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DELIVERABLE

D2.7 - Clinical Protocols for Clinical Studies in NUTRISHIELD

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Deliverable Leader:	HAROKOPIO UNIVERSITY (HUA)
Contributors:	OSPEDALE SAN RAFFAELE SRL (OSR) STICHTING KATHOLIEKE UNIVERSITEIT (RU) FUNDACION PARA LA INVESTIGACION DEL HOSPITAL UNIVERSITARIO LA FE DE LA CONUNIDAD VALENCIANA (HULAFE) WP3 Leader
Reviewers:	ALPES, INTRA
Approved by:	ALPES

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Executive Summary

Deliverable 2.7 includes a preliminary design of the clinical validation scenarios to be used for the validation of NUTRISHIELD, describing the process of clinical trials. The goal of this deliverable is to describe the general frame and provide specific details on the clinical studies in order to serve as a reference for the future NUTRISHIELD developments. Deliverable 2.7 provides the report on the clinical trial protocols, developed to study the efficacy of NUTRISHIELD technology. In specific, D2.7 provides a thorough and analytical description, to be ideally followed, of the materials and methods, procedures and expected outcomes of the 3 studies that will be conducted within the NUTRISHIELD framework. These studies are a core component of the NUTRISHIELD project, since the studies will validate knowledge produced under NUTRISHIELD, and their findings will be used to feed the NUTRISHIELD platform.

Table of Contents

Table of Figures

List of Tables

Definitions, Acronyms and Abbreviations

Acronym	Title
BMI	Body Mass Index
CEN	Complete Enteral Nutrition
CONSORT	Consolidated Standards for Reporting Clinical Trials
DHM	Donor Human Milk
ERDF	European Regional Development Fund
FFQ	Food Frequency Questionnaire
FT-OMM	Full term infants receiving OMM
HM	Human Milk
HULAFE	University and Polytechnic Hospital La Fe
IPAQ	International Physical Activity Questionnaire
KIDMED	Mediterranean Diet Quality Index
MedDietScore	Mediterranean Diet Score
OMM	Own Mother's Milk
PT-DHM	Pre-term infants fed with pasteurized DHM
PT-OMM	Pre-term infants fed with OMM
RBW	Recover of birth weight
T2DM	Type 2 Diabetes Mellitus
WP	Work Package
YAP	Youth Activity Profile

1. Preamble

The NUTRISHIELD platform will be validated through three clinical studies, where the efficacy of the NUTRISHIELD technology and methodology will be tested and validated. Three clinical studies, namely Study I, Study II and Study III, will be implemented in parts (Study III), or totally (Studies I and II) within NUTRISHIELD, each one aiming to produce input regarding different aspects of the NUTRISHIELD project. To this direction, different health conditions, population and settings will be used.

- Study I - Obesity/Diabetes, will validate the nutrition algorithm developed based on personalised data of children having obesity and/or diabetes, through an observational and an intervention protocol.
- Study II – Human milk, the results obtained from this observational study will be used to feed and then train the NUTRISHIELD personalized algorithm with data obtained from clinical settings, including both pre- and full-term infants, to be used as an asset for future reference.
- Study III- Cognitive decline, important associations of nutritional (abdominal fat measures) and other health-related parameters (epigenetics, inflammatory markers) will be revealed.

1.1. Objective of the deliverable

Deliverable D2.7 provides the report on the clinical trial protocols, developed to study the efficacy of NUTRISHIELD technology. The rationale of each study, the study design, involving a description of the study phases, sample characteristics, measurements and outcomes, where applicable, as well as the statistical analysis, and finally the expectations and role of each study within the context of the NUTRISHIELD project are recorded. The CONSORT 2010 (Consolidated Standards for Reporting Clinical Trials) checklist of information is used when reporting the protocols, as described in www.consort-statement.org. The study protocols serve as a model for the methodology to be ideally followed by the medical centers responsible for each study.

1.2. Content of the deliverable

The Deliverable D2.7 includes:

- Protocol proposed for Study I - Obesity/Diabetes
- Protocol proposed for Study II - Human milk
- Protocol proposed for Study III- Cognitive decline

2. Study I protocol: Personalised nutrition against obesity/diabetes for children

2.1. Background

Childhood obesity rates appear to have reached a plateau in western societies [1], yet the estimates remain alarmingly high. In the year 2016, some 124 million school-aged children worldwide were reported to be obese [1], a number possibly underestimated [2]. Excess body weight in childhood is associated with adverse health outcomes across the life span [3]. In light of the severe long-term effects of pediatric obesity [4], the disease calls for timely detection and therapy.

Having obesity is associated with metabolic abnormalities that predispose children to increased cardiometabolic risk [5]. This risk remains persistent during adulthood and is associated with an increased mortality risk [3]. Furthermore, obesity appears to consolidate during childhood and adolescence. Consequently, the obese child is highly likely to remain obese in adulthood [6].

Medical research has fairly recently suggested that children with obesity suffer from non-communicable diseases reportedly uncommon in childhood, that is type 2 Diabetes Mellitus (T2DM) [7, 8]. Other risk factors for the development of pediatric T2DM include familial history of the disease, being born to a mother with gestational diabetes and various metabolic abnormalities [9]. Unlike type 1 diabetes, T2DM is diagnosed at later stages and is associated with poor metabolic control [10], findings that could explain why youth with T2DM are in greater risk for developing earlier and more severe cardiovascular complications than children with type 1 diabetes [9].

Having diabetes, with or without concurrent obesity, calls for integrative lifestyle modification measures, with dietary management as cornerstone. Available guidelines for both youth obesity and diabetes agree on (i) the adherence to a healthful and culturally appropriate dietary pattern, (ii) the adoption of an active lifestyle, (iii) the promotion of adequate growth, and, when appropriate, (iv) weight reduction (up to 10% of initial weight) [11-13]. These modalities have been proven effective in the short term; lifestyle interventions in obese children have resulted in moderate weight reduction and ameliorated glycemic and cardiovascular indices [14-17]. Similar results have been produced for children

with diabetes [11, 18-20]. However, the effects of a personalized lifestyle intervention based on measured biomarkers in urine or feces of these patients, or how these measures correlate to obesity and/ or diabetes biomarkers remain to be explored.

2.2. Objectives

The objectives of the Study I are:

a) to assess the effectiveness of an intensive personalized dietary intervention in children with obesity and/or diabetes, versus a non-personalized one, in terms of health indices improvement, namely anthropometry, biochemical markers and dietary intake

b) to identify possible links between biomarkers measured in urine or feces with obesity/diabetes markers

2.3. Methods

Overall study design, participants, Study Phase I and Study Phase II, sampling and statistical analysis are described in the following sections.

2.3.1. Study design

The details of the study design where optimized following discussions with the NUTRISHIELD partners (during the meeting held on Athens 9-10/4/2019, mails exchange and teleconferences) to better serve the project purposes. Small deviations from the Description of Action were decided regarding the study design related to the groups and the timeline without altering the overall goals of the study.

The proposed study is a two-arm, parallel, randomized controlled study, with a dietary intervention for children (8-18 year old) with obesity or diabetes (type I or II). It will be carried out in Ospedale San Raffaele hospital between M19 to M41. The study protocol will be approved by the Ethics Committee, and written informed consent will be obtained from all caregivers and/or participants. All procedures regarding volunteers' recruitment and participation, data security and handling will be in absolute accordance with the principles set under Work Package (WP) 9, concerning Ethical Requirements, and more specifically stated on Tasks 7.1 and 7.2 and D 7.2.

The study will be divided into two phases:

1. **Phase I**: the **Observational** phase, lasting at least four months. During this period children will be recruited and their diet and biomarkers will be assessed once, as described below. At the end of the observational phase and recruitment period, the study will pause for nine months (from M23 to M31) in order to perform machine learning analyses, feed the platform and train the personalized nutritional algorithm with the results from the preceding assessments of the observational phase.
2. **Phase II**: the **Intervention** phase, lasting six months. During this period children recruited in the study will be randomly allocated to one of the two groups:
    - **Group A (Intervention)** will receive a NUTRISHIELD-derived personalized diet
    - **Group B (Control)** will follow the standard European dietary guidelines for obesity/diabetes, i.e. the usual care/control diet.

    Diet as well as biomarkers and clinical data will be evaluated at the beginning and at the end of the intervention phase.

2.3.1.1. Schematic study design

The study design is schematically presented in Figure 2.1, depicting Phase I, Phase II and their interval. Figure 2.2 presents the timeline of the study, beginning month 19th (M19) and expected to be completed by month 41st (M41).

Figure 2.1: Study I design

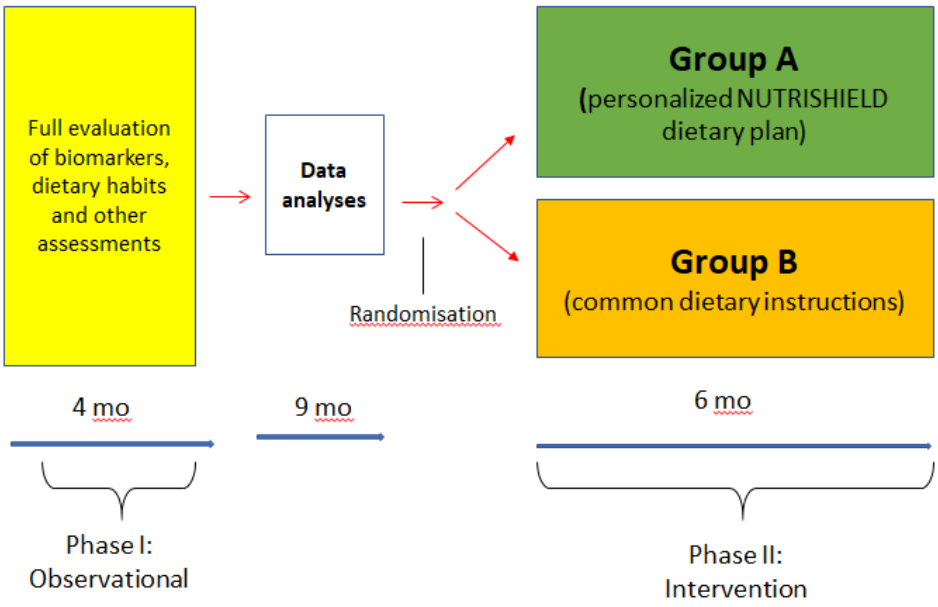
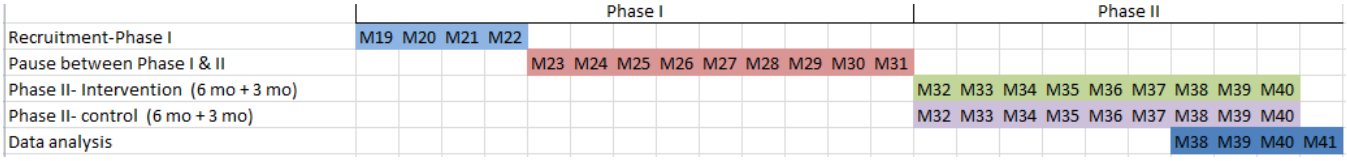


Figure 2.2: Study I timeline



2.3.2. Participants

Participants will be recruited at the San Raffaele Hospital (OSR). All visits and blood and urine sample collection will be held at the hospital. Fecal sample collection (for microbiome analysis) will be performed at home.

Inclusion criteria

- Children with obesity and/or diabetes, aged 8-18 years visiting hospital on an outpatient basis.

Exclusion criteria:

- Children with any acute or chronic disease (tumor, infection, others), gastrointestinal diseases, cardiovascular disease, chronic renal disease, diseases of the parathyroid, diseases necessitating regular phlebotomies, and other chronic diseases which could affect the results of the study
- Use of medicine which could affect the results of the study, such as systemic glucocorticoids, lipid-lowering or anti-hypertensive drugs
- Recent weight loss or weight gain (> 3 kg), within the previous 3 months of the beginning of the study
- Transfusion of blood in the last three months before Study Phase I
- Use of dietary supplements, including multivitamins, fish oil capsules, minerals, and trace elements (three months before and during the entire study period)
- Inability (physically or psychologically) to comply with the procedures required by the protocol.

2.3.3. Study Phase I

Phase I is the observational phase of the study, during which a thorough assessment of each participant will be performed.

2.3.3.1. Measurements

Measurements concern sociodemographic information, dietary and other lifestyle behaviors, anthropometry, medical history, genotype, metabolomics profile (in blood and urine), and microbiome.

- Sociodemographic information

Sociodemographic information will be collected from all children [21, 22]. In specific, the age (in years), the number of people living with (in the family and in the household), whether they attend school (yes/no), and whether the school is private (yes/no) will be recorded. Furthermore, information will be collected regarding parents' marital status (1; married, 2; unmarried/co-habiting, 3; single parent, 4; divorced or separated, 5; widowed), parent's origin (1; White, non-Hispanic; 2; Black, non-Hispanic; 3, Hispanic; 4, Asian; 5, other), years of education described by the number of educational years (mother and father separately), the maternal and the paternal occupation [1, unemployed; 2, employee in private sector; employee in public sector; 3, self-employed; 4, working without pay in family business; 5, on leave (excluding maternity); 6, pensioner]. Finally, the mother or the father will be asked to self-characterise their annual family and household income (ranging from 1, 0-4,999 € to 9, over 50,000 €) and whether the child has active health insurance (yes/no).

- Dietary assessment

Three methods of dietary assessment will be employed during Phase I, namely a 4-day food record supported by a mobile app (for food pictures uploading), an extensive semi-quantitative food frequency questionnaire (FFQ) and a short questionnaire assessing diet quality (KIDMED). We aim to cross-match intake data, and compare the validity of the FFQ against the 4-day food record. The dietary assessment modality that proves to be the most valid will be used in phase II and, finally, in the platform. The mobile app will be developed in the context of WP6, and will serve as an adjunct to the 4-day food record, so as to examine its additive value in improving dietary assessment of children. Finally, the KIDMED will be used as a quick index of dietary quality, which can be easily incorporated in the platform. All the dietary assessment means will be thoroughly described below.

Regarding the 4-day food record, all children with the help of their caregivers (for ages <9 years) will be asked to record all foods and drinks consumed for three consecutive weekdays and one day of the weekend and bring it to the hospital the day that Phase's I assessments are to be performed. Nutrients

intake will be calculated using a standard food analysis program [the Nutritionist Pro™ Diet Analysis software (Axxya Systems, Woodinville, WA, USA)]. The research team involved in the diet analysis is competent in using the proposed software, and HUA has a long history of analyzing dietary data through it. Another strength of the proposed software is that it comprises food databases from multiple countries, which can be used to extrapolate the most relevant and reliable reference. In order to extend and optimize the quantitative and qualitative information received from the food records, children will be asked to upload pictures of mixed foods and drinks to NUTRISHIELD application.

The second method of dietary assessment is the implementation of a semi-quantitative FFQ. This comprises 69 questions on the consumption of foods that are commonly eaten by various populations throughout a year, including dairy products, cereals, fruits, vegetables, meat, fish, legumes, added fats, alcoholic beverages, stimulants and sweets[23]. Using a 6-grade scale ("never/rarely", "1-3 times/month", "1-2 times/week", "3-6 times/week", "1 times/day", "≥2 times/day"), participants will be required to indicate the absolute frequency of consuming a certain amount of food, expressed in g, milliliters or in other common measures, such as slice, tablespoon or cup, depending on the food. The previous month will be set as the timeframe. The FFQ will be completed by the children with the help of the caregiver when needed.

Diet quality will be assessed by the KIDMED. The KIDMED was originally developed in an attempt to combine the Mediterranean diet characteristics as well as the general dietary guidelines for children in a single index [24]. It was based on the principles sustaining healthy, Mediterranean dietary patterns (e.g. daily fruit and vegetable consumption, weekly fish and legumes intake), as well as on those that undermine it (e.g. frequent fast-food intake, increased consumption of sweets).The index comprises 16 questions in the form of "yes or no": questions denoting a negative connotation are assigned a value of –1 and those with a positive aspect +1. Total score ranges from -4 to12 and it is divided into three levels: (1) ≥8, optimal Mediterranean diet; (2) 4-7, improvement needed to adjust intake to Mediterranean diet; (3) ≤3, very low diet quality. It has been used so far in variety of settings and countries [25-27].

- Other lifestyle behaviors assessment

Physical activity level of children will be evaluated using the self-administered 15-item Youth Activity Profile (YAP) [28]. Participants will be asked to report time spent on activities at school, leisure time activities outside of school, and sedentary behaviors. Participants will also report mean nocturnal sleep

duration (in hours/day) and whether they have a TV set in their room (yes/no). All questions regarding sedentary and physical activity behavior refer to the last week, except for sleep, which refers to the last month.

Smoking habits of the family will also be recorded [29]. The mothers of the children will be asked whether they smoked during pregnancy (yes/no) and whether they were exposed to smoke while pregnant more than 30 minutes/day (yes/no). What is more, children will be asked about themselves smoking: current smoking status (yes/no), smoking frequency (1, daily; 2, less than daily; 3, not at all), past smoking (yes/no), age of daily smoking initiation and date of smoking cessation.

Finally, intake of dietary supplements (e.g. vitamins) will be recorded and all children will be also asked whether they are medically diagnosed with dietary allergies or other diseases [1, lactose intolerance; 2, celiac disease; 3, medical diseases under restrictive dietary schemes (sub-question-please refer); 4, other (please refer)] and whether they follow a vegetarian (all sub-types) or vegan diet (yes/no). If the answer to the former question is positive they will be asked for further details [1, vegan; 2, lacto-vegetarian; 3, ovo-vegetarian; 4, lacto-ovo vegetarian; 5, other (please refer)].

- Anthropometric assessment

Children's weight will be measured on leveled platform scale to the nearest 0.5 kg and height on a wall-mounted stadiometer to the nearest 0.5 cm. Body Mass Index (BMI) (kg/m^2) will be calculated by dividing the weight in kilograms by the height in meters squared.

- Medical history assessment

Children's medical history will be recorded by asking all the chronic conditions that they may have.

- Genotype characterisation

A buccal swap sample will be collected from all children. In specific, standard buccal swab kits will be used to gather DNA sample for sample, as described in Deliverable 3.1.

- Metabolome characterisation

For the characterisation of the children's metabolic profile, urine and blood samples will be collected as described in Deliverables 2.2 and 3.1.

- Microbiome assessment

Fecal samples from all children will be collected for microbiome analyses. The protocol is described in Deliverable 2.1.

2.3.4. Study Phase II

2.3.4.1. Description of the Intervention

- **Personalized diet (Group A)**

This intervention includes a personalized dietary plan derived from NUTRISHIELD platform. This plan will be based on the personalized nutrition algorithm taking into account genome analysis, dietary assessment, biochemical markers, socioeconomic status as well as any input having possibly emerged from the machine learning analyses from the evaluations performed during Study Phase I. In specific, in the beginning of the intervention period, taking into account the results from the evaluations performed in the observational period, a 4-week personalized dietary plan, derived as described above will be given to the children. Then, every month, for the following six months, visits will be arranged in the hospital with a window of ± one week, in order to perform the evaluations described below and the children to receive the new 4-week algorithm-derived dietary plans based on the previous month's evaluation. The intervention will be based on dietary guidelines presented in Table 1 [30], modified accordingly to address personal dietary, genetic and other needs, if important input emerges from Phase I.

- **Usual care diet (Group B)**

The control dietary plan will be based on the same principles as the intervention diet [30], but without the use of the NUTRISHIELD platform and the personalized algorithm. Standard information and advice will be given instead by trained dieticians in face to face meetings. This phase will last six months and visits will be arranged once per month.

Table 2.1: Evidence-based guidelines on which intervention and usual care dietary plans will be based upon[30]

Guidelines
○ caloric restriction of 10% below energy needs
○ fat intake of 25-35% of total energy intake
○ saturated fatty acids <10% total energy intake ○ monounsaturated fatty acids >10% total energy intake ○ adequate mixture of n-6 and n-3 polyunsaturated fatty acids (PUFA, approximately 10 % of total energy intake)
○ fruit intake: ○ 1.5 cups per day for children 8-13 years old ○ 1.5 cups per day for girls aged 14-18 years ○ 2 cups for boys aged 14-18 years old
○ vegetables intake: ○ 1 cup per day for girls aged 8 years old ○ 1.5 cups per day for boys aged 8 years old ○ 2 cups per day for girls aged 9-13 years old ○ 2.5 cups for boys aged 9-13 years old ○ 2.5 cups per day for girls aged 14-18 years old ○ 3 cups per day for boys aged 14-18 years old
○ whole-grain breads and cereals intake: ○ 4 oz per day for girls aged 8 years old ○ 5 oz per day for boys aged 8 years old ○ 5 oz per day for girls aged 9-13 years old ○ 6 oz for boys aged 9-13 years old ○ 6 oz per day for girls aged 14-18 years old ○ 7 oz per day for boys aged 14-18 years old
○ lean meat and legumes consumption: ○ 3 oz per day for girls aged 8-13 years old ○ 4 oz per day for boys aged 8-13 years old ○ 5 oz per day for girls aged 14-18 years old ○ 6 oz per day for boys aged 14-18 years old
○ low fat and non-fat dairy intake: ○ 2 cups per day for children aged 8 years old ○ 3 cups per day for those aged 9-18 years old
○ reduction of salt intake, including salt from processed foods
○ reduction of intake of sugar-sweetened beverages and foods
○ at least 20minutes of moderate to vigorous physical activity daily, with a goal of 60 minutes per week

2.3.4.2. Measurements

All children will be thoroughly evaluated at baseline (before the intervention begins) and post-intervention (after the end of intervention). Evaluation includes sociodemographic profile (baseline only), dietary and lifestyle habits, anthropometric measurements, microbiome, metabolome and genotype (baseline only) profiling. Sociodemographic assessment and genotype profiling will be performed only in the observational phase and will not be repeated during the intervention Phase I.

- Sociodemographic information

Sociodemographic information will be collected from all children at baseline [21, 22], as described above.

- Dietary assessment

At baseline and post-intervention the most valid method as evaluated in study phase I will be used, as described above (*2.3.3 Study Phase I, Measurements: Dietary assessment).* Two methods of dietary assessment will be compared during Phase I, namely a 4-day food record (supported by a mobile app for food pictures uploading) and an extensive semi quantitative food frequency questionnaire (FFQ), primarily aiming at evaluating the validity of the FFQ against the 4-day food record.

Moreover, for the evaluation of adherence to the Mediterranean Diet, the KIDMED score will be used [24], as described above.

- Other lifestyle behaviors assessment

Physical activity level, sleep duration and smoking habits will be assessed as described above. Furthermore, children will report whether they have a TV set in their room, the intake of dietary supplements and whether they are medically diagnosed with dietary allergies or other diseases as described in the former section.

- Anthropometric assessment

Children's weight will be measured on leveled platform scale to the nearest 0.5 kg and height on a wall-mounted stadiometer to the nearest 0.5 cm. BMI (kg/m^2) will be calculated by dividing the weight in kilograms by the height in meters squared.

- Medical history assessment

Children's medical history will be recorded by asking all the chronic conditions that they may have.

- Genotype characterisation

A buccal swap sample will be collected from all children at baseline. In specific, standard buccal swab kits will be used to gather DNA sample for sequencing, as described in Deliverable 3.1.

- Metabolome characterisation

For the characterisation of the children's metabolic profile, urine and blood samples will be collected as described in Deliverables 2.2 and 3.1.

- Microbiome assessment

Fecal samples from all children will be collected repeatedly for microbiome analyses. The protocol is described in Deliverable 2.1.

2.3.4.3. Outcomes

To evaluate the effectiveness of the intensive personalized dietary intervention changes in dietary, lifestyle, growth-related and metabolic factors will be assessed, as described below and in Deliverable 2.8.

- Dietary factors

Changes in dietary factors are expected after the personalized intervention. In specific, energy intake, the composition of the diet in macro-nutrients, especially of saturated and trans-fatty acid intake [31] as well as specific micro-nutrients, such as vitamin D and iron [32, 33] are expected to improve. What is more, consumption of specific food groups, such as fruits and vegetables [34-37] and dairy products [37-40] is expected to change. Finally, adherence to the Mediterranean Diet is expected to increase [41].

- Other lifestyle factors

Levels of children's physical activity after the implementation of the platform-derived personalised diet are expected to increase [42-44].

- Growth related factors

Regarding children's weight, height and BMI status is expected to change and will be compared to the available growth charts proposed by the World Health Organization [45].

- Clinical outcomes

To test the effect of the personalised diet intervention on clinical diabetes we will evaluatepossible improvement in the metabolic profile of children such as blood glucose, insulin, A1C, HOMA-IR (Type II diabetes) and c-peptide (Type I diabetes) [46, 47].In addition, cardiometabolic risk factors such as total cholesterol, LDL cholesterol, HDL cholesterol, LDL/HDL cholesterol ratio and triglycerides will be also assessed[48].

- Microbiome-related factors

In the post intervention phase, changes in the gut microbiome related to dietary modifications are expected to occur [49]. We also expect to detect alteration of the metabolome profile at the systemic level (blood and urine), such as changes in the quantity of colonic short chain fatty acid [50].

2.3.5. Sample

2.3.5.1. Sample size

For **Study I phase I**, the working sample will be 200 children, 8-18 years old. By this sample size a statistical power of 92% will be achieved to evaluate two-sided correlation coefficients between the investigated measurements at the level of r = 0.33 [* power one correlation 0 .30, n(200)].

For **Study I phase II**, the required sample will be 80 children (40 per Group A or B) aged 8-18 years old, in order to achieve statistical power of at least 90% at 5% significance level of two-sided hypotheses for the evaluation of 0.75 standard deviations (SD) mean differences in the primary end-point of the Study [power two means 100, diff (15) sd (20) n (80)].

2.3.5.2. Randomisation

The randomisation, either to Group A or Group B, will be performed by an 2X2 block randomization on the basis of age of children (i.e., ≤12 and >12 years old, 1:1 ratio) and sex of children (1:1 ratio). The random allocation sequence (i.e., sequentially numbered containers), will be developed using STATA v15.0 software and relevant commands. Specifically, stream random-number generators will be used. One seed value will set and then using the software and particularly a stream version of the 64-bit Mersenne Twister, Stata's default pseudorandom-number generator, a random sequence of binary numbers will be created that allocate children to each study's Group.

2.3.5.3. Blinding

The physicians and all contributors to the project who will be responsible for baseline analysis and interpretation (apart from the clinical dietitians who will be involved in dietary counselling) along with the biostatisticians will be blind to the intervention groups. Considering that the blinding of clinical dietitians is not amendable, those will be randomly assigned to either the intervention or the control group in order to reduce the risk of bias.

2.3.6. Statistical analysis

Descriptive statistics will be used to present the characteristics of the participating children. Linear regression analyses will be performed with weight and BMI, as well as blood glucose, insulin, A1C, HOMA-IR as dependent variables and physical activity levels, children's and mother's smoking habits, nutritional status including energy intake, consumption of specific food groups and KIDMED score, medical history, genotype and metabolome as the independent variables.

Between Group comparisons for the baseline measurements of the phase II of Study I, will be performed using t-test or Mann-Whitney test, to ensure comparability of the Groups. Then, children

enrolled in the intervention diet will (Group A) be compared to those of the control diet (Group B), regarding their weight and BMI status as well as regarding the glycemic control as indicated by blood glucose, insulin, A1C, HOMA-IR (i.e., primary end points), using Repeated Measures Analysis of Variance (RMANOVA). Moreover, the 2Groups will be compared regarding various cardiometabolic risk factors such as total cholesterol, LDL cholesterol, HDL cholesterol, LDL/HDL cholesterol ratio and triglycerides, improvement in nutritional status including energy intake and consumption of specific food groups, as well as urine biomarkers, biomarkers of intestinal inflammation and microbiome profile, using the aforementioned method. All necessary adjustments (based on the baseline analyses) will be performed to ensure the robustness of the results.

The analyses will be performed by using STATA v15 software (STATA Corp LLL, College Station, TX, USA).

Finally, additional statistical analysis with the view of deriving additional insights and influences in personalised nutrition will be presented in D2.5.

2.4. Expectations and role to the project

From the observational and the interventional phases of the study, novel outcomes are expected to occur, of interest for both the NUTRISHIELD project as well as the scientific community.

The proposed study incorporates knowledge produced under NUTRISHIELD, as materials and methods designed under WP3 - 6 and WP9 that will be directly applied within its context. Findings from the observational phase are expected to highlight novel associations between several, not yet adequately studied, biomarkers and pediatric obesity/ diabetes progression. It will also underscore the most valid means of dietary assessment for this population, and the knowledge produced shall be directly applied to the interventional phase of the study.

Another major expected outcome of this study is the validation of the NUTRISHIELD project in clinical settings during the intervention phase, in essence describing how a theoretical framework designed and fed by the patients' data translates to clinical practice.

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2.6. Study I CONSORT 2010 checklist

Table 2.2: Study I CONSORT 1

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	Non-applicable
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	Non-applicable
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	7-8
	2b	Specific objectives or hypotheses	8
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	8
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	Non-applicable
Participants	4a	Eligibility criteria for participants	10
	4b	Settings and locations where the data were collected	10
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	14-15
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	16-19
	6b	Any changes to trial outcomes after the trial commenced, with reasons	Non-applicable
Sample size	7a	How sample size was determined	18-19
	7b	When applicable, explanation of any interim analyses and stopping guidelines	Non-applicable
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	19
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	19

Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	19
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	19
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	19
	11b	If relevant, description of the similarity of interventions	Non-applicable
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	19-20
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Non-applicable
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Non-applicable
	13b	For each group, losses and exclusions after randomisation, together with reasons	Non-applicable
Recruitment	14a	Dates defining the periods of recruitment and follow-up	Non-applicable
	14b	Why the trial ended or was stopped	Non-applicable
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Non-applicable
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Non-applicable
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Non-applicable
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Non-applicable
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Non-applicable
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Non-applicable
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Non-applicable
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Non-applicable
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Non-applicable
Other information			

Registration	23	Registration number and name of trial registry	Non-applicable
Protocol	24	Where the full trial protocol can be accessed, if available	Non-applicable
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Non-applicable

3. Study II protocol: Personalised nutrition for lactating mothers

3.1. Background

Breastfeeding is the optimal feeding practice for all infants, associated with numerous health benefits for the mother–infant dyad, being an ecologic practice as well [1]. The World Health Organization highly recommends exclusive BF for all infants up to 6 months, born either full–term [2, 3] or prematurely [4]. Human Milk (HM) is a dynamic fluid that meets offspring's needs [5-8], and is ample in nutrients and bio-active compounds intended to enhance the offspring's immunity and growth [9]. HM also hosts a complex ecosystem of microbiota [10], which is of paramount importance for the offspring's immunity [5].

During the last decades, the incidence of preterm deliveries (<37 weeks of gestation) has been steadily increasing, and so has the interest in pre-term and early infant nutrition. Associated birth complications are the leading cause of death among children under 5 years of age, responsible for ~1 million deaths per year [11]. Progress in medical interventions has allowed for enhancing survival of an increasing proportion of extremely low gestational age newborns and low birth weight infants. Early infant nutrition has become a major player in improving clinical outcomes of survivors [12]. HM is recommended for preterm infants based on an impressive array of benefits provided to this highly vulnerable population, including ameliorated immunological and gastrointestinal outcomes [13-19].

For mothers unable to produce sufficient milk quantities so as to exclusively or partially breastfeed, pasteurized donor human milk (DHM) is a viable option to avoid formula feeding, especially for low birth-weight children [20]. To date most studies focus on the benefits of using DHM over formula in pre-term infants, when own mother's milk (OMM) is limited or unavailable. There is clear scientific evidence indicating the superiority of OMM or DHM against formula feeding [21]. In relation to DHM, its use over infant formula protects against necrotizing enterocolitis [22]; but there are few reports comparing DHM and OMM. While DHM provides some bioactive agents, its consumption is associated with slower growth rates in comparison with the consumption of OMM or formula, although inconclusively [21].This

finding may be attributable to several factors. Most DHM is provided by women who have delivered at term and donate their milk in later stages of lactation several months after delivery. In comparison to preterm milk during the first weeks after delivery, this milk is low in protein, fat and other bioactive molecules [23]. Its composition is also affected by the processing of expressed milk, including stringent protocols applied in human milk banks, i.e. pasteurization, freezing, and storage (25, 26), necessary to circumvent the potential risk of transmitting infectious agents.

Beyond the physiological adaptations of HM composition, research has focused on the potential effects of the maternal diet on the HM composition. In an earlier report, HM's composition was found rather constant among mothers of diverse ethnic backgrounds, with different dietary habits [24]. The total fat content of HM has been found to be independent of the mother's diet [25], but responsive to the mother's fatty acids dietary intake [26, 27]. In a recent comprehensive systematic review of 104 observational and interventional studies, HM composition was found to relate to the maternal consumption of fatty acids, fat-soluble vitamins, vitamin B1 and vitamin C intakes [28, 29]. No similar relationships were found for dietary intake of iron, folate, calcium, selenium or proteins [28]. However, more studies are needed to conclude for other nutrients and little is known on how the maternal diet may also affect non-nutritive constituents of the HM, i.e. prebiotics content[30], interactions with gut microbiota [31, 32]. Finally, existing evidence is mainly based on studies on full-term infants; the impact of maternal nutrition on the composition of the HM for the pre-term offspring has not been adequately documented. Thus, it still remains unknown whether dietary advice to the nursing mother could enhance the premature offspring's growth rate, and if so, whether non-nutritive aspects of the HM play any part, or mediate this process.

3.2. Objectives

The objectives of the study are:

a) to evaluate associations between the mother's diet composition and HM composition in pre- and full-term infants, i.e. how the potential associations between mother's diet and HM nutrient composition is affected by prematurity.

b) to evaluate the effect of pasteurization/storage on DHM composition.

c) to evaluate associations between HM composition (OMM or DHM) on growth and other health parameters in pre- and full-term infants.

3.3. Methods

Overall study design, participants, study phases (baseline, observational), sampling and statistical analysis are described in the following sections.

3.3.1. Study design

The proposed study is an observational, parallel group, non-randomized study in lactating mothers. It will be carried out at the University and Polytechnic Hospital La Fe (HULAFE), beginning at Month 21. Previously, the study protocol will be approved by the Ethics Committee for Biomedical Research of the Health Research Institute La Fe (Valencia, Spain) and the study participants will sign a relevant consent form. All procedures regarding volunteers' recruitment and participation, data security and handling will be in absolute accordance with the principles set under WP 9, concerning Ethical Requirements, and more specifically stated on Tasks 7.1 and 7.2 and D 7.2.

To address the objectives mentioned above, three mother-infant groups will be examined. In specific:

- **Group A** will consist of 25 pre-term infants fed with OMM (PT-OMM) and their mothers.
- **Group B** will consist of 25 pre-term infants fed with pasteurized DHM (PT-DHM), in range of complete enteral nutrition (150 mL/kg/day), and their mothers.
- **Group C** will consist of 25 full term infants receiving OMM (FT-OMM) and their mothers.

The study will be divided into two phases:

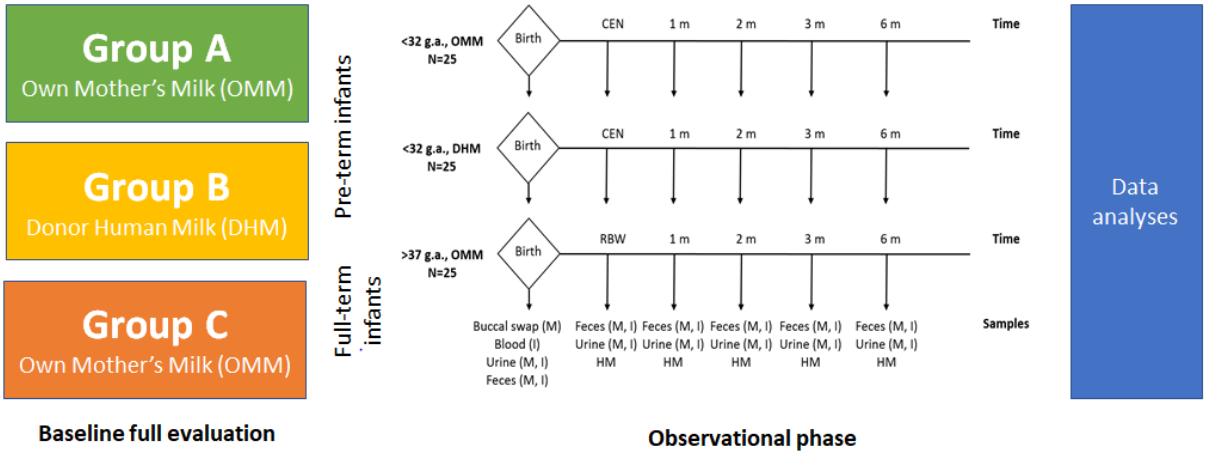
1. **A baseline phase**, referring to the period between the time of delivery and the time preterm infants receive complete enteral nutrition (CEN) and full term infants recover of birth weight (RBW). During this period, all infants and their mothers will be evaluated, as described in the next section.
2. **An observational phase**, lasting six months and referring to the period that infants are fed with HMand/or formulas orsolid foods. During this period, assessments, as described below,

will be arranged for all mother-infants dyad and the donors providing the DHM in the hospital or the participants' home.

3.3.1.1. Schematic study design

The study design and comparisons among groups to address the objectives of the study are presented schematically in Figures 3.1 and 3.2 below.

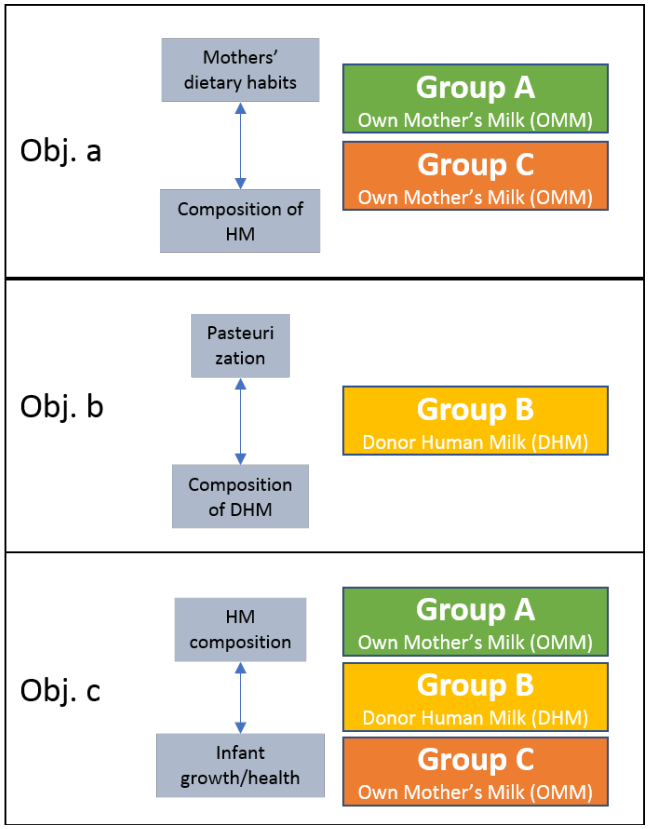
Figure 3.1: Study II design



Every point represents the time points where assessments are to be made

CEN: complete enteral nutrition, RBW: recovery of birth weight

Figure 3.2: Group comparisons in relation to the study objectives



Obj. a: objective a. Obj. b: objective b. Obj. c: objective c

3.3.2. Participants

Participants will be recruited in HULAFE. Visits at baseline, when preterm infants receive complete enteral nutrition (CEN; i.e. 150 mL/kg/day) and at months 1, 3, and 6 will be held at the hospital. Visits when full term infants recover of birth weight and at month 2 will be held at the participant's home.

The eligibility criteria for participating will be as follows:

Inclusion criteria

- Mother accepts to participate in the study and signs an informed consent form
- Gestational age < 32 weeks for the group of preterm infants and gestational age > 37 weeks for the full-term infants
- Exclusive consumption (i.e. >80% of intake) of OMM or DHM

Exclusion criteria:

- Mother requires special diet (e.g. celiac disease, diabetes)
- Mother consuming probiotics
- Infant needs intestinal surgery
- Severe congenital malformations
- Chromosomopathies

3.3.3. Baseline Study Phase

At the baseline, sociodemographic and medical history information will be recorded and lifestyle, dietary, anthropometric, genotype, metabolome and microtype assessments will be performed as described below. Detailed Standard Operating Procedures employed for sample collection, storage and outcome assessment will be provided in WP3. Results obtained from data analysis will be used to feed the NUTRISHIELD platform and train the NUTRISHIELD algorithm, to be used as an asset for future reference.

3.3.3.1. Measurements

During their stay at the hospital mothers of all three groups will receive nutritional advice based on current knowledge and following the standard protocols of HULAFE.

Measurements concern sociodemographic information, dietary and other lifestyle behaviors, anthropometry, medical history, genotype, metabolome, and microbiome.

- Sociodemographic information

Mothers of PT-OMM, PT-DHM and FT-OMM and donors providing the DHM will be asked to self-characterize their annual household income and their family income (ranging from 1, 0-4,999 € to 9, over 50,000 €), whether they have active health insurance (yes/no), their employment status (1, unemployed; 2, employee in private sector; employee in public sector; 3, self-employed; 4, working without pay in family business; 5, on leave (excluding maternity); 6, pensioner), whether they are on maternity leave (yes/no) and their marital status (1; married, 2; unmarried/co-habiting, 3; single parent, 4; divorced or separated, 5; widowed) [33, 34]. Education level will be described by the number of years

on education, while information about the number of people living with the family and in the household will also be included. Maternal or donor's age and origin will be recorded (1; White, non-Hispanic; 2; Black, non-Hispanic; 3, Hispanic; 4, Asian; 5, other).

- Dietary assessment

Mothers of PT-OMM, PT-DHM, and FT-OMM will complete a validated semi-quantitative food frequency questionnaire (FFQ) [35]. FFQ will be administered by trained personnel and it comprises 69 questions on the consumption of foods that are commonly eaten by various populations throughout a year, including dairy products, cereals, fruits, vegetables, meat, fish, legumes, added fats, alcoholic beverages, stimulants and sweets. Using a 6-grade scale ("never/rarely", "1-3 times/month", "1-2 times/week", "3-6 times/week", "1 times/day", "≥2 times/day"), participants will be required to indicate the absolute frequency of consuming a certain amount of food, expressed in g, milliliters or in other common measures, such as slice, tablespoon or cup, depending on the food. The previous month will be set as the timeframe.

According to the FFQ responses, adherence to the Mediterranean Diet will be evaluated by using the MedDietScore, an 11-item composite score calculated for each participant from the FFQ-based food consumption [36]. For food groups presumed to be part of the Mediterranean pattern (i.e. those with a recommended intake of 4 servings per week or more, such as non-refined cereals, fruits, vegetables, legumes, olive oil, fish, and potatoes) higher scores will be assigned when the consumption is according to the rationale of the Mediterranean pattern, while lower scores will be assigned when participants report no, rare, or moderate consumption. For the consumption of foods presumed not to be part of the Mediterranean pattern (i.e. consumption of meat and meat products, poultry, and full fat dairy products), scores will be assigned on a reverse scale. For alcohol intake, a score of 5 was assigned for consumption of less than 300 ml of wine/day, a score of 0 was assigned for no consumption or for consumption of 700 ml/day and scores of 4 to 1 were assigned for consumption of 600-700, 500-600, 400-500 and 300-400 mL/day, respectively. All alcoholic beverages will be converted into ml of wine, assuming that 12 g of ethanol correspond to 100 mL of wine. The theoretical range of the MedDietScore is between 0 and 55. Higher values of the score indicate greater adherence to the Mediterranean diet.

- Other lifestyle behaviors assessment

Physical activity levels of mothers of PT-OMM, FT-OMM and PT-DHM will be evaluated using the validated short version of the International Physical Activity Questionnaire (IPAQ) [37]. They will report the time spent on vigorous, moderate activities, walking (days/week, minutes/day) and sitting (hours/day) and their mean nocturnal sleep duration (in hours/day). All questions regarding sedentary and physical activity behavior refer to the last week, except for sleep, which refers to the last month.

Furthermore, the mothers will be asked regarding their smoking habits [38]. In specific current smoking status (yes/no), smoking frequency (1, daily; 2, less than daily; 3, not at all), smoking during pregnancy (yes/no), past smoking (yes/no), exposure to second hand smoking (yes/no), exposure to second hand smoking during pregnancy (yes/no), age of daily smoking initiation and the date of smoking cessation will be recorded.

- Anthropometric assessment

Weight and height of mothers of the PT-OMM, PT-DHM, and FT-OMM will be measured. Weight will be measured on a levelled platform scale to the nearest 0.5 kg and height on wall-mounted stadiometer, to the nearest 0.5 cm. Body Mass Index (BMI) (kg/m^2) will be calculated by dividing the weight in kilograms by the height in meters squared.

All infants will be evaluated regarding their growth by measuring weight, length and cranial perimeter upon every visit at the hospital. Weight and recumbent length will be measured on an electronic baby scale and on a neonatometer scale, to the nearest 0.5 kg and 0.5 cm respectively and cranial perimeter will be measured using a flexible inch tape to the nearest 0.5 cm.

- Medical history assessment

At observational phase PT-OMM, PT-DHM and FT-OMM mothers' medical history, i.e. all the chronic conditions that they may have as well as the use of medicines, will be recorded.

- Genotype characterisation

A buccal swap sample will be collected from mothers of PT-OMM, PT-DHM and FT-OMM at baseline. In specific, standard buccal swab kits will be used to gather DNA sample. Furthermore, umbilical cord

blood will be extracted from PT-OMM, PT-DHM, and FT-OMM newborns. The detailed protocol is described in Deliverable 3.1.

- Metabolomecharacterisation

For the characterization of metabolic profile urine samples will be collected from mothers of PT-OMM, PT-DHM and FT-OMM and their infants, as described in Deliverables 2.2 and 3.1.

- Microbiome assessment

Fecal samples from all mothers and their infants will be collected for studying the microbiome. The protocol followed is described in Deliverable 2.1.

3.3.4. Observational Study Phase

A thorough evaluation of the health status of the mother-infant dyad and the donors providing the DHM will be performed, including dietary and other lifestyle parameters assessment, medical history and evaluation, anthropometric measurements and microbiome profiling. A description of each of these aspects follows. Evaluations in the donors providing the DHM will be performed every time a batch is donated. Detailed Standard Operating Procedures employed for sample collection, storage and outcome assessment will be provided in WP3. Results obtained from data analysis will be used to feed the NUTRISHIELD platform and train the NUTRISHIELD algorithm.

3.3.4.1. Measurements

Measurements concern sociodemographic information, dietary and other lifestyle behaviors, anthropometry, medical history, genotype, metabolome, and microbiome.

- Dietary assessment

Upon every visit of the observational phase mothers of PT-OMM, PT-DHM, and FT-OMM as well as donors providing the DHM will complete a validated semi-quantitative food frequency questionnaire (FFQ) [35], from which MedDietScore, will be calculated [36], as described in section 3.3.3.

In addition, during the hospital visit at month 1 all mothers will be asked to return a 4-day food record, in which they will have recorded all foods and drinks consumed for three consecutive weekdays and one day of the weekend. Nutrients' intake will be calculated using the Nutritionist Pro™ Diet Analysis (Axxya Systems, Woodinville, WA, USA) software. The research team involved in the diet analysis is competent in using the proposed software, and HUA has a long history of analyzing dietary data through it. Another strength of the proposed software is that it comprises food databases from multiple countries, which can be used to extrapolate the most relevant and reliable reference. We aim to cross-match intake data, and compare the validity of the FFQ against the 4-day food record, in order for the most valid to be used in the platform.

- Other lifestyle behaviors assessment

Physical activity levels of mothers of PT-OMM, FT-OMM and PT-OMM as well as milk donors providing DHM will be evaluated at every visit, as described in section 3.3.3.

Furthermore, the mothers will be asked regarding their smoking habits [38]. In specific current smoking status (yes/no), smoking frequency (1, daily; 2, less than daily; 3, not at all) and exposure to second hand smoking (yes/no) will be recorded.

- Anthropometric assessment

Weight and height of mothers of the PT-OMM, PT-DHM, and FT-OMM, as well as milk donors providing DHM will be measured and recorded at every visit at the hospital, as described above.

All infants will be evaluated regarding their growth by measuring weight, length and cranial perimeter upon every visit at the hospital. Weight and recumbent length will be measured on an electronic baby scale and on a neonatometer scale, to the nearest 0.5 kg and 0.5 cm respectively and cranial perimeter will be measured using a flexible inch tape to the nearest 0.5 cm.

- Medical history assessment

Number of respiratory, gastrointestinal, and neurologic infections, number of hospital admissions due to infectious diseases as well as antibiotics use will be recorded for infants of all three groups upon every visit.

- Metabolome characterisation

For the characterization of metabolic profile urine samples will be collected from mothers of PT-OMM, PT-DHM and FT-OMM and their infants at each evaluation, as described in Deliverables 2.2 and 3.1.

- Human milk nutrient composition assessment

OMM will be collected at every visit. DHM aliquots of each milk batch will be collected before and after pasteurization and fortification of DHM will be recorded. The detailed protocol is described in Deliverable 2.3.

- Microbiome assessment

OMM and DHM before pasteurization will be analyzed for microorganism's content. Fecal samples from mothers of PT-OMM, PT-DHM and FT-OMM and their infants will be also collected for studying the microbiome at every visit. The protocol followed is described in Deliverable 2.1.

3.3.5. Sample size

In order to achieve statistical power of at least 95% at 5% significance level of two-sided hypotheses for the evaluation of 10% standardized mean differences of fat and protein composition of HM, as well as growth markers (weight, height), and health status (number of gastrointestinal and respiratory diseases) of preterm infants, a total of 75 mother-infant dyads (25 per Group of Study II) will be enrolled.

3.3.6. Statistical analysis

Associations between mothers' diet and HM composition will be examined with linear regression models. Associations with the macro- and micro-nutrients of the mothers' diet as the independent variable and HM macro- and micro-nutrient composition as dependent variable using prematurity, age and years of education as covariates, will also be examined using linear regression models. The analyses will be repeated separately from the groups of PT-OMM and FT-OMM. Furthermore, linear regression models will be performed with the HM macro- and micro-nutrient composition as the independent

variables and weight, length, cranial perimeter and number of diseases as the dependent using prematurity, age, years of education etc., as the covariates. The analyses will be repeated separately for the groups of preterm and full-term infants. Finally, composition of DHM in terms of nutrients and microorganisms content will be compared before and after pasteurization/storage using Student's t-test or Mann-Whitney test. The analyses will be performed by using STATA software (STATA Corp LLL, College Station, TX, USA).

Finally, additional statistical analysis with the view of deriving additional insights and influences in personalised nutrition will be presented in D2.5.

3.4. Expectations and role to the project

The results and the associations revealed from the study will be used to feed NUTRISHIELD platform and train the personalized nutritional algorithm with data obtained from clinical settings, including from both pre- and full-term infants as well as infants fed both with OMM and DHM. This will in essence validate the NUTRISHIELD platform, describing how a theoretical framework designed and fed by the patients' data translates to clinical practice. What is more, evaluations performed in DHM before and after pasteurization/storage will provide input to the human milk analyzer that is developed within WP4, with focus on the determination of milk proteins that may change with thermal treatment of the milk.

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3.6. Study CONSORT 2010 checklist

Table 3.1: Study II CONSORT 1

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	Non-applicable
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	Non-applicable
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	28-29
	2b	Specific objectives or hypotheses	28-29
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	29
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	Non-applicable
Participants	4a	Eligibility criteria for participants	31-32
	4b	Settings and locations where the data were collected	31
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	Non-applicable
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	32-37
	6b	Any changes to trial outcomes after the trial commenced, with reasons	Non-applicable
Sample size	7a	How sample size was determined	37
	7b	When applicable, explanation of any interim analyses and stopping guidelines	Non-applicable
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Non-applicable
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Non-applicable
Allocation	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers),	Non-applicable

concealment mechanism		describing any steps taken to conceal the sequence until interventions were assigned	
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	Non-applicable
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	Non-applicable
	11b	If relevant, description of the similarity of interventions	Non-applicable
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	37-38
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Non-applicable
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Non-applicable
	13b	For each group, losses and exclusions after randomisation, together with reasons	Non-applicable
Recruitment	14a	Dates defining the periods of recruitment and follow-up	Non-applicable
	14b	Why the trial ended or was stopped	Non-applicable
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Non-applicable
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Non-applicable
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Non-applicable
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Non-applicable
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Non-applicable
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Non-applicable
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Non-applicable
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Non-applicable
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Non-applicable
Other information			
Registration	23	Registration number and name of trial registry	Non-applicable

Protocol	24	Where the full trial protocol can be accessed, if available	Non-applicable
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Non-applicable

4. Study III protocol: Personalised nutrition against cognitive decline for children

4.1. Background

A diet of sufficient quantity and quality is crucial for children's growth and mental development. An excess in energy intake, along with reduced energy expenditure, has contributed to the modern epidemic of pediatric obesity [1], while in the same time estimates of undernutrition remain concerning [1]. This double burden of malnutrition poses great risk for children's current and future health and well being [2-5].

Beyond nutrient and energy intake per se, literature suggests that dietary quality has a major imprint on children's health as well. Lower dietary quality in children has been previously associated with worse sleep indices [6], higher prevalence of excess weight and central adiposity [7-12], breathing anomalies [13], and poor cardiometabolic health [14-16]. Furthermore, evidence suggests that higher adherence to a variety of healthful dietary patterns is associated with enhanced neurocognitive development [17], reportedly more profound in boys [18]. Last, better diet quality has been associated with improved academic performance [19].

Evidence is accumulating concerning the effects of dietary intake and quality of children in other health outcomes. For instance, the interrelationship between the diet and gut microbiota and its impact on children's health has been a potent area of interest in recent years. Diet has been described as a main driver of the composition and function of gut microbiota [20]; however, their relationship has been mostly studied in very specific pediatric populations (i.e. children with epilepsy, autism, gastrointestinal diseases) [21-23]. Some results from healthy children have indicated that children with worse diet quality have reduced colonic short chain fatty acid fermentation compared to children with better diet quality [24]. Similar results have been produced in children adhering to a Western dietary pattern, compared to a traditional one [25, 26]. Nevertheless, conclusions are yet to be drawn on how these findings affect pediatric development. In addition, cross-matching of dietary indices with other individual measures, such as breath markers, may also highlight potential targets for optimal growth.

4.2. Objectives

The objective of this study is:

a) to evaluate the association of diet with health-related parameters, i.e. how the quality of the diet affects health related parameters (i.e. gut microbiota, breath analyses)

4.3. Methods

Overall study design, participants, measurements, sampling and statistical analysis are described in the following sections.

4.3.1. Study design

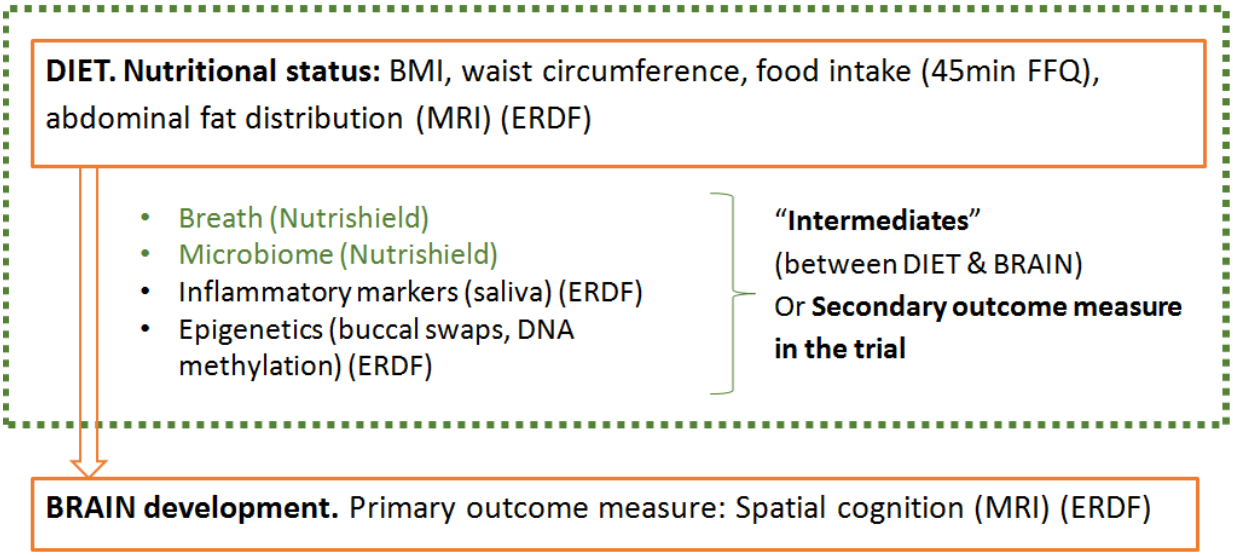
The proposed study is an observational trial, part of a trial in progress, performed by Stichting Kathoelike Universtiteit, in collaboration with Donders Institute, the Netherlands. It is entitled “Neural development of spatial cognition: an fMRI study on the effects of current and past nutritional status” (Principal Investigator: Dr. Esther Aarts, Donders Institute). The trial is aiming to examine the relationship of nutritional status with the neural development of spatial cognition in 8-10 year old children. The part of the trial under NUTRISHIELD will be observational, and takes place at its baseline. During recruitment (observational phase) participants will be assessed once, as described below.

The trial is funded by the European Regional Development Fund (ERDF, PROJ-00405). Ethical approval for the ERDF trial has been granted by the Medical Ethical Committee of Radboud University Medical Center (date of approval: 22/05/2018; registration number: 2017-3923; NL-number: NL64464.091.17). Written informed consent will be obtained prior to participation. All procedures regarding data security and handling in the context of NUTRISHIELD will be in absolute accordance with the principles set under WP 9, concerning Ethical Requirements, and more specifically stated on Tasks 7.1 and 7.2 and D 7.2.

4.3.1.1. *Schematic study design*

The design and the measures acquired at the baseline of the ERDF trial along with the role of NUTRISHIELD are presented schematically in Figure 4.1 below.

Figure 4.1: Role of NUTRISHIELD 1 within ERDF trial



The part of the joined action between NUTRISHIELD and ERDF highlighted in green.

4.3.2. Participants

The study sample will include 55 healthy children, aged 8-10 years old.

4.3.3. Baseline Study Phase

4.3.3.1. *Measurements*

Under the ERDF study protocol, all participants will be evaluated, including dietary intake, nutritional status, body composition, inflammatory markers and epigenetics, afterwards explored as intermediates in the relationship between the children's diet and spatial cognition. In collaboration with NUTRISHIELD, analyses of breath, and gut microbiome will be carried out in the study population.

- Sociodemographic information

ERDF trial.

- Dietary assessment

45-minute Food Frequency Questionnaire (ERDF trial).

- Anthropometric assessment

Weight, height, computation of BMI, measures of abdominal fat by Magnetic Resonance Tomography (ERDF trial).

- Inflammatory assessment

Measured in children's saliva (ERDF trial).Inflammatory assessment is funded by an external company. Thus, using these measures under NUTRISHIELD warrants the company's approval.

- Genotype characterisation

Buccal swaps, DNA methylation patterns (ERDF trial). Epigenetics assessment is funded by an external company. Thus, using these measures under NUTRISHIELD warrants the company's approval.

- Microbiome assessment

Fecal samples from children will be collected for studying the microbiome at baseline. The protocol followed is described in Deliverable 2.1.

- Breath analyses

Breath measures of children will be collected, and novel markers will be analyzed. The protocol followed is described in Deliverable 2.4.

4.3.4. Sample size

A sample size of 55 children, aged 8-10, has been found adequate to significantly detect expected outcomes of the ERDF trial.

4.3.5. Statistical analysis

Association of acquired measures with dietary measures will be explored. Additionally, the relationship in between acquired measures, other than dietary intake, will be studied. Categorical values will be explored with Chi-square analysis. Student's t-test will be employed to test associations of continuous, independent variables. Correlations between continuous variables will be tested with Spearman's r coefficient. A p-value of 0.05 is considered significant.

Statistical analyses will be performed with the use of STATA statistical software (STATA Corp LLL, College Station, TX, USA).

Finally, additional statistical analysis with the view of deriving additional insights and influences in personalised nutrition will be presented in D2.5.

4.4. Expectations and role to the project

The collaboration of the ERDF study and NUTRISHIELD is expected to bring added value to both projects. This collaboration allows for exploring associations of nutritional (abdominal fat measures) and other health-related parameters (epigenetics, inflammatory markers) not collected under NUTRISHIELD, with those studied under NUTRISHIELD. This joint approach is expected to greatly benefit the long-term vision of NUTRISHIELD, namely the evidence-based personalised nutrition for healthy youths.

4.5. References

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4.6. Study III CONSORT 2010 checklist

Table 4.1: Study III CONSORT 1

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	Non-applicable
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	Non-applicable
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	44
	2b	Specific objectives or hypotheses	45
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	45
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	Non-applicable
Participants	4a	Eligibility criteria for participants	Non-applicable
	4b	Settings and locations where the data were collected	Non-applicable
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	Non-applicable
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	Non-applicable

	6b	Any changes to trial outcomes after the trial commenced, with reasons	Non-applicable
Sample size	7a	How sample size was determined	47
	7b	When applicable, explanation of any interim analyses and stopping guidelines	Non-applicable
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Non-applicable
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Non-applicable
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	Non-applicable
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	Non-applicable
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	Non-applicable
	11b	If relevant, description of the similarity of interventions	Non-applicable
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	48
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	48
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Non-applicable
	13b	For each group, losses and exclusions after randomisation,together with reasons	Non-applicable
Recruitment	14a	Dates defining the periods of recruitment and follow-up	Non-applicable
	14b	Why the trial ended or was stopped	Non-

			applicable
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Non-applicable
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Non-applicable
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Non-applicable
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Non-applicable
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Non-applicable
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Non-applicable
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Non-applicable
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Non-applicable
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Non-applicable
Other information			
Registration	23	Registration number and name of trial registry	Non-applicable
Protocol	24	Where the full trial protocol can be accessed, if available	Non-applicable
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Non-applicable

5. Appendix

5.1. Sociodemographic Dictionary

Variable Name	Short Name	Description	Reference
General Information			
Date of study entry		DD/MM/YYYY	
Identification Number	ID	Unique identification code	
Given name	Name		
Surname	Surname		
Date of Birth	Date of Birth	DD/MM/YYYY	
Demographics (all participants, unless otherwise specified)			
Marital status (adults only)	Marital status	1; married, 2; unmarried/co-habiting, 3; single parent, 4; divorced or separated, 5; widowed 6. No response	Barrett &Wellings, 2002 [1] NHANES
Maternal origin	Maternal origin	1; White, non-Hispanic; 2; Black, non-Hispanic; 3, Hispanic; 4, Asian; 5, other, 6. No response	Kanakamedala et al., [2]
Paternal origin	Paternal origin	1; White, non-Hispanic; 2; Black, non-Hispanic; 3, Hispanic; 4, Asian; 5,	Kanakamedala et al., [2]

Variable Name	Short Name	Description	Reference
		other, 6. No response	
Number of persons in your family	Family members	Open-ended, No response	NHANES
Number of persons under 5 years old in your family	Family members under 5 years old	Open-ended, No response	NHANES
Number of persons aged 6-17 years old in your family	Family members aged 6-17 years old	Open-ended, No response	NHANES
Number of persons aged 18-59 years old in your family	Family members aged 18-59 years old	Open-ended, No response	NHANES
Number of persons aged >60 years old in your family	Family members >60 years old	Open-ended, No response	NHANES
Number of persons in your household	Household members	Open-ended, No response	NHANES
Number of persons under 5 years old in your household	Household members under 5 years old	Open-ended, No response	NHANES
Number of persons aged 6-17 years old in your household	Household members aged 6-17 years old	Open-ended, No response	NHANES
Number of persons aged 18-59 years old in your household	Household members aged 18-59 years old	Open-ended, No response	NHANES
Number of persons aged >60 years old in your household	Household members >60 years old	Open-ended, No response	NHANES
Household address	Address		
Postal code	Postal Code		

Variable Name	Short Name	Description	Reference
Socioeconomic status (all participants)			
Maternal Formal education	Maternal Education years	Open-ended, No response	
Paternal Formal education	Paternal Education years	Open-ended, No response	
Are you currently under health insurance	Active health insurance	0, no; 1, yes, 2, No response	NHANES [3]
Annual Household Income	Annual Household Income	1, 0-4,999 €; 2, 5,000-9,999 €; 3, 10,000-14,999 €; 15,000-19,999 €; 4, 20,000-24,999 €; 5, 25,000-29,999 €; 6, 30,000-34,999 €; 7 35,000-39,999 €; 8, 40,000 – 49,9999 €; 9, over 50,000 €; 10, do not know, 11, No response	NHANES
Annual Family Income	Annual Family Income	1, 0-4,999 €; 2, 5,000-9,999 €; 3, 10,000-14,999 €; 15,000-19,999 €; 4, 20,000-24,999 €; 5, 25,000-29,999 €; 6, 30,000-34,999 €; 7 35,000-39,999 €; 8, 40,000 – 49,9999 €; 9, over 50,000 €; 10, do not know; 11, No response	NHANES
Current maternal occupation	On maternity leave	0,no; 1, yes, 2, No response	

Variable Name	Short Name	Description	Reference
Maternal Occupation status	Maternal employment type	1, unemployed; 2, employee in private sector; employee in public sector; 3, self-employed; 4, working without pay in family business; 5, on leave (excluding maternity); 6, pensioner, 7, No response	Adapted from NHANES
Current paternal occupation	On paternity leave	0,no; 1, yes, 2, No response	
Paternal Occupation status	Paternal employment type	1, unemployed; 2, employee in private sector; employee in public sector; 3, self-employed; 4, working without pay in family business; 5, on paid leave (excluding paternity); 6, on unpaid leave; 7, pensioner, 8, No response	Adapted from NHANES
Children only (6 years old or more)			
Do you attend school?	School attendance	0, no; 1, yes, 2, No response	
Is your school private?		0, no; 1, yes, 2, No response	Proxy of SES
Lifestyle (adults only)			
Current smoking	Current tobacco	0, no; 1, yes, 2, No	WHO – GATS

Variable Name	Short Name	Description	Reference
	smoking	response	
Current tobacco smoking frequency	Do you currently smoke cigarettes?	1, daily; 2, less than daily; 3, not at all, 4, No response	
Current exposure to second-hand smoke (defined as >30min/ day)		0, no; 1, yes, 2, No response	
Past smoking	Previous tobacco use	0, no; 1, yes, 2, No response	
Age of daily smoking initiation		Open-ended, No response	
Smoking cessation date		DD/MM/YYYY	
Were you smoking during pregnancy?	Smoker during pregnancy	0, no; 1, yes, 2, No response	
Were you exposed to second-hand smoking while pregnant (defined as >30min/ day)?		0, no; 1, yes, 2, No response	
Lifestyle (youth only)			
Current smoking	Current tobacco smoking	0, no; 1, yes, 2, No response	
Current tobacco smoking frequency	Do you currently smoke cigarettes	1, daily; 2, less than daily; 3, not at all, 4, No response	
Current exposure to second-hand smoke (defined as >30min/ day)		0, no; 1, yes, 2, No response	

Variable Name	Short Name	Description	Reference
Past smoking	Previous tobacco use	0, no; 1, yes, 2, No response	
Age of daily smoking initiation		Open-ended	
Smoking cessation date		DD/MM/YYYY	
Specific dietary aspects (all participants)			
Medically diagnosed dietary allergies or other diseases	Have you changed your diet due to any of the following	1, lactose intolerance; 2, celiac disease; 3, medical diseases under restrictive dietary schemes (sub-question-please refer); 4, other (please refer), 5, No response	
Are you a vegetarian (all sub-types) or vegan?		0, no; 1, yes, 2, No response	
Type of vegetarian diet		1, vegan; 2, lacto-vegetarian; 3, ovo-vegetarian; 4, lacto-ovo vegetarian; 5, other (pleaserefer), 6, No response	

5.2. FFQ

The Harokopio Food Frequency Questionnaire		
Food or beverage	Portion size that used as a reference	Frequency of consumption measured
DAIRY		
Full fat milk/ yoghurt	1 cup (240mL)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Low fat milk/ yogurt	1 cup (240mL)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Yellow cheese/ cream cheese	30 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
White cheese (e.g. feta cheese)	30 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Low fat cheese (light/ cottage)	30 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Egg (boiled, fried, omelet)	50 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
CEREALS, STARCHY FOODS		
White bread / toast	1 slice (30 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Wholemeal bread/ rusk	1 slice (30 g), 2 pieces	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Burger-bread	1 piece (60 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Crisp breads	2 thin pieces (20 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Cereal/ cereal bars	1/2 cup (20 g), 1 piece	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
White rice	1 cup (160 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Brown rice	1cup (195 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Pasta/ pearl barley	1 cup (140 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Whole meal pasta	1 cup (140 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Potatoes boiled/ baked/ mashed	1 medium (90 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
French fried potatoes	1/2 portion (70 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
MEAT		
Veal (steak, filet)	150 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Burger/ meat balls/ minced-meat	120 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Chicken/ turkey (all kind)	150 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Pork (steak, filet)	150 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Lamb/ goat/ game/ lamb-chops	150 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Cold sliced meats	1 slice (30 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Sausage / bacon	1 medium, 2 slices (30 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Light/ no fat cold sliced meats	30 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
FISH		
Small fish	150 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Large fish	150 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Sea-food (octopus, sleeve-fish, prawns)	150 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
LEGUMES, TRADITIONAL DISHES		
Pulses (lentils, beans, chickpeas)	1 potion (300 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Spinach-rice/ cabbage-rice	1 potion (250 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Pastitsio/ mousakas/ papoutsakia	1 portion (150 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
VEGETABLES		
Petit pois (peas) , green beans, okra, artichoke	200 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Tomato, cucumber, carrot, pepper	100 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Lettuce, cabbage, spinach, rocket	80 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Broccoli, cauliflower, courgette	100 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Greens, celery, spinach	90 g	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d

FRUIT, NUTS		
Orange	1 medium (170 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Apple, pear	1 medium (140 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Other winter-fruits	1 piece, 1/2 cup (150 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Banana	1 medium (100 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Other summer – fruits	1 piece, 1/2 cup (150 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Fruit juice	1glass (240 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Dried fruits	1/4 cup (35 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Nuts	1 coffee cup (50 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
SNACKS		
Home made pies (e.g. Cheese-pie, spinach-pie)	1 piece (150 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Pies	1 piece (150 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Toasted sandwich, sandwich	1 piece (200 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
SWEETS, SAVORY SNACKS		
Sweets made in tray	1 piece (150 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Sweet preserves, stewed fruit, fruit - jelly	1 portion (100 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Gateau, tart	1 piece (150 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Croissant, gofer, cake , biscuits	1 item, 1 slice, 3-4 pieces	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Chocolate	1 medium (60 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Ice-cream, milk-shake, cream, rice pudding	1 piece	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Chips, pop-corn	1 bag (70 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Honey, marmalade, sugar	1 tsp (5 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Olives	10 small/ 5 large	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
BEVERAGES		
Wine	1 glass (125 mL)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Beer	1 glass (240 mL)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Other alcohol drink	1 glass (30 mL)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Soft drinks	1 can (330 mL)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Light soft drinks	1 can (330 mL)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Coffee	1 cup (240 mL)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Tea, other teas	1 cup (240 mL)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
FATS		
Mayonnaise, sauce	1 Tbsp (15 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Light mayonnaise, light sauce	1 Tbsp (15 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Olive oil	3 Tbsp (45 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Seed oil	3 Tbsp (45 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Margarine	1 Tbsp (15 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
Butter	1 Tbsp (15 g)	never/ rarely, 1-3 t/mo, 1-2 t/wk, 3-6 t/wk, 1 t/d, ≥2 t/d
t/mo: times per month; t/wk: times per week; t/d: times per day		
Bountziouka V, Bathrellou E, Giotopoulou A, Katsagoni C, Bonou M, Vallianou N, Barbetseas J, Avgerinos PC, Panagiotakos DB. Development, repeatability and validity regarding energy and macronutrient intake of a semi-quantitative food frequency questionnaire: methodological considerations. NutrMetab Cardiovasc Dis. 2012 Aug;22(8):659-67. doi: 10.1016/j.numecd.2010.10.015. Epub 2011 Jan 26. PubMed PMID: 21269818.		

5.3. KIDMED

	Scoring
Takes a fruit or fruit juice every day	+1
Has a second fruit every day	+1
Has fresh or cooked vegetables regularly once a day	+1
Has fresh or cooked vegetables more than once a day	+1
Consumes fish regularly (at least 2–3 times per week)	+1
Goes more than once a week to a fast-food (hamburger) restaurant	-1
Likes pulses and eats them more than once a week	+1
Consumes pasta or rice almost every day (5 or more timesper week)	+1
Has cereals or grains (bread, etc.) for breakfast	+1
Consumes nuts regularly (at least 2–3 times per week)	+1
Uses olive oil at home	+1
Skips breakfast	-1
Has a dairy product for breakfast (yoghurt, milk, etc.)	+1
Has commercially baked goods or pastries for breakfast	-1
Takes two yoghurts and/or some cheese (40 g) daily	+1
Takes sweets and candy several times every day	-1

5.4. IPAQ

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the vigorous activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, aerobics, or fast bicycling?

___ days per week

No vigorous physical activities Skip to question 3

2. How much time did you usually spend doing vigorous physical activities on one of those days?

   ___ hours per day

   ___ minutes per day

Don't know/Not sure

Think about all the moderate activities that you did in the last 7 days. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   ___ days per week

No moderate physical activities Skip to question 5

2. How much time did you usually spend doing moderate physical activities on one of those days?

   ___ hours per day

   ___ minutes per day

Don't know/Not sure

Think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

1. During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

   ___ days per week

No walking Skip to question 7

2. How much time did you usually spend walking on one of those days?

   ___ hours per day

   ___ minutes per day

Don't know/Not sure

The last question is about the time you spent sitting on weekdays during the last 7 days. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

3. During the last 7 days, how much time did you spend sitting on a week day?

   ___ hours per day

   ___ minutes per day

Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

5.5. Youth Activity Profile

Before you begin, it is important to get some basic information about your school and about you.

Circle your Gender:	Male	Female
Circle yourSchoolLevel:	Elementary	MiddleHigh
CircleyourGrade:	3 4 5 6 7 8 9 10 1112	

How many days each week do you have PE?
a. 0 days(never)
b. 1day
c. 2days
d. 3days
e. 4days
f. 5days

How many break/study hall periods do you have per day?
a. 0
b. 1
c. 2
d. 3
e. 4

How many times last week did you attend sessions or practices for sports or structured physical activities that were led by a coach, instructor or leader
a. 0
b. 1
c. 2
d. 3
e. 4
f. 5 ormore

The Youth Activity Profile will ask you about the time you spend being active (both in school and out of school) and the time you spend being sedentary.

- **Physicalactivities**arethingsthatinvolvealotofwalking,runningormovingaround. Itincludesbiking and dancing as well as sports or outdoor play that involves a lot of movingaround.
- **Sedentary activities** are things such as watching TV, or playing video games, computer games, or hand- held games that you do in your free time. It does NOT include the time you spend sitting while eating or while doinghomework.

Most questions will ask you only to think about the last 7 days but a few questions will ask about what you typically do (on a normal week). There are no right or wrong answers so provide honest answers.

1. **Activity To School:** How many days did you **walk or bike to school**? *(If you can't remember, try toestimate)*
a. 0 days(never)
b. 1day
c. 2days
d. 3days
e. 4-5 days (most everyday)

2. **ActivityduringPhysicalEducationClass:**During**physicaleducation**,howoftenwereyourunningandmoving as part of the planned games or activities? *(If you didn't have PE, choose "I didn't have physicaleducation")*
a. I didn't have physicaleducation
b. Almost none of thetime
c. A little bit of thetime
d. A moderate amount oftime
e. A lot of thetime
f. Almost all of thetime

3. **Activity during Breaks/Study Hall:** During **breaks/study hall**, how often were you playing sports, walking, running, or playing active games? *(If you didn't have a break at school, choose "I didn't have breaks/studyhall")*
a. I didn't have breaks/study hall
b. Almost none of thetime
c. A little bit of thetime
d. A moderate amount oftime
e. A lot of thetime
f. Almost all of thetime

Elementary Version:**Activity during Recess:** During **recess**, how often were you playing sports, walking, running, or playing active games? *(If you didn't have a recess at school, choose "I didn't have recess").*

Changed option a: I didn't have recess

4. **Activity during Lunch:** During **lunch break**, how often were you moving around, walking or playing? *(If you didn't have a lunch break at school, choose "I didn't have lunchbreaks")*
a. I didn't have lunchbreaks
b. Almost none of thetime
c. A little bit of thetime
d. A moderate amount oftime
e. A lot of thetime
f. Almost all of thetime

5. **Activity from School:** How many days often did you **walk or bike fromschool**? *(If you can't remember, try to estimate)*
a. 0 days(never)
b. 1day
c. 2days
d. 3days
e. 4-5 days (most everyday)

6. ActivitybeforeSchool: Howmanydays **beforeschool(6:00-8:00am)**didyoudosomeformofphysicalactivityfor at least 10 minutes? (This includes activity at home NOT walking or biking toschool)

a. 0days
b. 1day
c. 2days
d. 3days
e. 4 to 5days

7. Activity after School: How many days **after school (between 3:00 -6:00 pm)** did you do some form of physical activity for at least 10 minutes? (This can include playing with your friends/family, team practices or classes involving physical activity but *NOT walking or biking home fromschool*)
a. 0days
b. 1day
c. 2days
d. 3days
e. 4 to 5days

8. Activity on Weeknights: How many **school evenings (6:00-10:00 pm)** did you do some form of physical activity for at least 10 minutes? (This can include playing with your friends/family, team practices or classes involving physical activity but *NOT walking or biking home fromschool*)
a. 0days
b. 1day
c. 2days
d. 3days
e. 4 to 5days

9. **Activity on Saturday:** How much physical activity did you do last **Saturday**? (*This could be for exercise, work/chores, family outings, sports, dance, or play. If you don't remember, try toestimate)*
a. No activity (0minutes)
b. Small amount of activity (1 to 30minutes)
c. Small to Moderate amount activity (31 to 60minutes)
d. Moderate to Large amount of activity (1 to 2hours)
e. Large amount of activity (more than 2hours)

10. **ActivityonSunday:**Howmuchphysicalactivitydidyoudolast**Sunday**?(*This couldbefor exercise,work/chores, family outings, sports, dance, or play. If you don't remember, try toestimate)*
a. No activity (0minutes)
b. Small amount of activity (1 to 30minutes)
c. Small to Moderate amount activity (31 to 60minutes)
d. Moderate to Large amount of activity (1 to 2hours)
e. Large amount of activity (more than 2hours)

11. TV Time: How much time did you spend **watching TV** outside of school time (*This includes time spent watching movies or sports but NOT time spent playing videogames*).
a. I didn't watch TV atall
b. I watched less than 1 hour perday
c. I watched 1 to 2 hours perday
d. I watched 2 to 3 hours perday
e. I watched more than 3 hours perday

12. Video Game Time: How much time did you spend **playing video games** outside of school time? (*Thisincludes games on Nintendo DS, wii, Xbox, PlayStation, iTouch, iPad, or games on yourphone*)
a. Ididn'treallyplayatall

b. I played less than 1 hour perday
c. I played 1 to 2 hours perday
d. I played 2 to3 hours perday
e. I played more than 3 hours perday

13. Computer Time: How much time did you spend using **computers** outside of school time? (*This doesn't include home work time but includes time on Facebook as well as time spent surfing the internet, instant messaging, playing online video games or computergames*)
a. I didn't really use the computer atall
b. I used a computer less than 1 hour perday
c. I used a computer 1 to 2 hours perday
d. I used a computer 2 to3 hours perday
e. I used a computer more than 3 hours perday

14. Phone / Text Time: How much time did you spend using your **cell phone** after school? (*This includes time spent talking ortexting*).
a. I didn't really use a cell phone
b. I used a phone less than 1 hour perday
c. I used a phone 1 to 2 hours perday
d. I used a phone 2 to 3 hours perday
e. I used a phone more than 3 hours perday

***15.* Overall Sedentary Habits:** Which of the following best describes your **typical**sedentary habits at home?*(Try to think about a typical week and not just lastweek)*
a. I spent almost none of my free timesitting
b. I spent little time sitting during my freetime
c. I spent a moderate amount of time sitting during my freetime
d. I spent a lot of time sitting during my freetime
e. I spent almost all of my free timesitting

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