTION OF RICHNESS BETWEEN INTERVENTION AND COMPARATOR GROUP



Example taken from Birzele et al. Allergy 2016

13.2.2 DISTRIBUTION OF RELATIVE ABUNDANCE AT GIVEN TIME POINTS:



Example taken from Depner et al. Nat Med 2020

13.3 PROGRAM CODE

The program code will be published separately.