ZERO HIDDEN HUNGER EU

REDUCING MICRONUTRIENT DEFICIENCIES

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Deliverable

D.2.1 – MN Biomarkers data operations flowchart

ZERO HIDDEN HUNGER EU

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MN Biomarkers data operations flowchart

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Revision history

VERSION	DATE	REVIEWER	MODIFICATIONS
1.0	26/03/2024	Mairead Kiely and Kevin Cashman	Suggested minor edits
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2.0	14/12/2025	Kevin Cashman	Edits following RP1 review

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1. Executive Summary

The *Zero Hidden Hunger EU* project will provide a best-in-class collection of reliable data on population micronutrient status for Europe and inform hidden hunger prevalence estimates. This will be based on the collation of existing individual participant data on micronutrient status from national surveys and epidemiological cohorts of European children, adolescents, adults and elderly, including ethnic subgroups, pregnant women. It will also include data from new analysis on bio-banked samples from large population-based studies conducted among these and additional participant groups to generate new individual participant data on status of priority micronutrients. This will help bridge the gap between existing and required micronutrient status data.

This Deliverable (D2.1) report presents a micronutrient biomarkers data operations schematic flowchart which charts the collation and transfer of the existing individual participant data on micronutrient status, as well as throughput of biosample collation, *de novo* analysis and associated data transfer. The schematic provides a top-level visual summary of the process, decision tree, and workflow involved in the micronutrient biomarkers data operations. This is underpinned within the Deliverable report by additional information in relation to the variable data and/or bio-samples being sought, from which studies, and how they will be managed.

Deliverable D2.1 will help the WP2 Steering Group to monitor progress in relation to existing micronutrient data transfer and bio-sample analysis for micronutrient status markers within the project.

2. Schematic workflow charting throughput of biosample collation, analysis and data transfer

2.1. Orientation and context:

The true magnitude of micronutrient malnutrition in Europe is unknown because there has never been a concerted effort to collect and catalogue reliable and valid micronutrient status biomarker data and metadata characterising a broad diversity of population groups. Thus, Work Package (WP) 2 within the *Zero Hidden Hunger EU* project will provide the best-in-class collection of reliable data on population micronutrient status for Europe and inform hidden hunger prevalence estimates in WP4.

The best-in-class collection of reliable micronutrient data will be based on:

i) the collation of existing individual participant data on micronutrient status from national surveys and epidemiological cohorts of European children, adolescents, adults and elderly, including ethnic subgroups, pregnant women; and

ii) new analysis on bio-banked samples from large population-based studies conducted among these and additional participant groups to generate new individual participant data on status of priority micronutrients. This new data will bridge the gap between existing and required micronutrient status data.

The schematic workflow charting collation and transfer of existing individual participant data on micronutrient status as well as throughput of biosample collation, analysis and associated data transfer are shown in **Figure 1**. This represents a top-level visual summary of the micronutrient biomarkers data operations flowchart and is underpinned and supported by additional detail provided in Sections 2.2-2.5 of this Deliverable report.

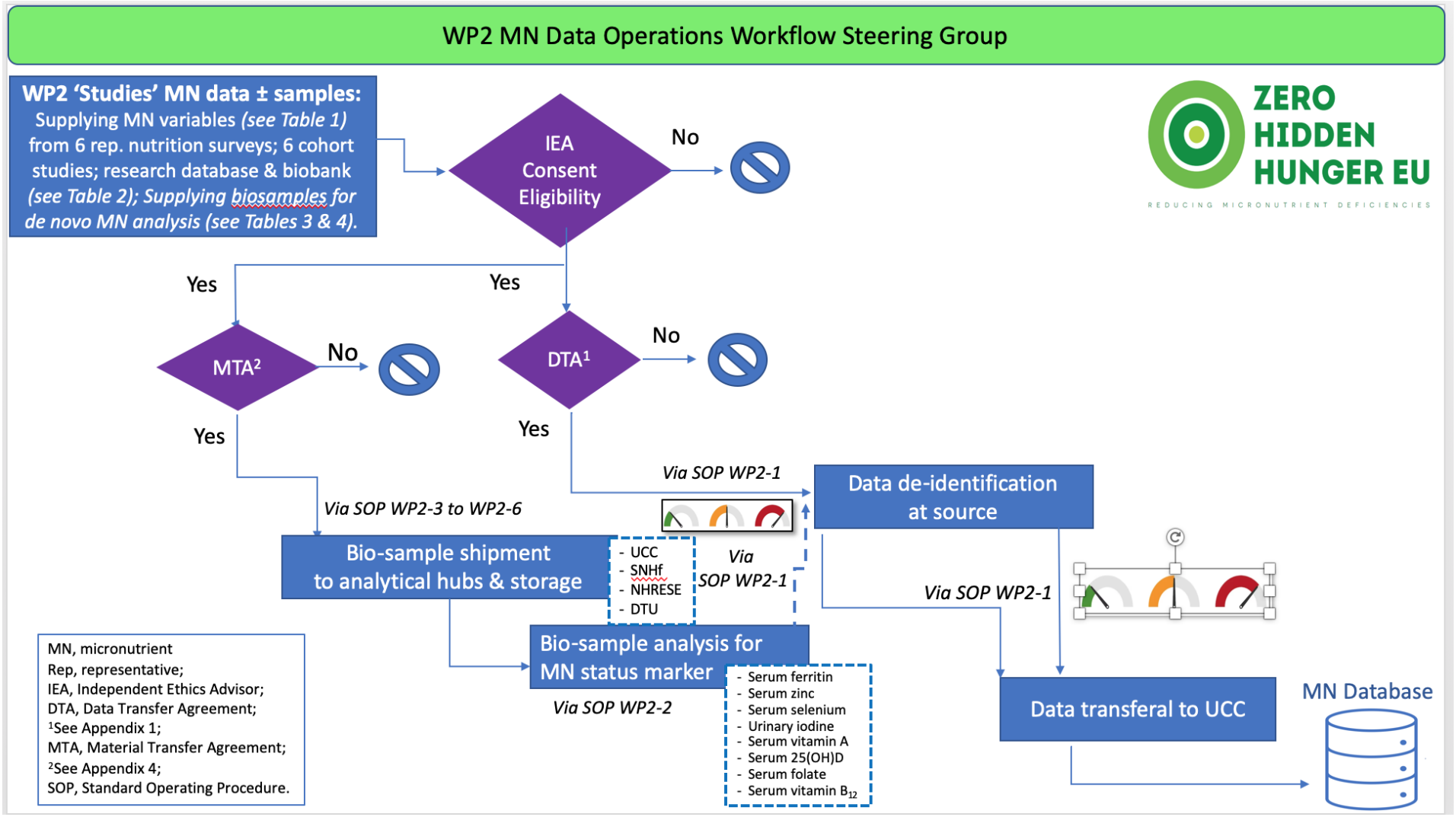

FIGURE 1. MICRONUTRIENT BIOMARKERS DATA OPERATIONS FLOWCHART

2.2. Existing micronutrient data for secondary analysis within *Zero Hidden Hunger EU*

As mentioned above, the collation of existing individual participant data on micronutrient status and intake from the prioritized at-risk population groups is a key aspect of generating the data to inform the prevalence of hidden hunger in Europe. The following provides a summary of the information being sought and how it will be managed:

2.2.1 Variables for existing micronutrient status and intake data within Zero Hidden Hunger EU.

The list of micronutrient-related variables for which existing data, at an individual participant-level, being gathered within *Zero Hidden Hunger EU* is shown in **Table 1**. These include the eight prioritized micronutrients for which a biomarker does exist (shown in 2nd column in the table), and daily average intake estimates for three micronutrients where a biomarker does not currently exist (shown in the last column in the table). There are also some additional analytes (shown in 3rd column in the table) which were used for adjustment of certain MN status biomarkers for inflammation, if deemed necessary (e.g., serum c-reactive protein [CRP] and/or serum alpha-1-acid glycoprotein [AGP]) or the normalisation of urinary iodine (e.g., urinary creatinine), as well as needed for analyses in another WP (e.g., haemoglobin which is needed for the estimation of prevalence of iron-deficiency anaemia in WP5).

TABLE 1: VARIABLES FOR EXISTING MICRONUTRIENT DATA WITHIN ZERO HIDDEN HUNGER EU

DEMOGRAPHIC	8 MN STATUS BIOMARKER	ADDITIONAL ANALYTES	3 MN INTAKE
Sex	Serum ferritin (µg/L)	Whole-blood haemoglobin (g/dL)	Calcium (mg/d)
Age (years)	Serum zinc (µmol/L)	Serum CRP (mg/L)	Magnesium (mg/d)
Weight (kg) Weight-for-length z-score [<36 months]	Serum selenium (µmol/L)	Serum AGP (g/L)	Potassium (mg/d)
BMI (kg/m^2)	Serum retinol (µmol/L) *Or* Serum retinol binding protein (µmol/L)	Urinary creatinine (mg/dL)	
Country	Serum vitamin B12 (pmol/L)		
Fasting status	Red blood cell folate (nmol/L) *Or* Serum folate (nmol/L)		
Blood-draw time	Serum total 25OHD (nmol/L)		
Employment status Highest Education level attained (adults) No. of individuals in household	Urinary Iodine (µg/L)		

CRP, C REACTIVE PROTEIN; AGP, ALPHA-1-ACID GLYCOPROTEIN; MN, MICRONUTRIENT

2.2.2 *Studies providing the existing data on micronutrient intake and status within Zero Hidden Hunger EU.*

Data for the population-appropriate, and available, micronutrient intake estimates and status marker concentrations (as per Table 1) will be gathered from 6 representative nutrition surveys; 6 epidemiological cohort studies, and a research database and biobank. Expected total *n* from this collection of WP2 studies ~54,000+.

2.2.3. *Process and management.*

The following is an overview of the process and constituent steps for processing and transferal of existing micronutrient status and intake data for secondary analysis within *Zero Hidden Hunger EU*:

i. To confirm that each WP2 study is eligible for inclusion based on previous informed consent provided, and prior to any transfer of data, each WP2 study Principal Investigator needs to provide the following documentation in relation to ethical requirements:

   a. Participant Information Leaflets (PILs) used in the study.
   b. Informed Consent Forms (ICFs) used in the study.

ii. These will be reviewed by the independent ethics advisor for the project, who will issue confirmation of eligibility for inclusion or otherwise.

iii. The independent ethics advisor will also ensure that adequate Data Transfer Agreement are in place and that they have been reviewed to ensure the requirements of Chapter V of Regulation 2016/679 (GDPR) are upheld and adhered to.

iv. Following confirmation of eligibility for inclusion, data will be de-identified using the Standard Operating Procedures (SOP) WP2-1 and prior to data transfer. De-identified data will be sent to UCC as the Data Controller via a secure method.

2.3. De novo analysis of micronutrient status markers in bio-banked samples

As mentioned above, the generation of new micronutrient status data from the *de novo* analysis on bio-banked samples from a number of key studies will help bridge the gap between existing and required micronutrient status data. The following provides a summary of the bio-samples and information being sought, from which studies and how it will be managed:

2.3.1. *Core analytical hubs for micronutrient status markers.*

SNHf, DTU, UCC, and NHRESE will provide the core analytical platforms for the 8 priority micronutrient status markers and associated analytes within the *Zero Hidden Hunger EU* project (**Table 3**). The details of the core methodology for each of these analytes are provided in SOP WP2-2 *(Appendix 1)*.

TABLE 2. CORE ANALYTICAL HUBS FOR 8 MICRONUTRIENT STATUS MARKERS WITHIN ZERO HIDDEN HUNGER EU, INCLUDING METHODOLOGY, AND MINIMUM VOLUME NEEDED FOR ANALYSIS.

Priority MN	Biomarker	Analytical lab.	Methodology	Min. sample volume
Iron	Serum ferritin	SNHf	CMIA	100 µl
Zinc	Serum zinc	SNHf	ICP-MS	100 µl
Selenium	Serum selenium	NHRESE	ICP-MS	100 µl
Iodine	Urinary iodine	DTU	ICP-MS	1.3 ml
Vitamin D	Serum 25(OH)D (WP2) Serum 25(OH)D (WP7)	UCC SNHf	LC-MS/MS ELISA	150 µl 50 µl
Vitamin A	Serum retinol *or* Serum retinol-binding protein	SNHf	LC-UV ELISA	100 µl 100 µl
Folate	Serum folate	SNHf	Microbiology	15 µl
Vitamin B_{12}	Serum B_{12}	SNHf	Microbiology	100 µl
-	Serum CRP	SNHf	Turbidimetry	100 µl
-	Urinary creatinine	DTU	Jaffe method	-*

MN, *micronutrient*; 25(OHD, *25-hydroxyvitamin D*; CRP, *C-reactive protein*; Min, *minimum*; LC-MS/MS, *liquid chromatography tandem mass spectrometry*; ICP-MS, *inductively coupled plasma mass spectrometry*; CMIA, *Chemiluminescent microparticle immune assay*; ELISA, *enzyme-linked immunoassay*; LC-UV, *liquid chromatography with ultraviolet detection*;

*covered in the volume required for urinary iodine analysis.

2.3.2. *Bio-banked samples for de novo analysis of micronutrient status.*

Bio-banked samples from 2 representative nutrition surveys; 6 epidemiological cohort studies, and a research biobank will be used for *de novo* analysis to generate new data on status of prioritized micronutrients. Target number of samples (by MN marker) are as follows: serum zinc, *n*=3,875; serum ferritin, *n*=7,376; serum retinol, *n*=3,514; serum vitamin B12, *n*=7,376; serum 25(OH)D, *n*=7,826; serum selenium, *n*=7,497, serum folate, *n*=4,007; urinary iodine, *n*=7,673.

2.3.3. *Process and management.*

The following is an overview of the process and constituent steps within the *de novo* analysis of micronutrient status in bio-banked samples:

i. As per 1.3 above, to confirm that each WP2 study providing bio-banked samples for *de novo* analysis of micronutrient status is eligible for inclusion based on previous informed consent provided, and prior to any transfer of samples, each WP2 study Principal Investigator needs to provide the following documentation in relation to ethical requirements:

   a. PILs used in the study.
   b. ICFs used in the study.

ii. These will be reviewed by the independent ethics advisor for the project, who will issue confirmation of eligibility for inclusion or otherwise.

iii. The independent ethics advisor will also ensure that adequate Material Transfer Agreement are in place and that they have been reviewed to ensure the requirements of Chapter V of Regulation 2016/679 (GDPR) are upheld and adhered to.

iv. Following confirmation of eligibility for inclusion, de-identified samples can be shipped to the relevant core analytical hubs using the SOP WP2-3 to SOP WP2-6.

The analytical hub(s) will return the results back directly to the providing WP2 study provider, who will have the ability to include this new MN status data in their own dataset together with other required existing data. The resulting final combined dataset will be de-identified using the Standard Operating Procedures (SOP) WP2-1 and prior to data transfer. De-identified data will be sent to UCC as the Data Controller via a secure method.

2.4. Schedule

The *WP2 MN Data Operations Workflow Steering Group* will coordinate micronutrient data operations from month 1 thought to month 30 of the project. The acquisition of existing micronutrient status and intake data will take place from month 1 to month 12. New micronutrient status data arising from the *de novo* analysis of micronutrient status markers during months 1 to 18, will be added to the existing collection of micronutrient status and intake data, all of which will be included in the MN database for month 24 of the project (Deliverable 2.2).

2.5. Workflow Monitoring

A bespoke online Excel-based workflow tracker (coordinated by UCC) which will allow for monitoring of progress in relation to i) existing data transfer and ii) bio-sample shipment to relevant analytical hubs and MN status marker analysis. This will be updated monthly starting at month 4 of the project. The progress will be reviewed against assigned targets within the 12-month data acquisition window and 18-month MN status analytical window.

Appendix 1: Core methodology used for de novo analysis of micronutrient status markers

SOP Number: WP2-2

Title: *Core methodology used for de novo analysis of micronutrient status marker concentrations in Zero Hidden Hunger EU*

Date: 20 March 2024

Authors: The Cork Centre for Vitamin D and Nutrition Research at University College Cork, the Swiss Nutrition and Health foundation; the Institute for Nutrition and Health Research Ltd, and the National Food Institute at the Technical University of Denmark.

Context: Four partners within the *Zero Hidden Hunger EU* project, namely University College Cork, the Swiss Nutrition and Health foundation, the Institute for Nutrition and Health Research Ltd, and the Technical University of Denmark will provide the core analytical platforms for the eight priority micronutrient status markers. This centralised approach will facilitate the *de novo* analysis of micronutrient status markers within Work package (WP) No. 2 of the project. The following sections provide a brief overview in relation to core methodology that will be applied to the WP2 bio-samples within the project:

1. Analysis of serum 25(OH)D_3 and 25(OH)D_2 by University College Cork

The concentration of total 25(OH)D (i.e. 25(OH)D_2 plus 25(OH)D_3) in serum will be measured by the *Cork Centre for Vitamin D and Nutrition Research* at University College Cork using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Full details of this method have been published (1). In brief, it is based on a liquid–liquid extraction and uses a Ultra-performance LC–MS/MS system (Waters Acquity UPLC (Triple Quadrupole) TQD®) for quantitation. This LC–MS/MS method measures the 3-epimer of 25(OH)D_3 (3-epi-25(OH)D_3), which is not chromatographically resolved from 25(OH)D_3 by most routine LC-MS/MS methods, in addition to measuring 25(OH)D_2 and 25(OH)D_3 in serum. The presence of 3-epimers of 25(OH)D can pose problems for LC–MS/MS methods because the mass and fragmentation patterns are the same as those for 25(OH)D, thus failure to account for these metabolites can result in the overestimation of 25(OH)D_2 and 25(OH)D_3 concentrations. Total 25(OH)D is calculated as 25(OH)D_3 plus 25(OH)D_2.

The method has been used in the CDC's *Vitamin D Standardization Program* (VDSP) (2) and included in the CDC's *Vitamin D Standardization-Certification Program* (VDSCP). It has also been used as core analytical platform for serum total 25(OH)D in the EC FP6 vitamin D *ODIN* project (3). The *Cork Centre for Vitamin D and Nutrition Research* is a participant in DEQAS.

In addition to this centralised analytical hub for serum 25(OH)D data in Workpackage (WP) 2, serum total 25(OH)D will also be analysed at the Swiss Nutrition and Health foundation (SNHf) in samples from the metabolomics cohorts in WP7. Details of this methodology are provided in Appendix A.

2. Analysis of serum vitamin A by the Swiss Nutrition and Health foundation

The concentration of vitamin A in serum will be measured by the SNHf using a liquid chromatographic (LC) method with UV detection following 2 pre-analytical steps. In brief, the proteins of the serum sample are precipitated with ethanol containing retinyl acetate as internal standard. The vitamin A together with the internal standard are extracted from the resulting supernatant with hexane. The extract is dried in a speed-vac and reconstituted in ethanol prior to injection on the LC system in reversed-phase mode with UV detection at a wavelength 325 nm. The vitamin A is quantified by area integration taking into account the internal standard area.

3. Analysis of serum folate by the Swiss Nutrition and Health foundation

The concentration of folate (vitamin B_9) in serum will be measured by the SNHf using a microbiological assay. In brief, the standards, controls and serum samples are diluted with ascorbate buffer. The assay bacteria (*Lactobacillus Rhamnosus* – ATCC 7469) are diluted in assay medium by gentle agitation and transferred to a 96-well plate. The standards, controls and serum samples are transferred in duplicate at different concentrations on the plate following a specific scheme. The plate is sealed with adhesive film and incubated 42 hours at 37°C in an incubator. After incubation, the plate is mixed for 30 minutes on an orbital shaker and immediately read on a microplate reader at a wavelength of 590 nm in end-point mode to measure the bacterial growth by turbidimetry. A dose response curve of absorbance unit (optical density [OD] at 590 nm) versus concentration is generated using the values obtained from the standard. Folate in the serum sample is determined directly from this curve.

4. Analysis of serum vitamin B_{12} by the Swiss Nutrition and Health foundation

The concentration of vitamin B_{12} in serum will be measured by the SNHf using a microbiological assay commercialised by Immundiagnostik AG (Bensheim, Germany). In brief, the standards, controls and serum samples are diluted with a provided buffer and then transferred into the wells of a microtiter plate coated with *Lactobacillus delbrueckii subsp. lactis*. The addition of vitamin B_{12} in either standards or serum samples gives a vitamin B_{12}-dependent growth response until vitamin B_{12} is consumed. After incubation at 37°C for 48 h, the growth of *Lactobacillus delbrueckii subsp. lactis* is measured turbidimetrically on microplate reader at a wavelength of 630 nm and a standard curve is generated from the dilution series. The amount of vitamin B_{12} is directly proportional to the turbidity.

5. Analysis of serum ferritin at the Swiss Nutrition and Health foundation

The concentration of ferritin in serum will be measured by the Laboratoires Salamin, Switzerland, on behalf of the SNHf (as a sub-contract within the project), using a Siemens Attlica analyser (Zürich, Switzerland). The serum samples will be introduced in the analyser without any treatment. The sample is measured with a CMIA (chemiluminescent magnetic microparticle immunoassay) method.

6. Analysis of serum zinc at the Swiss Nutrition and Health foundation

The concentration of zinc in serum will be measured by the Laboratoires Salamin, on behalf of the SNHf (as a sub-contract within the project), using an inductively coupled plasma - mass spectrometry (ICP-MS (ICP-MS) method. In brief, the serum sample diluted and digested in presence of nitric acid at a controlled temperature for 2 hours. Prior injection to ICP-MS, the sample is neutralised. A dose response curve versus concentration is generated using the values obtained from the standard. Zinc in the serum sample is determined directly from this curve.

For both serum zinc and ferritin, the concentrations will be adjusted for serum C-reactive protein (CRP) to account for the potential impact of inflammation (4). The concentration of CRP in serum will be measured by the SNHf using an Abbott Architect c4000 analyser (Baar, Switzerland). The serum samples will be introduced in the analyser without any treatment. The sample is measured with a turbidimetric method.

7. Analysis of serum selenium by the Institute for Nutrition and Health Research Ltd

Serum/plasma selenium concentration will be measured by ICP-MS using an Agilent 7800 ICP-MS system (Agilent Technologies Inc., Santa Clara, CA, USA) (5,6). In brief, 100 µl of serum sample is extracted using nitric acid solution. Germanium is used as an internal standard. On the ICP-MS m/z = 78 is scanned for selenium and m/z = 72 for germanium determination in enhanced He mode. The Institute for Reference Materials and Measurements (IRMM) certified reference material BCR®-638 has been used to validate the method. Results are submitted as csv files.

8. Analysis of urinary iodine by the National Food Institute at the Technical University of Denmark

The content of iodine in urine samples will be measured by the National Food Institute at the Technical University of Denmark (DTU Food). The method is based on the principles described by Huang *et al*. (7). Briefly, urine samples are diluted with an alkaline solution containing 0.5% (v/v) ammonia, 0.05% Triton X-100 and 100 µg/L of Tellurium stock solution, which is used as internal standard. The total mass concentration of iodine in the urine samples is determined by ICP-MS using an iCAP TQ ICP-MS (Thermo Fisher Scientific, Bremen, Germany) equipped with an ASX-560 autosampler and a ASXpress PLUS valve equipped with a 1 mL sample loop (Teledyne CETAC Technologies, Omaha, NE, USA). The analysis is performed using single quadrupole mode in no gas mode.

The iodine concentration in urine will be normalised against creatinine. Concentrations of creatinine will be measured at DTU Food. The method principle is based on the principles in the Jaffe method, which is an assay for creatinine determination based upon the reaction between creatinine and sodium picrate (8). Kinetic procedures based on the observed reaction rates between various substances, including creatinine, with alkaline picrate have been proposed by Fabing (9) and Soldin (10). This improved Jaffe chemistry is a kinetic procedure which does not require deproteinization of the sample and is formulated to reduce the interference in serum proteins.

Creatinine + alkaline picrate ⟶ creatinine-picrate complex (Janovsky complex)

At an alkaline pH, creatinine reacts with picrate to form Janovsky complex. The rate of increase in absorbance at 510 nm due to the formation of creatinine-picrate complex is directly proportional to the creatinine concentration present in the sample. For the creatinine determination a clinical chemistry analyzer Pentra C400 (Horiba ABX, Montpellier, France) is used using a standardised assay (11).

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Appendix A

Analysis of serum total 25(OH)D by the Swiss Nutrition and Health foundation

The concentrations of total 25(OH)D (i.e. 25(OH)D_2 plus 25(OH)D_3) in serum will be measured by the Swiss Nutrition and Health foundation using a commercial CE marked sandwich ELISA kit from Immundiagnostik AG (Bensheim, Germany). In brief, the standards, controls and serum samples are incubated with the first and second antibody in the microtiter plate for 20 minutes. The resulting vitamin D antibody-immune complex is detected with a third, peroxidase labelled antibody by adding tetramethylbenzidine (TMB). The colour development is stopped by the addition of stop solution, and the resulted yellow colour is measured at 450 nm using a microtiter plate reader. The colour intensity is positively proportional to the concentration of 25(OH)D in the sample. A dose response curve of the absorbance unit (optical density, OD at 450nm) versus concentration is generated using the values obtained from the standard. 25(OH)D in the serum sample is determined directly from this curve.