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Application and perspectives of non-invasive urinary biomarker measurements in epidemiological research on child nutrition: hydration and iodine status, two health-relevant examples

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“La tarde no se quería ir,
todo era agua agua agua.

-El niño reía-

Soltó el barco de vela,
de su boca brotó el viento
y comenzó a navegar.

Se iba, se iba, se iba,
sus ojitos detrás del barco
y él, dentro,
soñando, cantando
hasta que se hundió...

Una hoja más del cuaderno
y continuó su viaje
en otro barquito de papel.”

Humberto Ak´abal (poeta Guatemalteco1953-)

SUMMARY

Application and perspectives of non-invasive urinary biomarker measurements in epidemiological research on child nutrition: hydration and iodine status, two health-relevant examples.

Background and Aim: Non-invasive biomarkers of nutritional status provide a promising and alternative measure of dietary intakes in epidemiological research. Hydration and Iodine Status are two examples of important predictors of long-term health and cognitive performance, especially in children, for which urinary biomarkers exist. **The aim** of the present thesis was to exemplary examine the application of these urinary biomarkers for the investigation of the interactions with dietary patterns in children and also to methodologically check long-term stability of urinary parameters used for the present and for additional biomarker analyses. Databases for the four consecutively conducted studies were the prospective Dortmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Study, which collects data on diet, growth and metabolism in healthy children from birth until young adulthood.

Results: To provide information on possible analytical measurement errors, the stability and validity of ca. 20 chemical urinary analytes frequently measured in the DONALD Study were evaluated at baseline and after 12 or 15 yr of storage under moderate freezing conditions (-22º C) and without use of preservatives **(Study I: methodological pre-analysis)**. 24-h Urinary concentrations of most of the analyzed metabolites (e.g. creatinine, urea, iodine, nitrogen, sodium, potassium, magnesium, calcium, ammonium, bicarbonate, citric&uric acid*)* were stable after the particular collection and storage conditions. The application of the hydration status biomarker "free water reserve" (a parameter comprising osmolality, urine volume) was investigated in **Study II.** The physiological effect of consuming fruit and vegetables (F&V) on hydration status in healthy children was analysed in 4-10 y old DONALD participants (n= 424, with 1286 repeated measurements). The results showed that an additional intake of 100 g of F&V (in solid form), or 100 mL F&V (as juice) would increase the total body water by ~ 40 mL, independent of the intake of other important dietary water sources (i.e. plain water, water from beverages and milk). In **Studies III** and **IV**, iodine status assessment using urinary iodine excretion was explored. **Study III** assesses the suitability of the currently recommended epidemiological parameter urinary iodine concentration measured in spot urines in n=180 6-18 y-old children, who in parallel collected one spot and one 24-h urine sample. Results strongly suggest that spot urine iodine concentration relevantly depending on hydration status, reasonably reflects true 24-h iodine excretion only when scaled to parallel creatinine excretion. The longitudinal analyses of **Study IV** (n=516 6-12 y-olds, with 1959 repeated measurements) demonstrated that an increase in dietary animal to plant protein ratio was significantly associated with an increase in 24-h urinary iodine excretion. Although this association was partially mediated by salt intake, it underlines one of the positive aspects of a limited, not exclusively plant-based nutrition.

Conclusions: The results of the present thesis have shown in four studies the high potential but also the pitfalls in the application of urinary biomarker measurements in epidemiological research. The long term storage stability of most of the urinary analytes makes "urine" a suitable and reasonably valid tool in epidemiological settings for later quantification. In large epidemiological studies commonly only spot urines instead of 24-h urines can be collected. In this regard it could be shown that hydration status can strongly affect renal concentration parameters and requires a correction by creatinine measurement. A high F&V intake provides a high potential to improve hydration status of children, however at the same time, a more plant based diet may somehow negatively affect their iodine status. Since limited salt and increased intake of plant-based foods are part of a preferable healthy food pattern, effective nutrition political strategies will be required in the future to ensure appropriate iodine nutrition in adherent populations. Future application of the nutritional biomarkers (such as these examined here) in a broader context may open new possibilities for researchers to explore non-invasively the role of diet and prevention of diseases, and therefore contribute importantly in the area of nutritional epidemiology.

ZUSAMMENFASSUNG

Hintergrund und Zielsetzung: Nicht-invasive Biomarker des Ernährungsstatus sind ein vielversprechendes und alternatives Maß für die Ernährungszufuhr in der Epidemiologie. Hydratations- und Jodstatus sind Beispiele für wichtige Prädiktoren für eine langfristige Gesundheit und die kognitive Leistungsfähigkeit besonders für Kinder, für die es Urin-Biomarker gibt. **Das Ziel** der vorliegenden These war es, exemplarisch die Anwendung dieser Urin-Biomarker zu untersuchen um Interaktionen mit den Ernährungsgewohnheiten von Kindern festzustellen und die langfristige Stabilität der Urinparameter, die für diese und weitere Biomarker-Analysen genutzt wurden, zu überprüfen. Die Datengrundlage für die vier durchgeführten Studien war die Dortmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Studie, welche Daten zu Ernährung, Wachstum und Metabolismus von gesunden Kindern von der Geburt bis ins junge Erwachsenenalter sammelt.

Ergebnisse: Um Informationen über potentielle analytische Messfehler zu erlangen, wurden die Stabilität und die Validität von ca. 20 chemischen Urin-Analyten, welche häufig in der DONALD Studie gemessen werden zu Beginn und nach 12 oder 15 Jahren Lagerung unter moderaten Gefrier-Bedingungen (-22° C) und ohne Gebrauch von Konservierungsmitteln **(Studie 1: methodologische Voranalyse)** evaluiert. Die 24-Stunden Konzentrationen der meisten analysierten Metabolite (z.B. Kreatinin, Jod, Stickstoff, Natrium, Kalium, Calcium, Ammonium, Bicarbonat, Zitronen- und Harnsäure) waren nach der Sammlung zu gegebenen Lagerbedingungen stabil. Die Anwendung des Biomarkers für den Hydratations-Status, die „freie Wasser Reserve“ (ein Parameter, welcher die Osmolalität und das Urinvolumen umfasst) wurde in der **Studie II** untersucht. Der physiologische Effekt des Obst- und Gemüsekonsums (O&G) auf den Hydratations-Status von gesunden Kindern wurde bei 4-10-jährigen Teilnehmern der DONALD Studie (n = 424, mit 1286 Messwiederholungen) analysiert. Die Ergebnisse zeigten, dass ein zusätzlicher Verzehr von 100 g O&G (in fester Form) oder 100 mL O&G als Saft das Gesamt-Körperwasser um 40 mL erhöhen würde, unabhängig von der Aufnahme anderer für den Hydratations-Status wichtiger Nahrungsmittel (d.h. Trinkwasser, Wasser aus Getränken und Milch). In den **Studien III** und **IV** wurde die Messung des Jod-Status anhand der Jodausscheidung im Urin untersucht. **Studie III** überprüfte, ob die Jod-Konzentration im Urin, welche in n=180 Spontanurinen von 6-18-jährigen Kindern gemessen wurde, den aktuellen epidemiologischen Empfehlungen entspricht. Die Kinder sammelten parallel zum Spontan-Urin einen 24-Stunden-Urin. Die Ergebnisse lassen stark vermuten, dass die Jod-Konzentration im Spontan-Urin, welche vom Hydratations-Status abhängt, die wahre 24-Stunden-Jod-Ausscheidung nur reflektiert, wenn gleichzeitig die Kreatininausscheidung betrachtet wird. Die Analyse **der Studie IV** (n=516 6-12 jährige, mit 1959 Messwiederholungen) zeigte, dass ein Anstieg des Verhältnisses von tierischem zu pflanzlichem Protein signifikant in Zusammenhang mit einem Anstieg der Jod-Ausscheidung im 24-Stunden-Urin stand. Obwohl dieser Zusammenhang teilweise durch die Salz-Aufnahme erklärt werden konnte, unterstreicht er einen der positiven Aspekte einer limitierten, nicht nur pflanzen-basierten Ernährung.

Schlussfolgerungen: Die Ergebnisse konnten in vier Studien das große Potential, aber auch die Hindernisse in der Anwendung von Urin-Biomarkern in der Epidemiologie zeigen. Die Lagerstabilität über einen langen Zeitraum der meisten Urin-Analyten macht Urin zu einem angemessenen und guten Werkzeug in epidemiologischen Settings zur späteren Quantifizierung. In großen epidemiologischen Studien können für gewöhnlich nur Spontan-Urine, anstatt von 24-Stunden-Urinen, gesammelt werden. Es konnte gezeigt werden, dass sich der Hydratations-Status stark auf die renalen Konzentrations-Parameter auswirken kann und eine Korrektur durch die Kreatinin-Messung benötigt. Eine hohe Zufuhr an O&G zeigt großes Potential, den Hydratations-Status von Kindern zu verbessern. Gleichzeitig scheint sich eine eher pflanzenbasierte Ernährung negativ auf den Jod-Status auszuwirken. Da eine begrenzte Salz-Zufuhr und eine erhöhte Zufuhr pflanzlicher Nahrungsmittel zu einer zu bevorzugenden, gesunden Ernährungsweise zählen, werden effektive ernährungspolitische Strategien in der Zukunft nötig sein, um eine angemessene Jodversorgung besonders in diesen Populationen zu sichern. Die zukünftige Anwendung von Ernährungs-Biomarkern (wie die hier untersuchten) in einem größeren Kontext könnte neue Möglichkeiten für Wissenschaftler eröffnen, nicht-invasiv die Rolle der Ernährung und die Prävention von Krankheiten zu erforschen und folglich einen wichtigen Beitrag in dem Gebiet der Ernährungsepidemiologie leisten.

RESUMEN

Aplicación y perspectivas del uso no-invasivo de biomarcadores urinarios para la investigación epidemiológica en nutrición infantil: hidratación y yodo, dos ejemplos de nutrientes relevantes para la salud.

Antecedentes y objetivo: los biomarcadores no invasivos del estado nutricional son herramientas que proporcionan medidas más objetivas y alternativas de dieta en investigación epidemiológica. Estado de Hidratación y Yodo, son dos ejemplos de importantes predictores de salud a largo plazo y especialmente en los niños en el rendimiento cognitivo, y para los cuales existen biomarcadores urinarios. El objetivo de la presente tesis fue examinar, a través de ejemplos concretos, la aplicación de estos biomarcadores urinarios y sus interacciones con patrones dietéticos de los niños; y también para comprobar metodológicamente la estabilidad a largo plazo de los parámetros urinarios utilizados para el presente y para el análisis adicional de biomarcadores. La base de datos para los cuatro estudios realizados consecutivamente fue obtenida del "Estudio nutricional y antropométrico longitudinal de niños y adolescentes de Dortmund (DONALD Study)", un estudio observacional sobre dieta, crecimiento y el metabolismo en los niños sanos, desde el nacimiento hasta la edad adulta.

Resultados: Para proporcionar información sobre posibles errores de medición analíticos, la estabilidad y la validez de alrededor de 20 analitos urinarios químicos, frecuentemente medidos en el Estudio DONALD fueron evaluados al inicio del estudio y después de 12 o 15 años de almacenamiento en condiciones de congelación moderada (-22º C) y sin el uso de conservantes (**Estudio I: pre-análisis metodológico**). Las concentraciones urinarias de 24-h de la mayoría de los metabolitos analizados (Ej. creatinina, urea, yodo, nitrógeno, sodio, potasio, magnesio, calcio, amonio, bicarbonato, acido cítrico y ácido úrico) se mantuvieron estables después de las condiciones particulares de recolección y almacenamiento. La aplicación del biomarcador para estado de hidratación "Free Water Reserve" (un parámetro que combina la osmolalidad y volumen de orina) se investigó en el **Estudio II**. El efecto fisiológico de consumir frutas y verduras (F & V) en el estado de hidratación en los niños sanos se analizó en niños de 4 a10 años de edad participantes del estudio DONALD (n = 424, con 1286 mediciones repetidas). Los resultados demostraron que una ingesta adicional de 100g de F & V (en forma sólida), ó 100 ml F & V (como jugo) aumentaría el agua corporal total en ~ 40 ml, independiente de la ingesta de otras fuentes dietéticas de agua (es decir, agua pura, agua de bebidas y leche). En los **Estudios III y IV**, se exploró la evaluación del estado de yodo mediante la excreción urinaria de éste. El **Estudio III** evalúa la idoneidad del parámetro epidemiológico actualmente recomendado para evaluar estado nutricional de yodo (concentración de yodo en muestras de orina) en n = 180 niños y adolescentes de 6 a18 años de edad, que contaban con muestras de 24-h de orina, con una muestra espontánea de orina en paralelo. Los resultados sugieren que la concentración de yodo medida en orina espontánea es dependiente del estado de hidratación, y puede ser comparada razonablemente a la excreción de yodo en 24 horas - sólo cuando se corrige a la excreción de creatinina - usando un método escalonado. Los análisis longitudinales del **Estudio IV** (n = 516 de 6-12 años de edad, con 1959 mediciones repetidas) demostraron que un aumento en la proporción de relación de proteína animal/vegetal en la dieta está asociada significativamente con un aumento de la excreción urinaria de yodo en 24-h. Aunque esta asociación fue parcialmente mediada por la ingesta de sal, resalta uno de los aspectos positivos de una dieta limitada, no exclusiva nutrición basada en productos de origen vegetal.

Conclusiones: Los resultados de la presente tesis demuestran, en cuatro estudios, el alto potencial, así como las dificultades en la aplicación del uso de biomarcadores urinarios en la investigación epidemiológica. La estabilidad para el almacenamiento a largo plazo de la mayoría de los análisis urinarios hace "la orina" una herramienta adecuada y razonablemente válida para cuantificar mas tarde en entornos epidemiológicos. En grandes estudios epidemiológicos comúnmente sólo se recolectan muestras de orina espontánea en lugar de las muestras de 24 h. En este sentido, se pudo demostrar que el estado de hidratación puede afectar fuertemente los parámetros de concentración renal y requiere una corrección mediante la medición de la creatinina. Un alto consumo de F&V ofrece un alto potencial para mejorar el estado de hidratación de los niños. Sin embargo, al mismo tiempo, una dieta basada en más productos de origen vegetal puede afectar de alguna manera negativa su estado de yodo. Puesto que el uso limitado de la sal y el aumento de la ingesta de alimentos de origen vegetal son parte de un preferible patrón alimentario saludable, se requerirán estrategias políticas de nutrición eficaces en el futuro para garantizar una nutrición adecuada de yodo en las poblaciones adherentes. Futura aplicación de los biomarcadores nutricionales (como los examinados aquí) en un contexto más amplio, puede abrir nuevas posibilidades para que los investigadores puedan explorar de forma no invasiva el papel de la dieta y la prevención de las enfermedades, y por lo tanto, contribuir de manera importante en el área de la epidemiología nutricional.

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LIST OF ABBREVIATIONS

AI	Adequate Intake
ADH	Antidiuretic Hormone
95% CI	95% Confidence Interval
BMI	Body Mass Index
BSA	Body Surface Area
BMR	Basal Metabolic Rate
CR	Creatinine
CV	Coefficient of Variance
Cys	Cystacin C
DGE	Deutsche Gesellschaft für Ernährung e.V. (German Nutrition Society)
DONALD	Dortmund Nutritional and Anthropometric Longitudinally Designed
DRI	Dietary Reference Intakes
EAR	Estimated Average Requirement
EsKiMo	Eating Study as a KiGGS Module (Ernährungsstudie als KiGGSModul)
$est24h\text{-}UIE_{crea}$	Creatinine-scaled Estimate of 24 hour Iodine Excretion
$est24\text{-}UIE_{assumedVOL}$	Estimated 24 hour Urinary Iodine Excretion from average 24 hour Urine Volume
F&V	Fruit and Vegetables
$F\&V_{juice}$	Fruit and Vegetable Juice
$F\&V_{solid}$	Solid Fruit and Vegetables
FWR	Free Water Reserve
HCL	Hydrochloric Acid
HFG	Hepatocyte Growth Factor
HS	Hydration Status

I-CR	Iodine-Creatinine Ratio
IDDs	Iodine Deficiency Disorders
IL-18	Interleukin 18
IOM	Institute of Medicine
KiGGS	German Health Interview and Examination Survey for Children and Adolescents (Kinder- und Jugendgesundheitssurvey)
KIM-1	Kidney Injury Molecule-1
LEBTAB	In-house Food and Nutrient Database (LEBensmittelTABelle)
mEq/L	Miliequivalent per Liter
mmol/L	Milimol per Liter
mosmol/Kg	Miliosmol per Kilogram
µmol/L	Micromol per Liter
µg/L	Microgram per Liter
NAE	Renal Net Acid Excretion
$NaHCO_3$	Sodium Bicarbonate
NGAL	Neutrophil Gelatinase Associated Lipocain
6-OHMS	6-hydroxy Melatonin Sulfate
OR	Odds Ratios
POsm	Plasma Osmolality
Q1	Quartile 1
r	Correlation Coefficient
RDA	Recommended Dietary Allowance
SD	Standard Deviation
SDS	Standard Deviation Score

SE	Standard Error
T1	Tertile 1
TWI	Total Water Intake
UCP	Urinary C-petide
UIC	Urinary Iodine Concentration
UIE	Urinary Iodine Excretion
24-h	24 hour
24h-UIE	24 hour Urinary Iodine Excretion
Uosm	Urine Osmolality
USDA	US Department of Agriculture
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

1. Introduction

Application and perspectives of non-invasive urinary biomarker measurements in epidemiological research on child nutrition: hydration and iodine status, two health-relevant examples.

Child nutrition has a central role in the prevention of chronic-diseases. Thus, in epidemiological settings the ability to obtain data that helps understanding the relationship between diet and metabolism is crucial. The development of evidence-based clinical guidance, and effective programs and policies to achieve global health promotion and disease prevention, depends on the availability of valid and reliable data [(1)]. In this regard, the assessment of collected data frequently requires or depends on the use of objective biomarkers that reflect nutrient exposure, status, and functional effects [(2,3)]. Despite the rapidly advancing application of nutritional biomarkers as tools in nutritional research, the nutrition community has recognized the lack of appropriate nutritional biomarkers as one major gap in knowledge that requires further exploration [(2,4)].

Biomarkers determined from urine samples have emerged for detecting and predicting changes in nutritional status and nutrient intakes (e.g. iodine, protein, water, sodium, folate) [(5–10)], and are particularly attractive candidates for application in nutritional research because they are non-invasive and may be relatively easily accessible for large-scale protocols [(6,11)].

Water and *Iodine* are two examples of essential components of the human diet, crucial in child nutrition, for which established urinary biomarkers for nutritional status evaluation exist [(8,12–16)]. Although for hydration status controversy exists about the best biomarkers for its assessment [(17)], the free water reserve, a parameter combining urine osmolality and other urinary parameters, is probably one of the best markers for predicting *euhydration* [(16)]. The free water reserve as marker of hydration has proven its relevance in early studies for the development of adequate water intake recommendations [(15,18)]; however, systematic studies exploring long-term effect of fluids and food intake on hydration are limited. Iodine is a micronutrient of public health importance in both developed and developing countries [(19,20)]; thus Iodine is one of the nutrients that has been included and reviewed in the initiative called Biomarkers of Nutrition for Development (BOND) for application in nutritional research, policy and program development [(21)]. The latter and other body of literature supports the use of urinary iodine as the preferred biomarker for iodine status, however still challenges in the assessment and interpretation of this for potential use as biomarker exist [(13,21)]. For Iodine status, the ideal biomarker is the assessment of urinary iodine excretion over 24-h that reflects iodine intake the best; however collection of 24-h urine samples has limitations for

application especially in large-epidemiological settings[12]. The current recommended assessment of iodine status in populations involves the measurement of iodine concentration in spot urine samples [22], however, factors such as daily variation on hydration status among others might falsely under-or overestimate iodine deficiency prevalence in populations.

Therefore, to provide a better understanding of several issues related to the potential use and pitfalls in the application of biomarkers in epidemiological research in children, the overall **aim** of the present thesis was to exemplary examine the application of urinary biomarkers for hydration and iodine status and their interaction with dietary patterns. The database for this purpose was the DOrtmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Study, which prospectively collects information on diet, growth, and metabolism in healthy free-living children from birth until young adulthood. Furthermore, the potential influence of storage conditions (temperature -22 ºC and urine-preservative free) over time on urinary analytes was examined in a sub-set of urines from the DONALD urine biobank for the present and for additional biomarker analysis.

Outline

A general background is presented (**Chapter 2**) where the main concepts for nutritional biomarkers, especially the measurement of urinary biomarkers for hydration and iodine are summarised. Since the focus of this thesis was to illustrate with practical examples how the urinary parameters may be useful for nutritional status assessment, in this chapter also description of issues related to assessment of hydration and iodine status are included. The research questions are formulated in **Chapter 3**. A general methodology section (**Chapter 4**) describes the DONALD Study as well as specific methodological considerations relevant to all or the analysis included in this thesis. The research questions will be addressed in a series of analyses of DONALD sub-samples which are referred as **Studies I-IV**. These studies are individually presented in each sub-section of **Chapter 5**. The general Discussion (**Chapter 6**) summarizes and evaluates the main results of the studies in a broader context. Finally, **Chapter 7** provides overall conclusions and ideas for future research.

2. Theoretical Background

2.1 Nutritional biomarkers

A nutritional biomarker can be any biological specimen that is an indicator of nutritional status with respect to intake or metabolism of dietary constituents. A biomarker can be a biochemical, functional or clinical index of status of an essential nutrient or other dietary constituent[3]. The fundamental role of biochemical parameters in assessing nutritional status has been recognized since the early 1980s, and since that time there have been many technical advances in the area of biomarkers as well as breakthroughs in the areas of genetic and metabolism [23]. According to Potischman & Freundheim et al [3], nutritional biomarkers can be used as *1)* means of validation of dietary instruments; *2)* surrogate indicators of dietary intake; or 3) integrated measures of nutritional status for a nutrient; however many biomarkers can fall into more than one of these categories.

Nutritional biomarkers are basically applied in four different areas, the first main field is in "*general research*", including basic research and understanding the role of nutrition in biological systems e.g serum retinol for Vitamin A intake [24,25] or the effect of genetic polimorfism on ß-carotene conversion and vitamin A metabolism [26]. For a biomarker to be used for validation of a dietary instrument, it should have a strong direct relationship with dietary intakes and be an independent assessment of the dietary intake of the nutrient of interest, as for example, the use of urinary nitrogen as a marker of dietary protein [9]. Nutrients and food components can vary considerably for the same food depending on where or how the food was grown or how it was processed. In these cases, a biomarker may be a better indicator of dietary intake. Examples of this type of biomarker would include iodine [21]. The other field where nutritional biomarkers have application is in *clinical care*. Nutritional biomarkers are also use in surveillance to identify populations at risk, monitoring, and evaluation of public health programs, for example specific programs are in place to increase the intake of micronutrients from food and supplementary sources (eg, food fortification and promotion of dietary diversity) as it has been the case of Iron [27], and finally in the evaluation of the evidence base to make national or global policy about diet and health. Each use has its own specific user needs, as well as overlapping needs [2,3].

The ability to assess the health impacts of nutritional status as it has been noted by different authors, depends on the availability of accurate and reliable biomarkers that reflect nutrient exposure, status, and effect [2,4]. Biomarkers for nutrition application, are essential in this regard, however to date, there is no general consensus in their use and application [2,28]. This has been highlighted by other authors [2,28,29], as they have emphasized the lack of clarity in the definition of biomarkers and their application and purpose. The confusion arises from the limitations that biomarkers have, for instance a biomarker may be a useful index of

nutrient exposure but not necessarily reflect nutrient status [1,3].

Biomarkers are desirable for their ability to more accurately assess nutritional intake/status versus self-reported methods. They are also valuable in studies where it is necessary to validate self-reported intake measures, or to evaluate intake of dietary items when food composition databases are inadequate. For example dietary iodine intake is particularly difficult to quantify for the general public from food-composition databases, because iodine content from food depends on the soil content of iodine; the main source of iodine is iodized-salt, and the content of iodine varies depending on countrie's regulations and purchase of iodized salt for home consumption. None of these issues can be addressed with dietary assessment instruments. In addition, many processed foods that are major contributors of salt to the diet may also provide iodine depending on the source of salt (iodized/non iodized), and this information is also unavailable using dietary assessment techniques [21]. In a more epidemiological application, biomarkers provide the basis for studies associating dietary intakes with disease risk and nutritional status [4,23]. However, despite the objectivity and value of using biochemical markers of nutrients, it is necessary to consider the factors related to specific biochemical markers - and amount of nutrients present in the diet, e.g. variation between individuals in physiology and nutrient metabolism, and absorption [1,28].

Biomarkers can be categorized into short-term (reflecting intake over the past hours/days), medium-term (reflecting intake over weeks/months) and long-term markers (reflcting intake over months/years), with the type of sample used being a main determinant of time (e.g. urine, blood, hair, adipose tissue) [1]. Because nutritional biomarkers are of importance in clinical and epidemiological research, a growing body of literature referring to dietary biomarkers since the early eighties and more recently with the genomic era is evolving. A recent literature review by Hedrick et al. [4] has summarized the currently available information on the use of dietary biomarkers for nutritional status. According to this review, the lack of nutritional biomarkers represents a knowledge gap in nutritional sciences that requires further research. Specifically, as it is expressed in this review, the two main cores that need to expand upon dietary assessment methods, is the development of biomarkers that can predict functional outcomes and chronic diseases; and the need to improve dietary assessments and planning methods. Although the simplicity of the concept, dietary biomarkers are not without limitations, cost and degree of invasiveness, therefore the need for non-invasive, inexpensive and specific dietary markers is clear [4].

2.2 Urinary biomarkers in nutrition

Biobanks, for their use and value in the development of biomarkers are important and the quality of biological samples and data is essential. A variety of biologic specimens can be obtained to evaluate the nutritional status of the individual or population. Most of the commonly used biologic samples in nutritional sciences (e.g. blood, plasma, urine, and

feaces) could be suitable to be obtained even in large-scale studies [1]. However, the collection of some types of specimens for epidemiologic or surveillance studies are less feasible and unpractical leading to subject burden and logistic considerations. Thus, the choice of the biological specimen depends much on the purpose of the study and the different biological and methodological issues, which will not be addressed here in detail, since they have been amply discussed and cited in previous reviews by various authors [1,23,28,30].

In general, health researchers have long been interested in measures, including biomarkers that can be collected non-invasively, with minimal discomfort and subject burden. At the same time, such measures need to represent the biological mechanism or phenomena of interest [1,4,23,30]. For evaluation of nutritional issues, studies that require fecal or urine samples could be intuitively informative and diminish subject burden because they are non-invasive [1,3,31].

In nutritional research "urine" has become one of the more attractive bio-fluids for clinical [32] and epidemiological research [6,11,33–36]. Urine is rich in a variety of proteins, metabolites that are either filtered or secreted into, or shed by the urinary tract [37]. The physical properties and chemical composition of urine are highly variable and are determined in large measure by the quantity and the type of food consumed. The weight of solute particles is constituted mainly of urea (73.0%), chloride (5.4%), sodium (5.1%), potassium (2.4%), phosphate (2.0%), uric acid (1.7%), and sulfate (1.3%) [38]. Urine may be useful for investigating water-soluble nutrients, but one limitation of its general application is that urine output depends on nutrient saturation of tissues and dietary intake, so this measure may only be relevant for nutrients with a consistent intake [3]. However, there are biomarkers that are used primarily as b*iomarkers of the validity of dietary assessment*, in this respect some examples of the already outperformed biomarkers of nutrition examined in urine are: 24-h urinary sodium as marker of salt [5,6,39,40]; 24-h urine nitrogen, which is the most well-known biological marker of protein intake [9,41]; 24-h urinary iodine excretion as biomarker of iodine intake [7,12]; urine osmolality as marker of hydration [8,14].

For the urinary content of nutrients or their degradative products, a 24-h collection can be required, which is the so called "*reference standard*", however complete 24-h urines deserve intensive efforts and are mostly not practicable to conduct in large-settings or epidemiological studies [12,13]. Compared to 24-h urine samples, spot urine samples are the urinary specimen of choice for most large-scale studies. However, one of the limitations of using spot-urine samples in studies, is the known high dependency on fluid intake [7,12]. Thus, the development of methods that allow the hydration-status independent use of spot urines would be beneficial for large-scale studies of populations. To overcome the dependency of the analyte concentration value (measured in spot urine samples) different approaches have been proposed. One solution would be relating the measured concentration value to an "*expected*

24-h urine volume". However, this is in general no promising approach due to the daily individual variation of mean fluid intake (caused by e.g. varying physical activity, seasonality, temperature), or even due to the notable differences in fluid intake between age-groups in one population [42,43]. To control for this phenomenon, different methods have been suggested instead [43–45]. Vought and London were one of the first who recommended adjusting spot urine measurements for *creatinine* [31,46,47] due to its relatively constant excretion throughout the day, and within and across populations. Urinary creatinine is regarded to be one of the most stable analytes [48,49], and creatinine output is frequently used to check roughly the completeness of 24-h urine collections [37,50] or to estimate the 24-h excretion rates of certain analytes from the respective ratio of analyte to creatinine concentrations [43,44,46]. Creatinine, however, is also determined by anthropometric characteristics e.g. height, sex; thus the application of age- and- sex stratified 24-h creatinine reference values has been suggested as a more accurate approach to assess 24-h analyte excretions from analyte/creatinine ratios in spot urine samples in children. Remer et al, [50] showed the successful applicability of using this approach to estimate 24-h excretion rates of urinary analytes such as calcium, deoxypiridinoline and dehydroepiandrosterone sulfate quantified in spot urine samples.

Storage and laboratory considerations

As described by Blanck et al [23], in a review of the *Laboratory Issues for Nutritional Biomarkers,* there are critical methodological points in this context that need to be considered in order to reduce the measurement error associated with specimen collection and analytical measurements. According to Blanck et al, in general at least four methodological considerations should be taken into account when choosing an appropriate nutritional biomarker: 1) *validity* (how well the biomarker is measured in relation to its true value); 2) *precision* (how repeatable is the measure); 3) *sensitivity* (how well does the biomarker identify individuals with the condition); and 4) *specificity* (how well does the biomarker identify individuals without the condition) [23]. Measurement error can lead to bias in measuring the association between nutritional exposure and outcome. The specific "measurement error" types i.e definition, assessment, and effect on epidemiological studies, will not be described here, since it has been dealt with amply by other authors [23,28,51]. It has been suggested that for epidemiological studies ideally the coefficient of variation (CV) of the measurement of the respective nutrients should not be > 5% and the CV of the respective assay should be included in the publications [1,3,23,28]. For the objective of this thesis, we applied this minimal level of accuracy in general for the biochemical analytes and not just for the nutritional biomarkers here evaluated: i.e. iodine and osmolality.

Separately from issues of measurement errors, another important aspect on the use of biomarkers is the "quality control in long-term storage". For instance, investigators often do not know all of the potential analyses at the time point of urine sample collections and

measurements. For example in the case of urine collections, they simply store additional aliquots of the urine samples in the hope that the urine will be adequately stored for new hypotheses that will emerge [23]. One concrete example is that of the first National Food Consumption Survey of Germany performed between 1986 and 1988. In that study around two-thousand 24-h urine samples had been collected and several nutritional biomarkers have been analyzed. Years later, it became clear that the additional measurement of osmolality in the available aliquotes (along with information gathered with regard to nutritional and anthropometrical data) served to examine in detail the water balance through the adult life span [18].

2.3 Assessment of hydration status

Water is the largest single constituent of the human body and is essential for cellular homeostasis and life [15,17,52]. Water provides the solvent for biochemical reactions, is the medium for material transport, has unique physical properties (e.g., high specific heat) to absorb metabolic heat, and is essential to maintain blood volume to support cardiovascular function and renal filtration [53]. One review of the literature addressing water and hydration, has acknowledged the important role of "*wate*r" and adequate hydration to prevent a range of physiological disorders and diseases, especially in children [17].

The human body water content varies with body composition (lean and fat mass), for instance infants and children have higher body water- as percentage of body weight compared to adults, mainly because of the higher water content in the extracellular compartment in children. As body composition changes (observed in the first year of life), water content of the fat free mass decreases and protein and minerals are increasing [54]. Actual *Hydration status* is determined by the "Water Balance" described below.

Water balance

Under usual conditions of moderate ambient temperature (18–20 ºC) and with a moderate activity level, body water remains relatively constant. This implies a precise regulation of water balance: over a 24-h period, intake and loss of water must be equal. It has been estimated that water balance is regulated within 0.2% of body weight over a 24-h period [55].

The water balance is determined by the "water inputs" and "water outputs". *Water inputs* are composed of three major sources: drinking water, water from foods and water metabolically produced. Drinking water is essentially composed of water and other liquids with a high water content (85 to ~90%). Water content from foods comes from various foods with a wide range of water content (40 to~80%). Metabolic water results from the oxidation of macronutrients (endogenous or metabolic water) [16,53]. It is normally assumed that the contribution of food to total water intake is 20–30%, whereas 70–80% is provided by

beverages. This relationship is not fixed and depends on the type of beverages consumed and on the choice of foods [56]. For an individual at rest under temperate conditions, the volume that might be drunk in a day is on an average 1.5 L. This has to be adapted according to age, gender, climate and physical activity. The water content of foods can vary within a wide range, and consequently the amount of water contributed by foods can vary between 500 mL and 1 L a day. Endogenous or metabolic water represents about 250–350 mL a day in sedentary people [57]. The adequate total water intakes for children are dependent on age, physical activity, climate and solute renal load [16], as it will be later described in this thesis.

The *water outputs* are represented mainly via the body water losses through kidneys (obligatory renal water losses), skin and respiratory tract and in a very low level, through the digestive system. The water losses that are lost by evaporation through the skin are called "insensible perspiration" and they represent about 450 mL water per day (in a temperate environment) [14,16].

In its simplest form, the net body water balance is generally the "zero sum" of food (water and solute) and fluid intake, minus insensible and obligatory renal water losses. The water balance is highly regulated by subtle hormonal changes, inducing thirst sensation and water reabsorption in the kidneys. Under conditions of ordinary normal daily body water flux, osmotic constancy is determined by the secretion of the antidiuretic hormone (ADH), which directly influences renal water excretion and conservation in response to intravascular fluid shifts (that result from thermal and positional changes) and from the free intake of food and liquid [58]. Plasma osmolality (POsm) remains stable as the kidneys modify urine osmolality and water excretion in accordance with ordinary living conditions. When water losses exceed water intake, body POsm increases and blood volume decreases causing a compensatory water-conservation (renal) and water-acquisition (thirst) responses [53,58]. As a result the discriminatory power of renal excretion measures for the detection of dehydration is always secondary to changes in POsm.

ADH is synthesized in the hypothalamus and released from the posterior pituitary gland [53]. Basal ADH concentrations can fluctuate considerably in response to ordinary postural and skin-temperature (skin blood flow) shifts in blood volume. However a threshold reduction in blood volume >10% is required to elicit greater (compensatory) ADH secretion, whereas smaller reductions in blood volume primarily act to enhance the sensitivity of the ADH response to changes in POsm. The receptors that elicit thirst have an osmotic threshold higher than the osmoreceptors involved in ADH release. Thus, ADH can act on the kidneys to increase water reabsorption before thirst is elicited (**Figure 1)** [53,58]. Osmotic homeostasis (<1-2% deviation in POsm) is also maintained by basal ADH regulation, but compared to blood volume smaller thresholds increases in POsm (>2%) produce intracellular dehydration and compensatory increases in ADH secretion, renal water conservation and thirst [58]. The set point of POsm above which ADH secretion is stimulated is about 280 mosm/L, and the

sensitivity of ADH response to a rise in POsm is enhanced when the circulating blood volume is lowered [53].

Kidneys are the main regulators of water losses. When the net balance between water intake and output becomes negative (dehydration), renal water conservation is insufficient to restore fluid balance. The kidneys can modify the osmotic pressure of urine within a large range in response to minute changes in POsm. Obligatory renal water losses persist, and fluid acquisition must occur, to restore the body water balance. However, the POsm threshold for thirst is highly variable in people, and thirst mechanisms are subject of numerous influences unrelated to body water balance [59]. During rehydration, thirst can disappear before water balance is reached.

Acute changes in the hydration status (HS) are commonly assignated as "dehydration" or "rehydration". Differences in the steady-state HS are called *hypohydration, euhydration or hyperhydration*. However, there are no universal definitions or laboratory methods to characterise the different forms of HS [8,16]. In this thesis, the differences in euhydration characterised by urine osmolality (Uosm) and the physiological based parameter to characterise euhydration (Free Water Reserve, FWR) will be addressed.

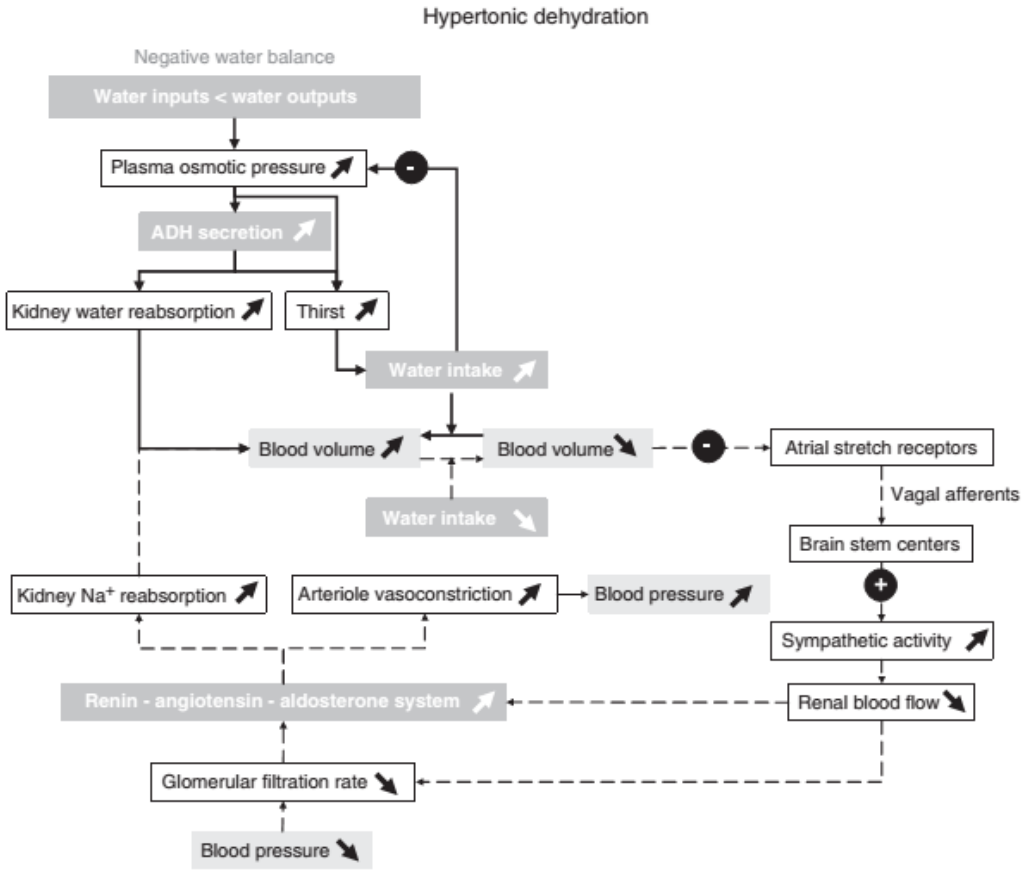

Figure 1. Physiology of hydration. [Adapted from Jequier&Constant[53]] Feedback from loops for water balance: main perturbations and physiological responses to hypertonic dehydration due to a negative water balance. Solid arrows show the responses induced by osmoreceptors when POsm

increases. Dashed arrows show the corrective mechanism induced by insufficient water intake and decreased blood volume to restores blood volume and blood pressure. In hypotonic dehydration due to a positive water balance, the physiological responses occur in the reverse direction.

Markers of hydration status

A "normal HS" (euhydration) is the condition of healthy individuals who maintain their water balance. Many indices have been investigated to establish their potential as markers of HS. Because euhydration (normal body water content) is a dynamic process, and the water balance changes constantly, there are no accurate and precise laboratory and field techniques to evaluate human hydration status [8,16]. The commonly used technique to measure changes in HS is the measurement of body weight (changes that occur during short periods of times); the tracer techniques (deuterium oxide); bioelectrical impedance; osmolarity measured in plasma or serum; plasma indices and urine indices [8,14]. In **Table 1** the hydration assessment techniques are summarized.

Free water reserve as marker of hydration

As exposed in Table 1, the hydration status assessment techniques are most effective in laboratory settings. During experimental phases, where the postural, activity, dietary and environmental factors are controlled, TBW, volume of fluid compartments and extracellular fluid concentration are stable. However, the process of selecting an appropriate technique for the laboratory setting is different than from selecting one for daily activities. The knowledge about the various variables that determine HS (water intake and water output, and dietary solute load) led to the concept of the "*Free Water Reserve*" (FWR), introduced by Manz et al [15,16] in the late 1990s. FWR is a physiological concept to characterize 24-h HS in an individual and to represent the balance between available body water (measured by urine volume) and water requirements based on an individual's solute load and the maximum urine osmolality (Uosm).

In a subject, maximum and minimum Uosm define the range of *euhydration*. Defining the data of maximum and minimum Uosm on a logarithmic scale, the two functional capacities are almost equidistant from plasma osmolality, allowing the kidney to overcome differences in urinary water excretion rates up to a factor of 20. This is illustrated in **Figure 2.**

If in a particular life stage and gender group values of maximum and minimum Uosm are known in a representative subgroup of subjects, three categories of 24-h hydration can be characterized using data of Uosm: *risk of hypohydration* (Uosm≥ mean -2 s.d. value of maximum Uosm), *euhydration* (mean -2 s.d value of maximum Uosm > Uosm > mean+ 2 s.d. value of minimum Uosm) and *risk of hyperhydration* (Uosm ≤ mean+ 2 s.d. value of minimum Uosm). Thus, in groups of healthy subjects mean -2 s.d. value of maximum Uosm may be used as a physiologically based criterion for the "safe" upper level of euhydration ensuring euhydration in 97.7 of the subjects [15,16]. In a subject of this life stage and gender

group diagnosis of hypo (hyper)-hydration presumes, however additional clinical o biochemical signs of hypo (hyper)-hydration.

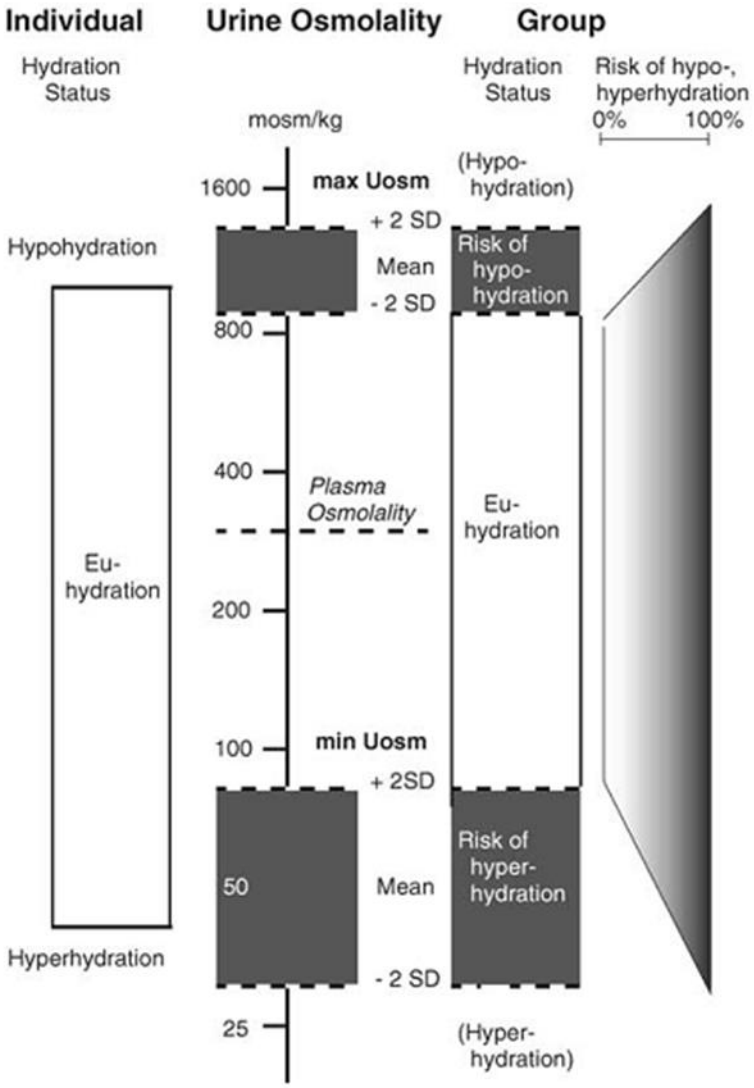

Figure 2. Definitions of 24-h hydration status for an individual and group [Adapted from Manz et al [(16)]]. In a subject individual minimum and maximum 24-h urine osmolality characterise 24-h hydration status of hypohydration, euhydration and hyperhydration. In a group in which only mean and standard deviation of minimum and maximum urine osmolality of a representative subgroup of subjects are known, three categories of 24-h hydration can be characterised using data of Uosm: risk of hypohydration (Uosm≥ mean -2 s.d. value of maximum Uosm), euhydration (mean -2 s.d value of maximum Uosm > Uosm > mean+ 2 s.d. value of minimum Uosm) and risk of hyperhydration (Usom ≤ mean+ 2 s.d. value of minimum Uosm). Additional clinical or biochemical signs of hypo (hyper)-hydration are necessary to diagnose hypo (hyper)-hydration in a subject of this life stage and gender group.

Osmolality is a measure of concentration. The FWR (mL/24-h) has been defined as a quantitative measure of individual 24-h euhydration [(15)]. Renal solutes excretion (mOsm/ 24-h) corresponds to the product of urine osmolality (mOsm/kg) and 24-h urine volume (L/d), assuming 1 kg water corresponds to 1 L. The solute load is mainly determined by urinary concentration of nitrogen, sodium, potassium and phosphorus from the diet. Obligatory urine

volume is defined as the "water volume necessary to excrete 24-h urine solutes at the age-related lower limit of maximum urine osmolality (mean-2 s.d)". Based on literature data of standardised tests of renal concentration capacity in subjects of industrialized countries, consuming a typical Western diet, with high intake of protein, fat, and sodium chloride and relatively low intake of complex carbohydrates from starch-and fiber-containing foods, this value is ~ 830 mOsm/L [15]. The calculation of FWR for children is:

$$\textit{Obligatory urine volume (L/d)} = \frac{\text{24-h urine solutes (mOsm/d) [measured in urine]}}{\text{830 mosm/L}^{-1}\text{ [assuming 1 kg = 1L]}}$$

FWR (L/d) = 24-h urine volume (L/d) [measured] – obligatory urine volume (L/d) [estimated]

Positive values of FWR are defined as *euhydration;* negative values of FWR denote "*risk of hypohydration*" (Figure 2). If almost all subjects (mean + 2 s.d. or 97.7%) of a population show 24-h Uosm below the criterion of water requirement (e.g. 830 msom/kg) or *positive FWR values*, then the population can be classified as adequately hydrated [15,16].

In the practical application FWR essentially helps to establish the Adequate Total Water Intake (AI) values for populations. By definition, in a population, euhydration is ensured if at least 97% of the subjects show positive values of FWR [15]. As exemplified by Manz et al [15] in one group of 4-7 y old the DONALD Study, with the obtained values for TWI and FWR was possible to estimate the AI as follows: the median TWI for these children was 1310 mL/24-h, the FWR value was 11 mL/24-h and the third percentile -156 mL/ 24-h. Thus the theoretically required increase to estimate the AI would be represented by the estimated median TWI plus the calculated third percentile value of FWR (1310 + 156= 1446 mL/24-h), to ensure euhydration in 97.7 % of these children and it would result in a predicted median Uosm of 598 mosm/kg, as it was previously applied in children and in adults [15,60].

Table 1. Hydration assessment techniques.

Hydration assessment technique	Outcome variable	Description/advantages/limitations
Urine indices		
Urine osmolality	urine concentration	Non-invasive. Direct measurement in urine. High variable depending on time of the day, additionally depends on solute excretion.
24-h urine volume	daily flow rate	Non-invasive. Highly variable depending on solutes and water intake.
Urine specific gravity	relative density of urine vs water	Non-invasive. Urine specific gravity increases with water deficit; however, considerable individual variability exists. Although a urine specific gravity greater than 1.03 indicates probable dehydration, the magnitude of the water deficit cannot be determined.
Urine conductivity	electrical conductivity	Non-invasive.
Urine color	urochrome concentration	Non-invasive. The color of urine darkens or lightens with low or high output levels (because the solute load is either concentrated or diluted, respectively). However, no precise relationship between urine color and hydration level exists. Furthermore, diet, medications, and vitamin use may affect the color. Can be used when high precision may not be needed.
Other markers		
Body mass change	body water loss or gain	Non-invasive direct measurement, inference is based on physiological changes involving water loos or gain. Measure changes of ± 1 kg (± 0.1 L of TBW). Excellent for brief elapsed time, poor for longer time (day to months).
Plasma osmolality	extracellular volume concentration	Invasive, requires collection of blood sample. Direct method that requires standard solutions with known osmolalities.
% plasma volume change	hematrocrit and hemoglobin	Invasive, requires collection of blood sample and the previous standardisation of posture for a time prior to blood collection to distinguish between postural changes in blood volume, and change due to water loss or gain.

Table 1. Hydration assessment techniques (*Continued*)

Hydration assessment technique	Outcome variable	Description/advantages/limitations
Isotope dilution	TBW volume	Calculation based on whole body dilution. Impractical as it requires 3 to 5 hours for internal isotope equilibration and analysis. Overestimates TBW 1-5%.
Neutron activation analysis	Fluid volumes and whole body ion content	Calculation is based on known gamma ray emission properties of elements. It is considered as the reference standard for all elements identification.
Bioelectrical impedance spectroscopy (BIS)	TBW, extracellular volume and intracellular volume	TBW and extracellular fluid are measured and allows calculation of intracellular fluid volume. The TBW measurement resolution obf about 0-8-1.0 L (out of a TBW of 42 L for a 70 kg individual) and therefore is not appropriate when dehydration is less than 800-1000 mL.
Salivary flow rate, osmolality, total protein	Flow rate, osmolality, protein concentrtion	They have been proposed as HS markers. However, few studies have evaluated changes of those variables. In dehydration (-3% body weight), salivary flow is reduced
Rating of thirst	Perception based on extracellular fluid concentration	Subjective. Renal, thirst and sweat glands are involved to varying degrees depending on the prevailing activities. This approach is , however, of limited value in elderly individuals and young children who have a blunted sensation of thirst

This table is based on published references [8,14].
Abbreviations: TBW, total body water; 24-h, 24 hour

2.4 Assessment of iodine status

As an integral part, Iodine is essential for the function and production of the thyroidal hormones: tetratiodothyronine (T4) and triiodothyronine (T3)[61]. Amongst the most important roles of the thyroid hormones are the regulation of numerous physiologic processes, including growth, neurological development and reproductive functions [61–63].

The numerous effects of iodine deficiency on growth and development are known collectively as Iodine Deficiency Disorders (IDDs) [64]. The obvious and familiar form of IDD is the Goiter, the enlargment of the thyroid. Severe iodine deficiency in early stages of life is associated to congenital anomalies, perinatal mortality and endemic cretinism. The important role of an adequate iodine supply during the period of growth and development is based on the essential need of an adequate thyroid hormone production for a number of processes involved in the development and function of glia cells and neurons [65]. Therefore, also in children and adolescents iodine deficiency is associated with negative effects on cognitive outcomes and physical performance [21,66,67]. The adverse effects associated with IDD represent some of the most important and common human diseases [61].

Because iodine intake mainly depends exclusively on the dietary sources (see section below on dietary iodine) which are not always sufficient, there are different efforts to eradicate IDD. The most common and effective measure is the fortification of salt with potassium iodide or sodium iodide. This is the global strategy recommended by the WHO as public health strategy since the early 1950's. Despite the local, regional, and global efforts to eradicate IDD, iodine deficiency still remains a global health problem [20,68].

Although assessment of salt iodization can serve as a useful proxy for iodine intake under defined circumstances, the quantification of iodine content in table salt is in general not sufficient in assessing iodine intake as the main source of salt (and therefore also iodized salt), today are processed foods [21,69,70]. Thus, different methods to assess iodine status are needed. Accordingly, iodine is one of the nutrients that has been included and reviewed in the initiative called Biomarkers of Nutrition for Development (BOND) for application in nutritional research, policy and program development [2].

Biomarkers of iodine status

The current available biomarkers for the assessment of iodine nutrition and thyroid health are summarized in **Table 2.**

Urinary parameters for the assessment of iodine status

Compared to dietary assessments (including the assessment of salt consumption) and other markers described in Table 2, iodine excretion measured in urine is considered an objective biomarker of exposure, and it is considered an excellent indicator of recent iodine intake because ≥ 92% of dietary iodine is absorbed and, in healthy iodine-replete adults ≥85% is excreted in the urine within 24-48 h [13,21]. Urinary iodine can be expressed as 24-h excretion (24-UIE, µg/d), concentration (UIC, µg/L) or in relation to creatinine excretion (I-CR ratio). Each method is described below and they are not-interchangeable methods.

24-h urinary iodine excretion

The collection of 24-h urines and measurement of 24h-UIE is considered to be quasy "reference standard" for the measurement of the iodine intake in an individual, as it incorporates the daily variability of the 24-h urine volume, and is thus more precise than using spot urine samples [12,71]. Furthermore, 24h-UIE measurement are often used to validate other methods for the measurements of iodine intake, like dietary assessment methods [12,72]. One of the limitations of this method is however, the dependency on the demanding and elaborate collection of 24-h urine samples. Especially at population level and field studies 24-h urine collections are impractical and bear the risk of lower compliance, compromising data quality [21]. However whenever feasible, 24-h UI (µg/d) should be the preferred method to determine iodine status [73].

Urinary iodine concentration measured in spot urines

The most common way to assess iodine status of a population is by determining median urinary iodine concentration, obtained from spot urine samples [13]. One of the reasons that makes the measurement of UIC as popular is the relatively simplicity compared to 24-h urine collections especially in field studies, thus a major number of individuals can participate. As urinary iodine concentration in a population is usually not normally distributed but skewed to the right, the World Health Organization (WHO) recommends that the median values of UIC are reported and used for the evaluation of the iodine status of a population [22]. Currently, a population's median urinary iodine concentration range of 100-299 µg/L is suggested as indicator of iodine sufficiency [19,22].

One disadvantage for the general application of this method is for the known variation in hydration status between individuals that affect the daily urine volume and thus iodine concentration. Urinary iodine concentration measurements consequently bear the risk of falsely under- or overestimated iodine deficiency prevalence [12,13,74]. However, it has been proposed that in a sufficient number of samples the median UIC in spot samples correlates well with that from 24-h samples and inter and intra-individual urine volume variations are levelled out [22]. The number of samples that is sufficient to contrarest those hydration

variations is still under discussion. For instance, Andersen et al. calculated that for an individual's estimate of iodine excretion with a precision range of 20%, at least 12 separate urine samples are needed [75], whereas for populations, the suggested sufficient sample size varies from n=30 [22] up to n=500 [75], as some authors suggest. Additionally, Remer et al. [74] in an earlier study involving urinary iodine excretion in a large sample of 6-12 y old healthy children (n ~ 1000), clearly showed that changes of urine osmolality over time even in a large population not necessarily level out and may significantly affect median iodine concentration.

Iodine-creatinine ratio (I-CR ratio)

Because of the known dependency of iodine concentration on urine volume, some authors in early times suggested the use of creatinine concentration as a correction method. This method was thought to obtain more reliable values since creatinine is known to be excreted at a relatively constant rate in 24-h [46,76]. This method however, was shown to be unsuccessful when applied in children, because of the observed physiological strong-age-dependent increase in muscularity and hence creatinine production during growth [77]. The pitfalls of the I-CR ratio in children were also confirmed later in the German Health Interview and Examination Survey for Children and Adolescents (KiGGS), in which by means of I-CR ratio >90% of the 0-2 y old children were categorized as adequately iodine supplied whereas it were only 55% when UIC was considered [78].

Although the I-CR ratio was commonly reported in the literature, especially in adult populations [12,79], the WHO considers that the additional measurement of creatinine is unnecessary and unreliable. The reasons for this consideration are that creatinine measurement may be expensive especially for some developing countries, and for the known creatinine excretion variation depending on sex, age, racial/ethnic, body mass index and dietary differences in populations (especially in animal protein intake) [13,22]. Other authors, have also confirmed the potential error of using I-CR ratio as an index if the creatinine is not corrected by age [80].

Estimates of 24-h iodine excretion

In an effort to obtain more reliable values for iodine status assessments, different alternative methods have been proposed. One approach to approximate 24-h analyte excretions from concentration measurements is the correction with parallel creatinine measurements and – importantly – subsequent scaling to population-appropriate 24-h creatinine reference values, as creatinine is known to be relatively constant over 24-h. Literature on the application of this method using creatinine reference values, refers mostly to studies conducted in adults from industrialized countries [21,44,72,81]. However, in children no studies that specifically apply the I-CR corrected approach exist.

Another method to simplify the calculation of daily iodine intake from iodine concentration measurements, it is the equation proposed by the IOM:

$$Iodine\ intake\ (\mu g/d) = UIC\ \left(\mu \frac{g}{L}\right) \div 0.92\ \times 0.0009\ (L \cdot h\text{-}1 \cdot kg \cdot 24\text{-}h \cdot d) \times \text{weight (kg)}$$

Where, 0.92 refers to 92% of bioavailability of dietary iodine and 0.0009 L refers to the excreted urine volume based on studies in pre-adolescent girls [82]. Although its simplicity this method uses approximated values and therefore represents an approximation without considering the inter- and intra-individual variations which can be one of the disadvantages of its application.

Table 2. Biomarkers and assessment of iodine nutrition and thyroid health.

Iodine assessment technique	Analytical	Description/advantages/limitations
Urine indices	Include: 24-h UIE, UIC (µg/L), or iodine/creatinine ratio.	Non-invasive. Relatively easy to collect in most population groups (except neonates and infants). Urinary iodine can be measured in spot urines or 24-h urine collections. The methods are not interchangeable (See the above section on urinary parameters for the assessment of iodine status, for broader description of each category).
Other techniques		
Dietary assessment	FFQ diaries, 24-h food intake and weighed food records	Non-invasive. Provides a broader picture beyond the household salt iodine content. This information is useful to design or adapt iodine intervention strategies. Dietary assessment methods do not accurately quantify the usual iodine intake. In most cases, no comprehensive and locally adapted food composition databases are available; analysis of iodine content in food matrices may require sophisticated methods.
Thyroidal measurements	TSH; Thyroglobulin; T3/T4	All the measurements are markers of "thyroid disorders" thus indirect iodine status. TSH serum levels are regarded as the most sensitive marker indicating thyroid function. Thyroid disorders may be either sub-clinical or below, given reference limits, however thyroid hormones are within the normal range. These methods require relatively sophisticated equipment (or can be invasive as they need blood e.g. thyroglobulin, T3/T4).
Goiter	Neck inspection and palpation; thyroid ultrasonography	Non-invasive. Poor sensitivity and specificity. Both are subjective and require judgment and experience. Differences in techniques can produce large inter-observer errors in thyroid volume. Ultrasound could be feasible using portable equipment and references ranges for thyroid volume by ultrasound are available for school-aged children.

Table adapted from Rohner et al. [21]

Abbreviations: 24h-UIE, 24 h urinary iodine excretion; UIC, urinary iodine concentration; FFQ, food-frequency questionnaires; TSH, thyroid-stimulating hormone; T3 triiodothyronine; T4, thyroxine.

Dietary iodine intake

Unlike most other essential dietary nutrients, iodine status is not linked to socioeconomic status but rather to geography. The iodine content of local foods is highly dependent on the environment, i.e. for plant foods iodine content depends on the iodine content of soil, and for animal foods, it is mainly the iodine content of the animal feed [83]. Although iodine (as iodide) is present in soils, the content may fluctuate widely within and across regions as a result of a number of factors. However, the natural iodine content of most foods is low because of the iodine depletion of most surface soils and therefore is usually insufficient to meet daily iodine requirements [83]. Only foods of marine origin – like saltwater fish – naturally have a higher iodine content (>50 µg/100 g food) because of their ability to concentrate iodine from seawater [61]. However, they do not contribute substantially to dietary iodine intake unless consumed regularly [84,85]. Iodine content in some seaweed is also relatively high, and populations consuming seaweed may obtain high iodine concentrations through their diet. Other source of iodine could be the drinking water drawn from certain aquifers or water disinfected with iodine [61].

Iodine from iodized salt: Iodized salt used for cooking and at the table in households nowadays only accounts for < 30 % of daily salt consumption. In industrialized countries, about 80 - 90 % of salt consumed comes from purchased processed foods and therefore provides the major source of iodine [13,86].
Iodine can be added to the salt as potassium iodide or iodate or sodium iodide. The global WHO-Universal Salt Iodization Program recommends the addition of iodine in a range of 20-40 mg iodine per kilogram salt [64]. However, initiatives for regulation of fortification and use of iodized salt in the majority of European countries still are on a voluntarily basis [21].

In Germany, a particularly improved iodine status in the population has been observed since 1993 parallel to legislation amendment, facilitating the use of iodized salt in all processed foods [74,87]. However, the iodization of salt used in households or for food production is still on a voluntarily basis. During the last years (starting in 2004), the use of iodized salt by the food industry has decreased in Germany, and by now encompasses only < 30% of total added salt, leading to a negative trend in iodine status [84,88].

Iodine from milk: In addition to iodized salt, milk and other dairy products are good sources of iodine [66,83–85,88–93]. In traditionally dairy consumer countries such as US, Canada, Switzerland and Germany, to mention some, the dairy products represent an important contributor of iodine, especially in children, not just because of the iodine content in milk, but also because of the relatively high daily intakes [66,83–85]. The iodine content of the latter is

mainly dependent on the iodine content of feeds for the dairy herds [94] and perhaps the iodine residues in milk from the disinfecting agents used in dairying [95] contribute to dairy iodine. In Germany, however the permitted disinfestations meanwhile only have marginal iodine content.

In some European countries such as the UK, and Norway, for example, iodine from the milk represents one of the main iodine sources [66,85]. In Germany, milk iodine content in the last 20 years increased continuously up to a mean iodine content of currently 110 µg/L, attributable to the increase of iodine content in cattle feed [90,92]. Despite the seasonal variations in iodine content due to the changes in feeding practices of cattle, milk in Germany contributes about 30% of the daily iodine supply [90]. The iodine content of cheese is not associated to the iodine content of the milk from which it was produced. This is due to the extraction process in the cheese manufacturer, and most of the iodine content of the milk is in the whey fraction, thus the added salt –iodised or not- determines the iodine content of cheese [83].

Iodine from other animal sources: Similar to milk, iodine content of eggs is highly dependent on the iodine supply of the hens. The transfer of iodine from feed into eggs can be up to 30%. The iodine content of meat on the other hand, is less affected by the iodine content of feed, with an estimated transfer of less than 1% of supplemented iodine [96].

2.5 Nutrient adequacy and dietary factors to be considered in hydration and iodine nutrition

Hydration status: water contribution from solid foods

Despite varying water needs, healthy humans regulate their daily water balance with precision (Chapter 2.3). Total water intake corresponds to the sum of beverages, metabolic water and water in food, which is usually estimated from dietary intakes. Numerous facts on the effect of food intake on HS, i.e. liquid sources and high water content diets [97], are known. However, to date no studies have evaluated the possible compensatory effect on the water balance that a diet rich in water food sources , i.e. fruits and vegetables (~70-95 % water content comparable to ~ 85-95% water content of beverages) can have [98].

Iodine nutrition: healthy food patterns and dietary iodine

Evidence from studies on the effect of diets containing little to no animal food products on iodine status is limited, but overall, the literature in this topic suggests that lower intakes of animal food can contribute to inadequate iodine intakes [99–101].

Although there is still controversy about the impact of reducing dietary protein intake in children and potential health outcomes [102], current dietary guidelines for healthy eating, for example, the "New American Plate" (NAP) from the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR), advise the limitation in the intake of animal protein and to increase the plant-based foods to prevent chronic diseases [103,104]. The potential presence of *Goitrogens* in more plant based foods may inhibit the uptake of iodine. Goitrogens are natural compounds of plant foods such as broccoli, brussels sprotus, cabbage, cauliflower, cassava [105].

In addition, reducing sodium intake in children is also part of the global health initiatives in the prevention of chronic diseases [106–109].

Concerns of the public health strategies for reducing salt consumption on the iodine nutrition have been also recently evaluated. They have concluded that programs aiming at salt reduction and iodine intake prophylactic measurements need to be carefully monitored in order to avoid re-emergency of iodine deficiency problems [68].

Assessing nutrient adequacy

The Dietary Reference Intakes (DRIs) established by the Institute of Medicine (IOM) refer to a set of four nutrient-based reference values: Estimated Average Requirement (EAR), Recommended Dietary Allowance (RDA), Adequate Intake (AI), and Tolerable Upper Intake Level (UL). The definitions of key categories and their use, in the derivation of the current dietary recommendations, are below described.

The *Recommended Dietary Allowance (RDA)* is the average daily dietary intake level that is sufficient to meet the nutrient requirements of nearly all (97 to 98 percent) healthy individuals in a particular life- stage (age/ gender) group. It can be used as a reference point for the daily nutrient intake of individuals. The equivalent reference value of the German-speaking nutrition societies (D-A-CH) is the "Zufuhrempfehlung" [110].

The *Estimated Average Requirement (EAR)* is the daily intake value that is estimated to meet the requirements in half of the apparently healthy individuals in a particular life-stage (age/gender) group [111]. This category is not used for individual assessment but can be used for population/group analysis.

When the scientific evidence is not sufficient to calculate the EAR, a reference intake called *Adequate Intake (AI)* is provided instead of the RDA. The AI is a value based on experimentally derived intake levels or approximations of observed mean nutrient intakes by a group (or groups) of apparently healthy people presumed to have adequate intakes. Because the AI is intended to define the amount of a nutrient needed in "essentially all" individuals in a target group, it can be used as a goal for individual intake [111].

The *Tolerable Upper Intake Level (UL)* is the highest average daily intake of nutrient that poses no risk of adverse health effect to almost all individuals in an otherwise healthy population. The UL is used as a reference for safety [111].

In addition to the IOM terminology, the joint FAO/WHO committee has published definitions which are similar to the IOM, and some representing similar or equivalent concepts, for example, the *Recommended Nutrient Intake (RNI*), defined as the "intake estimated to cover the needs of nearly all healthy individuals in a specific age/gender group"[112]. The RNI would correspond to the RDA definition of the IOM.

Adequate total water intake

As previously described, the natural drinking behavior or natural range of Uosm in man is influenced by water access and cultural context [16,52]. Water requirements vary between individuals and according to environmental conditions. Therefore adequate intakes have been defined for specific age groups, with a combination of observed intakes in population groups and desirable osmolarity values of urine and desirable water volumes per energy unit consumed [15,56,107,110]. In **Table 3** the AIs for total water intake are described. The reference values provided by the German-speaking nutrition societies (D-A-CH) have different age-groups categories than those from the EFSA and IOM and include the metabolic water.

Table 3. Dietary reference values for total water intake in children (mL/d).

	Adequate Intakes			**Guidance values**
Age-group[1]	**EFSA[2]**	**IOM[3]**	**Age-group**	**D-A-CH[4]**
4-8 y	1600	1700	**4 - <7 y**	1600
9-13 y	boys 2100	boys 2400	**7 - <10 y**	1800
	girls 1900	girls 2100	**10 - <13 y**	2150
			13 - <15 y	2450

[1] Adolescents of 14 y and older are considered adults with respect to Ais and adult values apply.
[2] European Food Safety Authority, EFSA [56].
[3]Institute of Medicine, IOM [107].
[4] German-speaking societies, D-A-CH [110]. TWI, include values supplied from foods and metabolic water.

Requirements for Iodine

The specific intake recommendations by the IOM and WHO for iodine are presented in **Table 4.**

Table 4. Recommendations for iodine intake for children and adolescents (µg/d).

	IOM[1]		UL			WHO
Age-group	**RDA**	**EAR**	**IOM[1]**	**EC[2]**	**Age-group**	**RNI[3]**
4 - <7 y	90	65	300	250	**0-5 y**	90
7 - <10 y	120	65	300	300	**6-12 y**	120
10 - <13 y	120	73	600	450	**Adults >12 y**	150
13 - <15 y	150	73	900	500		
15 - <19 y	150	95	900	500		

[1] Institute of Medicine [82].
[2] European Commission. Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Iodine [113].
[2] RNI (recommended nutrient intake) from WHO per definition corresponds to the RDA concept (i.e. suggested to meet the requirements of nearly 98% of the population) [22]

2.6 Interim conclusion

As it has been outlined in Chapter 2.2.1 and 2.2.2, Biomarkers in Nutrition are desirable for their ability to more accurately assess nutritional intake and status versus self-reported methods (e.g. dietary records, food frequency questionnaires or 24-h diet recalls). In general, the lack of nutritional biomarkers is recognized as a knowledge gap requiring further research in the field of nutrition. Hence, the present thesis aims to address with examples some of the open questions for the potential application of use of biomarkers in epidemiological research in children.

Separate from the essential knowledge on the physiological meaning of nutritional biomarkers, an important aspect is to understand how clinical chemical analytes for further use as biomarkers are affected by sampling and laboratory procedures, which are normally referred as “measurement error” and classified in different categories (Chapter 2.2). Studies that have examined the long-term stability of urinary analytes are rare. Particularly the long-term influence on recovery under conditions of low-temperature storage for urine samples collected and strored without the use of preservatives is unknown.

Water is quantitatively the most important nutrient in human nutrition. Hydration Status is mainly determined by the balance of water intake (from foods and beverages) and water output (renal and non-renal water losses, e.g. sweating, faeces). The high and precise regulation of this balance in a range of ± 0.2% of body weight over a 24-h period is maintained by subtle hormonal changes, inducing thirst sensation and water reabsorption of the kidneys (Chapter 2.3). Many indices have been investigated to establish their potential as markers of hydration status, and the current evidence and opinion tend to favour urine indices. A new physiological term *Free Water Reserve*, as a result of combining urianry parameters, has been defined as a quantitative measure of individual 24-h euhydration (Chapter 2.3). In how far the highly regulated mechanism of water balance is affected by the intake of *fruit and vegetables*, which are high in water content, to date has not been systematically explored. A confirmation that F&V in fact have a positive effect on hydration status would support strategies promoting F&V intake especially in children to – amongst others - reach adequate intakes of water.

Despite the increasing implementation of iodized salt fortification programs, iodine deficiency remains a common global health problem. The most accurate available measurement of dietary iodine intake is the 24-h Iodine Excretion (reference standard). However in large epidemiological studies, spot urines might be more feasible (Chapter 2.4). Currently, the commonly used measurement to assess iodine status in population is urinary iodine concentration (UIC µg/L), measured in spot urines according to the WHO recommendation. However, as described in Chapter 2.4, this indicator alone may be highly affected by hydration status. To overcome this problem, different approaches are recommended (Chapter 2.4). However, the approach of using predicted 24-h creatinine values for the estimation of 24-h iodine has not been considered by now in children.

Studies on the effect of a vegetarian-type diet suggest an association between lower animal food intake and decreased iodine intake in adults [99–101]. Currently, dietary guidelines for healthy eating advise to increase consumption of plant-based foods and limiting intake of salt (in its iodized form the most important dietary iodine source) to prevent chronic diseases (Chapter 2.5). Data on the association between lower consumption of animal food products (animal protein) and iodine excretion in healthy children consuming a typical Western-type diet is currently missing.

3. Research Questions

As has been summarized (interim conclusion in Chapter 2.6), application of urinary biomarkers are of potential applicability as non-invasive approaches for nutrition studies in children. The overall aim of the present thesis was to illustrate with three examples the potential application of urinary parameters as biomarkers for hydration and iodine status. The following research questions-that have been examined in four consecutive studies were formulated for this thesis.

Study I- Methodological pre-analysis on long term stability of clinical chemical urine parameters stored at -22 ºC

Urinary analytes, such as ions, creatinine, iodine, organic acids, among others, are important to evaluate distinct metabolic functions, and used both for clinical diagnostic and scientific research (Chapter 2.2). However, in how far storage of urines under specific conditions (low-temperature, preservative free) can affect their concentration after long-time periods has not been examined yet. The obtained information in this area is an essential requirement for the correct interpretation of urinary measurements as reliable values for application in further epidemiological studies as well as for the following research purposes. Therefore the first research question of this thesis was:

How stable are the concentration values of specific analytes measured in urines and how valid are the values of the same parameters when measured after 12 or 15 yr of storage at -22° C?

Study II- Effect of consumption of high water content foods (fruit and vegetables) on "Free Water Reserve" as marker of hydration status

The Free Water Reserve (FWR) is a physiological concept accepted as one of the most accurate measurement of water in the body, as it accounts for the 24-h changes in water metabolism (Chapter 2.3). The water balance is determined basically from the difference between the water intake and water excretion. It is assumed that food rich in water such as Fruits and Vegetables (F&V) (~ 50-80% water content) will provide higher water intake to the body. However, in how far the total body water may be affected by the intake of fruit and vegetables, i.e. whether higher intake of F&V will have an overall positive effect on hydration; or whether the highly regulated mechanism of water balance, e.g., in children consuming more F&V decline the intake of other water sources, has not been examined yet. This led to the following research questions:

1) ***Do children with a higher F&V intake have better hydration status than those children consuming less F&V?***

2) ***Could the higher water content from F&V result in a compensational reduction in water intake from other sources, eventually keeping the water balance more or less constant?***

3) ***To what extent adding F&V (solid or liquid) to the common diet may finally increase the FWR when other important water sources are kept constant?***

Study III + IV- Urinary biomarkers for iodine status

Study III- 24-h h iodine excretion and estimates of 24-h iodine from spot urines using a creatinine scaling method.

The most accurate available measurement of dietary iodine intake is the 24-h Iodine Excretion (24h-UIE, µg/d) (reference standard). However, in large epidemiological studies, spot urines might be more feasible to analyse (Chapter 2.4). The urinary iodine concentration (UIC µg/L), measured in spot urines is currently recommended as the parameter to assess the iodine status in populations. However, the UIC is affected by daily urine volume variation. As a need to obtain spot urine estimates that are appropriately comparable to true 24h-UIE in children, a creatinine scaling methods that involves published 24-h creatinine reference values is proposed. The research questions for this study were:

1. ***Is the UIC measured in spot urines a reliable approach when compared to 24-h Iodine excretion for iodine status? Or could it be relevantly affected by changes in the hydration status (using osmolality as hydration index)?***

2. ***Can an estimate of 24-h Iodine excretion (using the 24-h CR scaling method) obtained from spot urines be adequately indicative of 24-h urinary iodine?***

Study IV- 24-h iodine excretion long-term effect on iodine status of diets predominately animal or plant based.

Limiting animal protein and salt intake are part of the current dietary recommendations to prevent chronic diseases (Chapter 2.5). Two important iodine dietary sources include animal

products and iodized salt. Diets with low animal intake in adults have been observed to be associated with decreased iodine intake, the concurrent effect of both dietary factors i.e. low animal food diets and salt intake in children has not been investigated so far. Therefore the research questions for this study were:

1) ***Are changes in the dietary animal to plant protein ratio associated with urinary iodine excretion in children?***

2) ***Could the potential positive dietary animal to plant protein association with iodine nutrition be mainly mediated by salt intake?***

The four research questions formulated for this thesis and the methodological considerations regarding the application of urinary analytes as non-invasive biomarkers for specific nutrition studies were addressed using data from the longitudinal DONALD Study, which provides repeated detailed assessments on dietary intake, metabolism and growth in healthy children from birth until young adulthood (as described in Chapter 4.1). A set of four analyses, referred to as **methodological pre-analysis (Study I)** and **Studies II-IV** have been carried out and will be presented in the subsequent chapters.

4. General Methodology

4.1 Population and design of the DONALD Study

The **DO**rtmund **N**utritional and **A**nthropometric **L**ongitudinally **D**esigned (DONALD) Study is an ongoing longitudinal (open cohort) study, started in 1985 gathering information about diet, growth and metabolism between infancy and adulthood in healthy participants [114,115]. Every year, approximately 40 infants are recruited from the city of Dortmund and surrounding communities via personal contacts, maternity wards or paediatric practices. The participating children are first examined at the age of three months and are then followed until adulthood in regular visits at the DONALD Study Centre (formerly Research Institute of Child Nutrition) in Dortmund. In the first two years of life examinations take place quarterly or half-yearly, afterwards annually, preferably around the children's birthday. The regular assessments include 3-d weighed records of dietary intake and behaviour, anthropometry, interviews on life-style and health-related issues, a medical examination and from the age of 3 years onward 24-h urine sampling. Since 2005, from participants ≥18 years, fasting blood samples are taken at each visit. Design of the DONALD Study is summarized in **Figure 3.**

Ethical considerations

The DONALD Study is exclusively observational throughout and non-invasive until the age of 18. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects, including regular 24-h urine sampling, were approved by the Ethics Committee of the University of Bonn (Germany). All examinations and assessments are performed with parental, and later on with the participants' written consent.

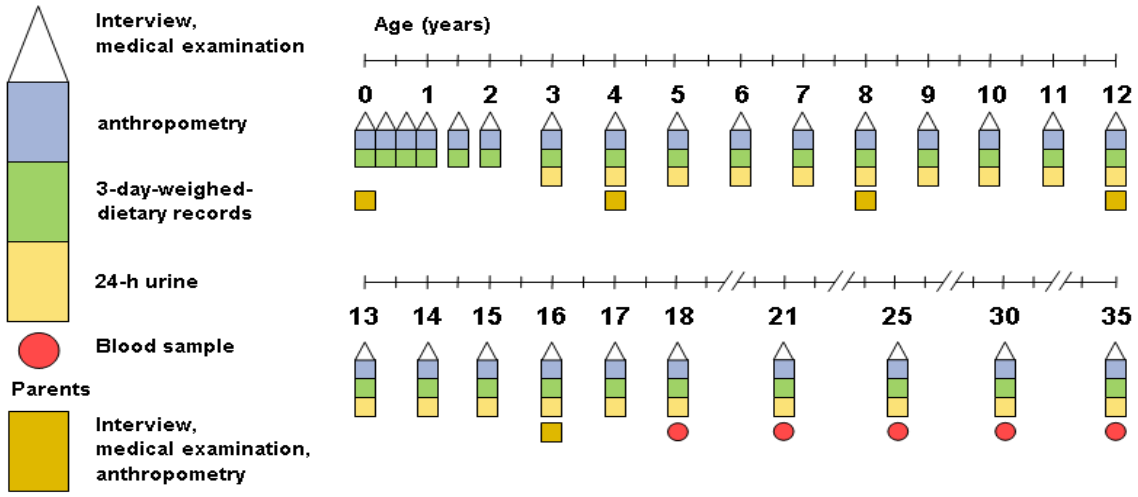

Figure 3. Design of the DONALD Study (adapted fromn Buyken et al [115])

4.2 Anthropometric assessment

Anthropometric measurements of the DONALD participants are performed at each visit by trained personnel following standard procedures [116], with children only dressed in underwear and barefoot. Annual quality control check for intra- and inter-observer agreement is monitored regularly for the nurses. The instruments are routinely calibrated as well as part of the DONALD protocol.

The number of the anthropometric measurements that are performed depends on the age of the participant (Figure 3). Relevant for this thesis are weight and height, obtained at the time of the dietary recording. Body weight was measured using an electronic scale (Seca 753 E; Seca Weighing and Measuring System, Hamburg, Germany) to the nearest 0.1 kg. Height was measured in a standing position to the nearest 0.1 cm using a digital telescopic stadiometer (Harpenden, Crymych, UK).

Body Mass Index (BMI) was calculated from the obtained measurements using the formula: BMI= body weight (kg) / height (m) squared. Per definition, sex-and age-independent BMI standard deviation scores (BMI-SDS) indicates how many standard deviations a child's individual BMI value lies above or below the mean of the reference. The differences were calculated using the German National Reference data [117]. Body surface area (BSA) was calculated according to the formula of DuBois & Dubois as follows: BSA (m^2) = weight (kg) $^{0.425}$ x height (cm) $^{0.725}$ x 0.007184 [118].

4.3 Medical examination, parental information and additional variables

Medical examination includes a physical examination and routine questions related to current or previous acute illness status and use of medical services or medicaments. If children are found to suffer from a disease known to impact their growth or development, they are excluded from the study.

On a child's entry to the study, parents are asked to provide information about family characteristics, such as educational background and employment. In this thesis a higher maternal education was defined as mothers with ≥ 12 years of schooling. The parents' weight and height are also measured by the same trained nurses who performed the anthropometric measurements of the participating children and using the same equipment.

For study II, physical activity (active, moderately active and inactive) was assessed by a questionnaire on daily organized and un-organized activities, which is an adaptation of the Adolescent Physical Activity Recall Questionnaire [119].

4.4 Dietary assessment

To estimate the individual food and nutrient intake, in the DONALD Study 3-d weighed

dietary records are used. Parents of the children or the older children themselves keep the weighed dietary records, where they registered and weighed all the foods and fluids consumed as well as leftovers during three consecutive days. A digital regularly calibrated food scale is used [initially Soehnle Digita 8000, Leifheit SG, Nassau, Germany; currently, WEDO digi 2000, (Werner Dorsch GmbH, Muenster/Dieburg, Germany) ± 1 g]. Recipes for meals prepared at home are recorded. The packaging of commercial food products is usually included in the records. Semi-quantitative recording is accepted when exact weighing was not possible (e.g. number of spoons, scoops). At the end of the 3-d record period, a dietitian visits the family and checks the records for completeness and accuracy, using a structured questionnaire to complete information on unusual events which might have affected the normal eating behaviour during the data collection period.

Energy and nutrient values are obtained from the continuously updated in-house nutrient database LEBTAB, which incorporates information from standard nutrient tables, product labels, or recipe simulation based on the labelled ingredients and nutrients [120]. By 2012, LEBTAB contained 13,200 entries of foods and beverages, additives, supplements and medicine including 1200 basic food items [115]. Data on total daily energy (MJ/d) and nutrient intakes (g/d) are calculated as individual means of the three recorded days.

Intake of specific micronutrients using table salt as vehicle (e.g. iodine, sodium) was addressed by questionnaire in the case of iodine. However, records do not provide the amount of salt used at the table, thus sodium and salt intake are assessed by the urinary sodium excretion values (1g NaCl= 0.4 g Na).

Plausibility check

Self-reported food intakes often underestimate habitual energy intake (underreporting). To evaluate the overall quality of dietary recording, reported was related to basal metabolic rate (BMR). BMR was estimated using the equations of Schofield [121] that include age, sex, and body weight and height. A ratio below an age- and sex-specific cut-off (based on levels of light physical activity) [122] was considered as an indicator of an implausible individual measurement of energy intake. For Studies I and III, only dietary records considered plausible were included.

Food groups

For practical purposes all reported food items were classified into 15 food groups on the basis of nutrient profiles and usage, described in **Table 5.** Two main categories were distinguished: (1) Beverages and (2) Foods, according to the usual way of how foods are consumed. This food group classification was used for dietary analysis in Studies II and IV.

Table 5. Food groups and their components.

Category	Contents
Beverages	
1. Drinking water and water from beverages	Plain water (tap water, bottled noncarbonated and mineral water,) and the water component from infusions (coffee, tea), regular and diet soft drinks (carbonated and noncarbonated) sugar-sweetened drinks (lemonades, iced tea) fruit flavoured drinks, sports drinks and energy drinks
2. Fruit and vegetable juices (F&V juices)	100% fruit and/or vegetable juices, homemade or commercial
Foods	
3. Fruits	all fruit fresh, dried, canned (without syrup) and frozen
4. Vegetables	all vegetables raw, boiled, canned and frozen
5. Potatoes	boiled, roasted, flour
6. Legumes	beans, chickpeas, lentils, peas
7. Cereals	all kind bread, flour and flour products, crackers, biscuits, fortified and non-fortified hot or cold cereals, rice (all types), pasta (all types), couscous, cereal bars
8. Milk and whey-based milk products	whole fluid milk, flavoured milk, partly-skimmed and skimmed milk, yoghurt, yoghurt shake, buttermilk, quark, sour cream, condensed milk, cottage cheese
9. Cheese	all types processed cheese: cheddar type, cream cheese and creamy cheese bases
10. Soja	meats substitutes (soya), soya powder (from milk) and soja products
11. Meats/Poultry	beef, lamb, pork, chicken, all processed meats (sausages, ham, pate, etc).
12. Seafood	fish and seafood
13. Eggs	eggs
14. Fats	all type vegetable oils, margarine, butter and animal fat, spreadable butter, lard, nuts and seeds
15. Diverse	sugar (reported as sugar table or sugar from beverages and recipes), sweets (hard candy), chocolate, cocoa, crisps, condiments

The nature of the LEBTAB database allows disaggregating each food item into its basic components when it comes from recipes. For example, the “fruit group” or “vegetable group”, included the content of fruit, vegetable or both of composite dishes with meat, fish, pasta, rice and eggs, or pizza, breakfast cereals, yogurts, dairy desserts, soups, puddings and fruit pies. As result of disaggregate all food items to the ingredient level, the category “drinking water” comprised plain water and the water component of other beverages, i.e. water added as ingredient in processed or home-made beverages. Composite dishes were disaggregated into their corresponding components, e.g. “pizza” into wheat, cheese, water, etc, and assigned to a food group category (e.g.: cereals, legumes, fruits, vegetables, milk, cheese, meat, fish, etc.).

Further combination of food groups were based upon the goals of the specific research question and specified in the studies.

4.5 Urinary assessment

4.5.1 Collections assessment

24-h urine samples

Starting at age 3–4 years when the child has learned to use the toilet, 24-hour urine collections are performed usually on the third day of the 3-day weighed dietary record according to standardized procedures. Parents and children receive personal and written instructions on how to collect complete 24-h urine samples. Children are asked to void their bladder upon getting up in the morning. This micturition is completely discarded. The time of this micturition is registered and defines the start of the 24-h collection which ends with the first micturition on the following morning. All micturitions are stored immediately in preservative-free, Extran-cleaned (Extran, MA03; Merck Darmstadt, Germany) 1 litre plastic containers at temperatures below –12 °C before transfer to the research institute. The dietician picking up the dietary record and urine samples inquires parents and children about the completeness of the urine samples and adds this information to the protocol sheet. Information on date of the urine collection, illness, or if urines are left unfrozen at home, are also included in the protocol for later quality control check. At the institute the containers are stored at –22 °C without addition of any preservative or chemicals until analyzed.

Spot urine samples

Additionally to the 24-h urine samples, one or more spot urine samples are collected in each visit usually parallel to the day of the anthropometric measurements. Routine analyses in spot urines include: pH, glucose, creatinine, among others. Aliquotes of the spot urines are also stored at –22 °C without addition of any preservative of chemicals for further analysis.

4.5.2 Analytical methods

A standardized laboratory protocol is used for each analyte in almost all cases, followed under strict and in the possible uniform conditions. The protocol includes use of the same chemicals, calibrator materials, and quality control parameters (mostly from identical suppliers), and if possible use of the same (original) measurement instruments.

After thawing and stirring, all urine samples undergo routine check using a commercial test strip (combur 9, Roche Diagnostics GmbH, Mannheim, Germany), while strictly avoiding that any dipping of the test strip into the original urine sample occurs. The latter is necessary due to a considerable liberation of iodine from the strips [123]. Total urine volume, pH, osmolality and creatinine are determined in all samples. Further parameters are

regularly quantified in numerous samples, i.e. urea, anions, cations, acid-base, organic acids, nitrogen and iodine – partly depending on specific research projects. From each total 24-h urine collection several aliquots of 20 ml each are stored at –22 °C for respective analyses and further reserve in the DONALD urine bank. The analytical methods for the urinary parameters (analytes) commonly measured in the DONALD Study and used in this thesis are detailed in **Table 6.**

Quality control of the analytical methods

In case that certain reagents or chemicals are no longer available (or were non-purchasable for a certain time) from the standard supplier- as it has been the case for perchloric acid (used in iodine analysis), or high purity acids and bases (acid-base tritration)- identical chemicals with the same analytical purity are obtained from alternative producers. To ensure analytical reliability also with respect to reagents' stability, test kits (like that of uric acid or citrate) are regularly ordered only few weeks in advance of schedule measurements or in case of long-term stable assays or reagents, substances are rigorously discarded when they reached their expiration date. Working solutions, buffers, and substrate stock solutions are stored at room temperature (in the case of certain diluting reagents of pure acid and base solutions) or are stored at 4 ºC in dark bottles if specified in the respective analytical instructions (e.g., for uric acid, urea, citrate analysis). Buffers for anion chromatography, are freshly prepared for each analysis. Reagents stability is periodically indirectly tested by measuring singe urine samples (together with the per se analysed controls) two times: first, in one analytical run with longer-term stored reagents and again during the following analytical run with freshly reconstituted reagents.

Quality control of urines

To avoid measurement errors due to incomplete urine collections, completeness of the 24-h urine was ascertained via sex-and age-specific, body-weight-related reference values of creatinine[50]. To ascertain completeness of urines in studies II-IV samples with a daily creatinine excretion rate below 0.1 mmol/kg body weight were not considered for analysis.

Further exclusion criteria for 24-h urine samples were: total collection time < 20-h or > 26-h, missing night urine collection, collection errors, no cooling of the urine sample, impurities (blood, faeces, etc.), missing collection time due to omitted micturition > 240 min, or disease of the child.

Table 6. Parameters measured in urines and their analytical method.

Parameter	Unit	Analytical method	Instrument
Urine volume	mL		Standard graduated cylinder.
Key analytes			
Creatinine	mmol/L	Photometric by the kinetic Jaffe Procedure [124].	Creatinine analyzer Beckman-2; Beckman Instruments Inc., Fullerton, CA.
Urea	mmol/L	Photometric with the urease-Berthelot method (Randox Laboratories Ltd; Crumlin, UK)	Photometer PM2DL Zeiss, Oberkochen, Germany
Osmolality	mosm/kg	Osmometer (freezing point depression)	Osmometer OM 802-D Vogel, Giessen, Germany.
Anions			
Chloride, Phosphate, Sulfate	mmol/L	Ion chromatography	Dionex 2000i/SP ion chromathography with an ion Pac AS4A column (Dionex GmbH, Idstein, Germany)
Cations			
Sodium, Potassium, Calcium, Magnesium	mmol/L	Flame atomic absorption spectrometry	Perkin-Elmer 1100 Spectrometer, Perkin-Elmer GmbH, Überlingen, Germany
Acid-base analytes			
pH		Three-phasic acid/base titration [125]	Mettler Toledo) end point tritrator (Giessen, Germany)
Titratable acid	mEq/L		
Ammonium, Bicarbonate	mmol/L		
Renal net acid excretion	mEq/L	Value estimated from the measured acid base analytes (titratable acid + ammonium – bicarbonate) [126].	
Organic Acids			
Total titrated organic acids	mmol/L	Titration by the van Slyke and Palmer method [127].	
Citrate	mmol/L	Photometric by enzymatic conversion of citrate via oxaloacetate to L-lactate , by a citrate kit from Boehringer, Manheim according to the principle described by Moellerin and Gruber [128].	Photometer PM2DL Zeiss, Oberkochen, Germany.

Table 6 Parameters measured in urines and their analytical method (*Continued)*.

Parameter	Unit	Analytical method	Instrument
Uric acid	mmol/L	Photometric by the uricase method with the Uric Acid plus kit (Roche Diagnostics GmbH, Mannheim Germany)	Photometer PM2DL Zeiss, Oberkochen, Germany.
Oxalate	µmol/L	Ion chromatography	Dionex 2000i/SP ion chromathograph with an ion Pac AS4A column (Dionex GmbH, Idstein, Germany)
Other analytes			
Iodine	µg/dL	Photometric (modified Sandell-Kolthoff) after wet ashing of the samples [129].	
Nitrogen	mmol/L	Kjeldahl technique	Buechi 430 Digestor and Buechi Distillation unit B-324

Influence of storage duration

As a preparatory work, we checked the frequently examined chemical parameters quantified in the 24-h urine samples and if they might be relevantly affected by the duration of storage of the urine samples at -22 ºC. For this the preparatory methodological analysis was conducted **(described in Chapter 5.1).** A reasonably stability was observed for the chemical analytes used in the DONALD Study, demonstrating that at least for this metabolites no statistical adjustment for varying storage duration was required in the respective data analysis.

4.6 Statistical considerations

All statistical analyses for the **Studies I-IV** were performed with Statistical Analyses System (SAS) procedures (version 9.1.3, SAS Institute, Cary, NC, USA). A *P*-value of <0.05 was considered statistically significant, except for analysis of interaction, where $P<0.1$ was considered statistically relevant.

The exposure and outcome variables together with sample size, study designs and applied statistical methods are summarized in **Table 7**.

Table 7. Overview on the conducted studies for this thesis.

Study	Sample size	Design	Age group	Exposures	Outcomes	Statistical method
Study I ***Methodological pre-analysis***	10 urine samples	Cross-sectional		• Time • Temperature	Concentration of 21 selected urinary analytes[1]	• Linear correlation • T-test
Study II	477 children 1589 observations	Longitudinal	4-10 y	• F&V intake • Other water sources	Free water reserve[1]	• Linear mixed effects regression models (PROC MIXED)
Study III	180 children	Cross-sectional	6-18 y	• Iodine excretion from spot urines[3]	Measured 24-h urinary iodine excretion	• Correlation coefficients • Cross-classification • Bland-Altman plots
Study IV	516 children 1959 observations	Longitudinal	6-12 y	• Dietary protein sources • Salt intake	24-h urinary iodine excretion	• Linear mixed effects regression models (PROC MIXED)

[1] Examined analytes are: creatinine, urea, osmolality, iodine, nitrogen, anions, cations, organic acids and acid-base parameters (see Chapter 4. 5)
[2]As marker of hydration status (see Chapter 2.3)
[3]Comparison of methods include: UIC (µg/L), 24-h iodine excretion using 24-h CR reference value.

4.6.1 Statistical models

Linear regression models

The method of linear regression is used to estimate the best fitting straight line to describe an association [130]. Multivariable regression analysis is a statistical approach for determining the relative contribution of different causes to a single event or outcome and allows to simultaneously assessing the impact of multiple independent variables on the outcome [131].

Linear mixed effects regression models (PROC MIXED in SAS)

Due to the longitudinal design of the DONALD Study, repeated measurements of individuals exist. Such repeated measurements are usually correlated and often exhibit heterogeneous variability. Thus, the use of mixed effects regression models is an appropriate approach for repeated assessed outcomes, since those models have a multilevel, hierarchical structure. The allowed inclusion of fixed and random effects in the models, accounts for the fact that observations on different subjects are independent while measurements within the same subject at different time points are dependent [132,133]. The PROC MIXED procedure considers all available measurements rather than using only participants with complete follow-up data, i.e. unbalanced data, assuming that any missing data is missing at random [132].

Time-varying predictors in longitudinal studies provide information on different sources of variation in the outcome, which can be separated by an approach called within context centering [132]. With this approach, the time-varying variables are decomposed into a time-invariant component that differs between persons, and a time-specific component that differs within persons [133,134]. Two methods of decomposing a time-varying predictor are 1) time-1 centering, in which the initial value for each person as well as the deviation from this initial value at each subsequent time point are included in the model, and 2) within-person centering, in which the time-varying predictor is decomposed into the average value for each person over time, and the deviation at each time point from this average value [132]. For the longitudinal analysis in **Studies II** and **IV** of the present thesis, the method of within-person centering was used. During the model construction progress, the best model and the best covariance structure of the data were selected based on the Akaike Information Criterion (AIC), acting as index of relative goodness-of-fit. For models with equal AIC, the Bayesian information Criterion (BIC) was also considered [132]. Further details on the statistical modelling are provided in the method section of Chapters 5.2 and 5.4.

4.6.2 Confounding, mediators and effect modification

The term *confounding* refers to a situation in which an association between an exposure and an outcome is observed as a result of the influence of a third variable, the confounder. Confounder can be described as a "bias" in the estimated association between an exposure and an outcome [135]. To be a confounder, per definition, the variable must be associated with the risk factor and causally related to the outcome. In order to qualify as a confounder, a variable must follow the following criteria: i) it must be causally associated with the outcome (even in the absence of the exposure of interest); ii) must be associated with the exposure of interest (casually or non-casually); and iii) the confounder must not lie on the causal pathway between the between exposure and the outcome (as an intermediate variable or mediator). As confounding cannot always be determined based on the data at hand, identification of confounders should be based on prior knowledge [136].

A *mediator* is an intermediate variable lying on the pathway between exposure and outcome and can serve to explain the mechanisms of association. For this reason, adjusting for intermediary variables has to be treated cautiously and should only be conducted in separated steps. If an association is eliminated by adjustment for a mediator, a potential mechanism involving the mediator is suggested, but does not question the result obtained before (at difference of the confounder). An *interaction (or "effect modification")* occurs when the effect of the exposure on the outcome is changed by the value of a third variable [131]. Since the association of interest is therefore not the same at different levels of an effect modifier, it needs to be explicitly described.

In the present thesis, potential confounders, mediators and interactions were evaluated in all studies. Details on the statistical modelling for each research question can be found in the Method sections of the specific studies described in Chapter 5.

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5. Studies and Results

5.1 Study I: Methodological pre-analysis on long term stability of clinical urine parameters stored at -22 ºC[1]

5.1.1 Summary

Measurement of clinical chemistry urine analytes is crucial in medical diagnostics and physiological and epidemiological biomarker research, but information on how urine storage under definite conditions (low temperature, preservative free), affects analyte concentrations in the long –term is rare. Therefore our aim was to examine the long-term stability and validity of analyte concentrations of 21 clinical biochemistry parameters in 24-h urine samples stores for 12 or 15 yr at -22°C and preservative free. Healthy children's 24-h urine samples in which the respective analytes had been measured shortly after sample collection (baseline) were reanalyzed. Second measurement was performed after 12yr (organic acids) and 15 yr (creatinine, urea, osmolality, iodine, nitrogen, anions, cations, acid-base parameters) with the same analytical methodology. Paired comparison and correlations between the baseline and repeated measurements were done. Recovery rates were calculated. More than half of the analytes (creatinine, urea, iodine, nitrogen, sodium, potassium, magnesium, calcium, ammonium, bicarbonate, citric and uric acid) showed measurement values after >10yr of storage not significantly different from baseline. 15 of the 21 parameters were highly correlated (r=0.99) between baseline and second measurement. Poorest correlation was r=0.77 for oxalate. Recovery ranged from 73% (oxalate) to 105% (phosphate). In conclusion, our results suggest high long-term stability and measurement validity for numerous clinical chemistry parameters stored at -22°C without addition of any urine preservative. Prospective storage of urine aliquots at -22°C for periods even exceeding 10yr, appears to be an acceptable and valid tool in epidemiological settings for later quantification of several urine analytes.

5.2.2 Introduction

As it has been reviewed in Chapter 2.2, urinary measurement of metabolites is a common and widely used non-invasive tool for assessment of health status in clinical [32] and epidemiological [11,33–35] settings. Prospective and longitudinal studies require a particular methodological attention regarding stability of metabolites, because often not all analyses can be performed immediately after collection. The stability of analytes in urine is dependent on many factors, such as way of collection procedures of the samples and storage temperature [36,137–139]; addition of preservatives to keep the urines free of bacteria [140,141]; time between collection and analysis; and number of thaw-cycles before analysis, among others.

[1]Resulting publication: Remer T, Montenegro-Bethancourt G, Shi L. Long-term urine biobanking: Storage stability of clinical chemical parameters under moderate freezing conditions without use of preservatives. Clin Biochem. 2014; 47: 307-311

Despite the importance to know such methodological characteristics for certain key analytes in clinical and epidemiological research [33], studies that have examined the long-term stability of urinary analytes like creatinine, urea, osmolality, iodine, nitrogen, anions, cations, acid-base parameters, or organic acids are rare. Particularly the long-term influence on recovery under conditions of low temperature storage for urine samples collected and stored without preservatives is unknown. As it was previously described (Chapter 4.5), the DONALD urine-biobank to date stores almost 8000 aliquots of 24-h urine samples from 3 to > 18 yr old participants. Therefore the aim of this **methodological pre-analytical study** was to examine the stability of selected urinary analytes measured in the DONALD Study, and the validity of reanalysing these parameters after 12 or 15 yr of storage at -22 °C. This may provide valuable information for a number of current as well as future epidemiological and/or intervention studies. The examined analytes include: creatinine, urea, osmolality, iodine, nitrogen, chloride, phosphate, sulfate, sodium, potassium, calcium, magnesium, components of renal net acid excretion, pH, tritratable acidity, ammonium, bicarbonate, and the organic acids citrate, uric acid, oxalate, and total (tritrated) organic acids.

5.2.3 Methods

Sample size for the methodological pre-analytical study

For baseline analysis, 10 urine samples (from 5 boys and 5 girls aged 4-10 y old) collected between 1993 and 1998 were randomly selected from the DONALD urine-data bank. Repeated measurements were done after 12 yr for organic acids (total titrated organic acid, citrate and uric acid) and 15 yr for the rest of metabolites. Mean age of the children was 6.7 yr (SD ±1.8) at the time of collection.

Urine analyses

Urine collection, storage and analysis were done as described in Chapter 4.2. The analytical method and instrument used to examine the included analytes: creatinine, urea, osmolality, iodine, nitrogen, chloride, phosphate, sulphate, sodium, potassium, calcium, magnesium, components of renal net acid excretion, pH, titratable acidity, ammonium, bicarbonate, and the organic acids citrate, uric acid, oxalate, and total (titrated) organic acids as done as described in Table 6 (Chapter 4.5). The clinical biochemical analyses at baseline were done as specified in Chapter 4.5 without further treatment apart from short stirring before pipetting the sample for the particular measurement.

For the repeat measurements (12 or 15 yr later), a separate aliquote was used that underwent the same procedure as at baseline. Urine aliquots used in the study went through one freeze-thaw cycle both before baseline and repeat measurement.

Statistical analyses

Baseline values were the values measured at the first analysis. For comparison, a repeated analysis was conducted in a separate aliquot of the same urine sample after 12 or 15

yr of storage at -22° C as described. Normal distribution for each variable (at baseline and repeated measurement) was tested with a Kolmogorov-Smirnov test. Descriptive statistics of the study sample are presented as means and SD, or medians with the 25^{th} and 75^{th} percentile, for the normal and non-normal distributed variables respectively. Values that equal zero, were treated as missing values and excluded from calculation, which exclusively applied to the parameter bicarbonate when urine pH fell below 6.2.

To determine the long-term stability of the concentration values for each analyte, paired t-test for normal distributed variables, or Wilcoxon's signed-rank test for non-normal distributed variables were used to compare the concentration values at baseline and after 12 or 15 yr. Associations between the measurements were assessed by Pearson's correlation in normal, and Spearman's rank correlation test in non-normal distributed variables.

Analytical recoveries were calculated as the ratio of repeated to baseline measurements and expressed as percentage.

Intra-assay coefficient of variance (CV) was determined by 6-10 measurements of the same urine in one-day; inter-assay CV was calculated from repeated measurements in the same urine sample analyzed at 10-12 different days. A *P* value<0.05 was considered to indicate statistical significance.

5.1.4 Results

Values of the analyzed metabolites at baseline and after 12 or 15 years are presented in **Table 8.** Significant differences were observed for osmolality, anions, titratable acid, and oxalate after 15 yr of storage. After 12 yr of storage only total titrated organic acids showed significantly different concentrations. The lowest recovery over time emerged for oxalate. With the exception of bicarbonate (-8%), total organic titrated acids (-11%), titratable acid (-19%), and oxalate (-27%), the mean percent changes in concentration over 15 yr (and 12 yr in the case of organic acids) were generally not substantial (± 5%).

Figure 4 shows the percent recovery difference for each metabolite from 100. After 12 or 15 years of storage, the correlations (r) ranged from 0.94 to 0.99, except for oxalate (0.77).

Intra and inter-assay CV (%) of the analyzed metabolites are also given in **Table 8** and were found ≤10% for all analytes except for bicarbonate and oxalate

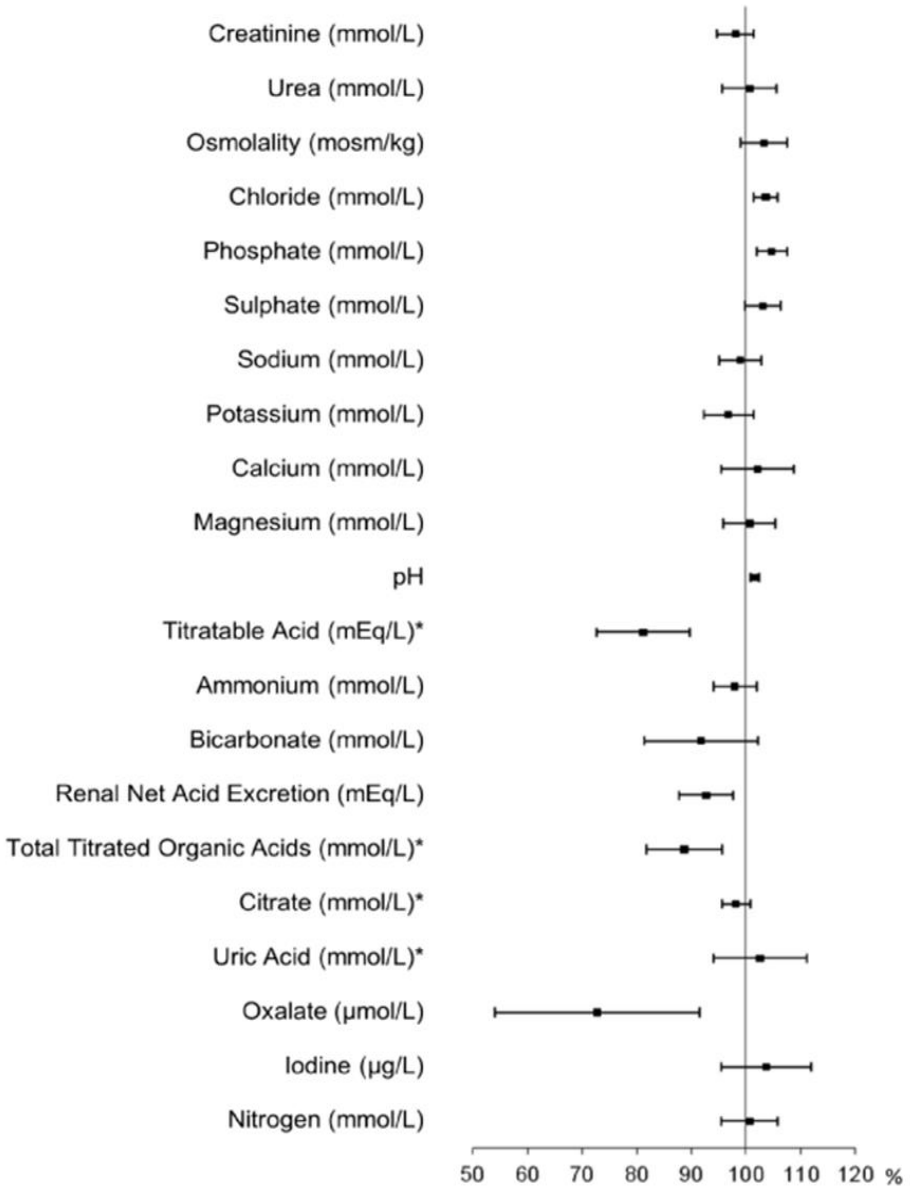
Table 8. Measurements and intra- and inter- assay coefficients of variance of the examined urinary analytes of Study I.

Parameter	Measurements		% Recovery[3]	r[4]	Intra-assay		Inter-assay	
	Baseline	15 yr (12 yr [2])			Mean ±SD[5]	CV%	Mean ±SD	CV%
Key analytes								
Creatinine (mmol/L)	6.4 ± 2.4	6.3 ± 2.2	98	0.99	6.3 ± 0.1	1.7	6.1 ± 0.2	3.5
Urea (mmol/L)	332.3 ± 91.7	334.4 ± 96.4	101	0.98	266.0 ± 12.2	4.6	267.0 ± 13.2	4.9
Osmolality (mosm/kg)	690.5 ± 185.9	713.0 ± 193.3	103*	0.99	585.0 ± 3.9	0.7	576.0 ± 16.2	2.8
Anions								
Chloride (mmol/L)	120.2 ± 44.6	124.8 ± 47.6	104*	0.99	82.0 ± 0.9	1.0	83.2 ± 3.2	3.8
Phosphate (mmol/L)	21.6 (17.0, 27.9)	22.2 (18.1,27.8)	105*	0.99	27.1 ± 0.3	1.1	27.6 ± 1.1	4.0
Sulfate (mmol/L)	17.7 ± 6.2	18.3 ± 6.6	103*	0.99	14.7 ± 0.1	0.9	15.0 ± 0.5	3.4
Cations								
Sodium (mmol/L)	118.7 ± 40.5	117.6 ± 41.1	99	0.99	93.7 ± 2.8	3.0	90.6 ± 3.2	3.6
Potassium (mmol/L)	60.3 ± 23.5	58.8 ± 24.7	97	0.99	59.0 ± 1.7	2.9	57.1 ± 2.5	4.4
Calcium (mmol/L)	2.2 ± 1.5	2.2 ± 1.4	102	0.99	0.6 ± 0.1	7.6	0.6 ± 0.1	8.0
Magnesium (mmol/L)	4.5 ± 1.1	4.5 ± 1.0	101	0.99	3.8 ± 0.1	2.4	3.7 ± 0.1	3.4
Acid-base								
pH	6.3 ± 0.4	6.4 ± 0.4	102*	0.99	6.8 ± 0.0	0.2	6.8 ±0.1	0.8
Titratable acid (mEq/L)	15.1 (11.1, 22.5)	11.4 (8.1, 20.7)	81*	0.98	11.9 ± 0.5	3.8	10.5 ± 1.1	10.1
Ammonium (mmol/L)	35.3 ± 12.1	34.7 ± 12.3	98	0.99	34.8 ± 0.8	2.4	35.5 ± 1.0	2.9
Bicarbonate (mmol/L)[6]	7.7 ± 2.6	6.9 ± 1.8	92	0.99	7.2 ± 0.9	11.9	6.9 ± 1.4	20.3
Renal NAE (mEq/L)[7]	37.1 (29.9, 61.6)	35.3 (27.1, 61.6)	93*	0.99	--	--	39.5 ± 1.1	2.7

Table 8. Measurements and intra- and inter- assay coefficients of variance of the examined urinary analytes of Study I (*Continued*).

Organic Acids (OA)								
Total titrated OA(mmol/L)	49.4 ± 11.8	44.2 ± 13.0[2]	89*	0.98	38.6 ± 0.8	2.0	35.9 ± 1.4	3.9
Citrate (mmol/L)	3.1 ± 1.6	3.0 ± 1.6[2]	98	0.99	2.2 ± 0.0	1.8	2.3 ± 0.1	2.1
Uric acid (mmol/L)	3.3 ± 0.9	3.4 ± 0.9[2]	103	0.96	2.3 ± 0.0	1.7	2.5 ± 0.1	5.4
Oxalate (µmol/L)	631.5 (330, 664)	399.0(316, 479)	73*	0.77	245.0 ± 17.3	7.0	295.8 ± 58.7	19.8
Other analytes								
Iodine (µg/dL)	6.0 (4.9, 9.1)	6.2 (5.5, 9.4)	104	0.94	7.2 ± 0.2	3.1	7.5 ± 0.5	7.1
Nitrogen (mmol/L)	833.6 ± 279.2	836.2 ± 274.7	101	0.99	571.0 ± 17.3	3.0	577.5 ± 18.5	3.2

[1]Data are arithmetic means ± SD; or median values with 25th and 75th percentiles in parentheses.
[2] Repeated measurement at 12 years after baseline.
[3] % recovery was calculated with the concentration values at 15 yr (12 yr) divided by the baseline values multiplied by 100.
[*]*P* significance for differences between the repeat measurement at 15 yr (12 yr) and baseline. Differences were tested with paired t-test for normal distributed variables and Wilcoxon signed rank test for non-normal distributed variables.
[4] Correlation coefficients (Pearson's in normal and Spearman's in none normal distributed parameters) for baseline *vs* repeated values.
[5] Mean concentration ± SD of the analytes for the intra- and inter-assay determination.
[6] In analysis for bicarbonate n=3 samples were excluded (pH<6.2).
[7] Renal NAE was calculated as: titratable acid + ammonium- bicarbonate.



[Taken from: Remer et al. Clin Biochem. 2014; 47:307-11].

Figure 4. Recovery percentage of the examined urine analytes of Study I.
Values are mean and SD for the recovery % of each analyte. Recovery % was calculated with the concentration values at 15 yr (12 yr *) divided by the baseline values multiplied by 100

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5.1.5 Discussion

To the best of our knowledge, stabilities of clinical chemical routine urine analytes and specific metabolites in urinary samples, stored at a temperature of about -20 °C for more than one decade have not been systematically investigated up to now. This study show for a number of select relevant renal excretion parameters an appropriate stability and validity if samples collected without added preservatives are stored at -22°C for long-term periods over 10 yr

The findings from this study can provide valuable information for observational and intervention studies in which urine collections are performed and samples are stored for a longer time period. The examined analytes from this **methodological pre-analytical study**, represent basic nutritional and /or physiological variables of which some (e.g. approved dietary biomarkers like iodine, urea, or the mineral anions or cations) may be analyzed directly after completion of respective study-specific urine collections; whereas others (like acid-base, stone risk-, or hydration parameters) may become important not until additional crucial research questions turn out at a later time point. A concrete example is that of the first National Food Consumption Survey of Germany performed between 1986 and 1988. In that study around two-thousand 24-h urine samples had been analyzed. Many years later it became clear that the additional measurement of osmolality in the available aliquots (along with the gathered nutritional and anthropometric data) would allow examining in detail water balance trough the adult life span [60]. Other examples can be found in the DONALD investigations which provide increasing evidence that nutrition-dependent acid-base balance may play a larger role than previously thought for bone health [142,143] and blood pressure development [144,145] in healthy children. However, acid-base specific NAE tritrations or related potential renal acid measurements [143,145] are not performed in the first place in most studies that collect urine samples.

Existent literature on the stability of urinary metabolites in long-term stored samples is also limited for other commonly analyzed, but rather more clinically relevant biomarkers such as kidney injury molecule-1 (KIM-1), neutrophil gelatinase associated lipocain (NGAL), interleukin-18 (IL-18), hepatocyte growth factor (HFG), cystatin C (Cys), or vascular endothelial growth factor (VEGF). It appears from the literature that these proteome-based biomarkers [146] need lower storage temperatures than -22 °C for appropriate molecule preservation during biobanking [147]. However, for those urine metabolites additional research is required to clarify specific aspects of storage conditions and duration. The term “urine-metabolites” groups a wide range of analytes and excretion products that are measured in urine, i.e. cells, complex proteins, organic molecules such as glucose, peptides, phenols, alkaloids, hormones and a variety of inorganic components (cations, anions, nitrogen) among others. Thus, depending on the analytes of interest, analytical factors related to laboratory techniques and assay systems need to be considered [32,36]. In this regard the intrinsic molecule

stability depending on the specific metabolite structure and the sample matrix plays a role, apart from intra- and inter-individual biological variabilities [148,149]. Previously, studies using urine samples and data from the DONALD Study addressed the importance of long-term stability of certain metabolites (at storage temperature of -22 °C) for use in retrospective analysis. Buyken et al. [150], evaluated the long-term stability of urinary C-peptide (UCP) for at least 12 yr, and Griefahn et al. [149] determined if the concentration of 6-hydroxy melatonin sulfate (6-OHMS) remained stable after 15 yr. In both examinations, stability of the analyzed hormones was found acceptable. However, stability checks could not be done by repeated measurements in the same samples, since the analytical methodology had not been used in the DONALD-lab before the research questions came up. Accordingly, comparative analyses with age-, sex-, body size- and nutrition-matched samples from different group of children (urine collection: recently and definite time-points in the past), were carried out. What is lacking up to now, are examinations in the same samples both initially measured and re-measured after a certain time period with the same methodology.

Urinary creatinine is regarded to be one of the most stable analytes, unaffected by short-and longer storage time (30 d to 2.5 yr) and various temperatures (ranging from <55 °C for short time to –4°C, -20°C, -70°C for longer time periods) [48,49]. With this **methodological pre-analytical study**, is confirmed a high degree of validity and stability for the analyte creatinine kept at -22 °C for even a longer time period (15 yr). In contrast to the former, oxalate is one of the most varying urinary metabolites in terms of reproducibility depending on analytical methods and interactions of the molecule with other urine components [151,152]. In accord with these literature findings, oxalate proved to be the analyte with the lowest mean percent recovery and with the highest variation between baseline and repeated measurement. Therefore, valid repeat measurements are not possible for oxalate after years of storage at around -20 °C and careful consideration has to be given when oxalate data with a varying storage history shall be used.

The need to add “stabilizers” or preservatives to urine samples in order to diminish bacterial degradation or certain metabolites is discussed controversially. Ferraz et al. [140] found no important differences for the metabolites: oxalate, calcium, magnesium, citrate, creatinine, and uric acid between urines either kept preservative free, acidified (HCL), or alkalized ($NaHCO_3$). Yilmaz et al. [141], also reported a relatively high stability for calcium, magnesium, phosphate, and uric acid when comparing measurement results of urine samples without added preservatives vs. those with HCL, sodium bicarbonate, or heat treatment. The authors concluded that for these metabolites no preservative addition or further treatment is needs. As an additional advantage, it is mentionable that no pH-dependent interference with the analytical methods has to be feared. Results from **Study I**, expand the aforementioned findings in as much as the high stability of these analytes over years of storage was shown to be present for the normal range of urine pH variation (pH 5 to 7) present in both children and adults.

Analyte stability over years of storage, even at -80 °C, needs to be determined for each biomarker of interest [153], or has- at least- to be considered during data analyses (in regression models) as an indirect, independent variable covering the storage duration for each individual sample. The longitudinal design of the DONALD Study along with its long-term urine biobanking allowed us to address this important methodological stability question directly and specifically. Because the initial measurements in **Study I** were-according to the DONALD protocol (detailed described in Chapter 4.5)- not done on freshly collected, unfrozen urines, but after one freeze-thaw cycle (freeze at home-thaw in lab), a possible initial "first-freeze" degradation or loss of examined metabolites could be eliminated.

Despite the large number of possible pre-and post-analytical laboratory errors [51,154], most of the analytes examined in this study, showed an appropriately low intra-and inter-assay precision (CV) of <5%. Interestingly, for almost all parameters (except calcium) with an intra-assay CV>5% and/or inter-assay CV>10% (ammonium, bicarbonate, oxalate), recoveries deviate more than 5% from baseline levels. This may indicate that at least some part of these poorer recoveries could be due to lower measurement precisions and not exclusively to decreasing analyte stabilities.

In conclusion, the results from **Study I** strongly suggest that urine samples stored at a temperature level of about -20 °C for a period up to 15 yr have a high stability for creatinine, urea, cations (sodium, potassium, calcium, magnesium), ammonium, citrate, uric acid, iodine and nitrogen. Less stable or more prone to (mostly small) measurement errors over time are osmolality, anions (chloride, phosphate, sulfate), tritratable acidity, bicarbonate, and total organic acids. The most erroneous and least stable analyte was oxalate. The very high correlations between almost all baseline and repeated measurements along with partly excellent or at least good recoveries for most of the analytes examined, underline measurement validity as well as eligibility for later analyses in clinical or epidemiological long-term studies. Furthermore, addition of urine preservatives appears to be not a must to appropriately preserve the respective urinary parameters in biobank settings of long-term epidemiological studies.

5.2 Study II: Effect of consumption of high water content foods (fruit and vegetables) on "Free Water Reserve" as marker of hydration status[2]

5.2.1 Summary

The specific effects of fruit and vegetable (F&V) intake on water balance and consequently on 24-h hydration status (HS) are unknown. The main objective of **Study II** was therefore to examine whether a higher F&V intake per se is associated with improved HS and attempted to quantify the influence of greater consumption of F&Vs on HS. We longitudinally examined relations of F&V with HS in 442 healthy children (4- to 10-y-olds) providing 1286 complete 3-d weighed dietary records and parallel 24-h urine samples. Free water reserve (FWR) served as an HS biomarker. Median FWR and water balance variables were analyzed in different categories of solid-F&Vintakes. Repeated-measures regression models (PROC MIXED), adjusted for all other dietary water sources, were used to quantify the separate effects of solid-F&V and F&V-juice consumption on FWR. Negative FWR values, which indicated risk of hypohydration, were observed in 22% of children. FWR was significantly higher in solid-F&V consumers with high intakes than in those with low intakes *(P < 0.0001).* PROC MIXED models predicted an increase of 46 mL in FWR (average in boys and girls) when increasing solid-F&V intake by 100 g. Similar results were observed for F&V juice (ß= 43, *P<0.0001*). Drinking water and milk were the other significant dietary predictors of FWR. Solid F&Vs and F&V juices contributed 12% and 10%, respectively, to total water intake. In conclusion, these data confirm that regular intake of F&Vs may relevantly improve HS in children. Dietary interventions to increase F&V intake may be a promising strategy to achieve positive water balance in this population.

5.2.2 Introduction

As reviewed in Chapter 2.3 Hydration Status (HS) is an important determinant of human health [17,53,56,155]. Adequate hydration may reduce the risk of a range of physiologic disorders and diseases, such as headache, urothialisis, and constipation [17]. HS is mainly determined by the balance of water intake (from foods and beverages) and water output (renal and nonrenal losses; eg, sweating, feces). The high and precise regulation of this balance in a range of ±0.2% of body weight over a 24-h period [55] is maintained by subtle hormonal changes, inducing thirst sensation and water reabsorption in the kidneys. How effective this regulation works was recently observed even in the elderly, a population at high risk of dehydration [60,156].

The knowledge about the various variables that determine HS (water intake, water output, and dietary solute load) led to the concept of the "free water reserve" (FWR), introduced by Manz et al [15,16] in the late 1990s. The FWR (reviewed in Chapter 2.3), is a

[2] Resulting publication: Montenegro-Bethancourt G, Johner SA, Remer T. Contribution of fruit and vegetable intake to hydration status in schoolchildren. Am J Clin Nutr. 2013; 98 (4):1103-12.

physiologic concept to characterize 24-h HS in an individual and represents the balance between available body water (measured by urine volume) and water requirements (based on an individual's solute load and the theoretical maximum urine osmolality); therefore, an FWR >0 mL indicates "euhydration" (Chapter 2.4, Figure 2). Although there is no universally accepted method for measuring HS to date, it is the biomarker most appropriate to characterize individual hydration in a 24-h period [14,16].

The role of fruit and vegetables (F&Vs) as important food groups in the prevention of chronic diseases has been recognized [157–160], and the intake of F&Vs is part of dietary recommendations in many countries [159,161,162]. As a result of the high water content of F&Vs (~70–95%, comparable to the 85–100% water content from beverages) [98], an increase in F&V intake can relevantly contribute to enhance water intake. However, to our knowledge, so far no study has investigated the impact of the latter on the physiologically well-regulated HS. This fact becomes relevant when it is hypothesized that HS might influence an individual's food intake, i.e., that a water deficit may induce an individual to prefer a diet with higher water content, potentially as a compensatory mechanism to counteract the deficit [97]. To date, no study has investigated that hypothesis in free-living children. Therefore, **Study II** addressed **research question 2,** and aimed to 1) examine whether a higher F&V intake per se leads to better HS or whether the higher water intake from F&Vs instead results in a compensational reduction in water intake from other sources, eventually keeping the water balance constant, and 2) to quantify the effect that adding F&Vs (solid or liquid) to the common diet has on the FWR as a measure of 24-h HS when other important water sources are controlled. For this purpose we analyzed longitudinal data collected from 2000 to 2010 in 4- to 10- y old participants of the DONALD Study.

5.2.3 Methods

Sample population of Study II

The sample for analysis of **Study II** consisted of a subgroup of DONALD participants between 4 and 10 y of age who had at least one 24-h urine sample collected between January 2000 to December 2010 with a parallel dietary record (encompassing the day of urine collection) (n= 477 children providing n= 1589 urine samples). Within this period, each subject underwent a minimum of 1 and a maximum of 7 potential measurements. The general inclusion and exclusion criteria had to be fulfilled 1) for urines 2) and plausibility of dietary records (Chapter 4.2-4.5). The employment of this criteria resulted in the rejection of 53 children (11%) and their corresponding urine samples and dietary records. The final sample consisted of 424 children who provided 1286 complete 24-h urine samples with corresponding plausible dietary records.

Urine sampling and analysis

The 24-h urine collection was generally carried out on the third day of dietary

recording. See Chapter 4.5 for a complete description of collection methods. From the 24-hour urine samples FWR (mL/24 h) was determined as described in Chapter 2.3 and used as marker of HS. The general concepts described in the general background were applied for this analysis. Positive values of FWR were defined as "euhydration"; negative values of FWR denote "risk of hypohydration" [15,97]. Furthermore, we estimated the Adequate Intake of total water (mL/d) for our population [15]. On the basis of the definition of the Recommended Dietary Allowance ["the average daily dietary nutrient intake level sufficient to meet the nutrient requirements of nearly all (97–98%) healthy individuals in a particular life stage and sex group" [111], (see Chapter 2.5). Adequate Intake was calculated as the difference of the observed median total water intake (TWI; in mL/d) and the third percentile value of the FWR, as previously applied [15,60].

Dietary assessment

For the purpose of this study, to estimate individual food and nutrient intake, 3-d weighed dietary records were used. Dietary analysis is described in Chapter 4.4. Data on total daily energy (MJ/d) and nutrient intakes (g/d) were calculated for each participant by using 2 different approaches: 1) the mean of the 3-d recording and 2) the value only from the day of the urine collection.

All reported food items were classified into the 12 food groups described in Table 5, of the general methodology (section 4.4), and analyses was based on the two main categories 1) beverages and 2) foods. Total Water Intake (TWI) corresponds to the total available water from beverages and foods additionally including the metabolic water. Metabolic water was calculated with the following formula recommended to estimate the water from oxidation [97]: fat intake (g/d) 31.07 + carbohydrate intake (g/d) 30.55 + protein intake (g/d) 30.41.

Anthropometric and additional variables

Weight and height were obtained at the time of the dietary recording following the standard procedures described in Chapter 4.2. BMI was calculated as body weight (in kg) divided by height (in m) squared. Sex-and age independent BMI SD scores were calculated by using German national reference data [117].

Maternal characteristic i.e. level of education and anthropometrical measurements were also assessed as described in Chapter 4.3. Maternal overweight was set to a BMI ($kg/m^{2)}$ > 25.

Physical activity (active, moderately active, and inactive) was assessed by a questionnaire on daily organized and unorganized activities, which is an adaptation of the Adolescent Physical Activity Recall Questionnaire [119].

Statistical analysis

Anthropometric, dietary, and urinary variables were tested for normality by the Shapiro-Wilcoxon and Kolmogorov-Smirnov tests. Data was stratified by sex and age-group (4-10 y). Outliers of TWI and FWR were defined as lying within 1.5 times the IQR below the first quartile or above the third quartile. Variables are described as medians and IQRs as they were not normally distributed. Stratification by sex was based on the known sex differences in urinary osmolality [(163)]; age groups were set according to the German reference values for nutrient intakes [(110)]. Differences between age groups and sex were tested with unadjusted linear mixed-effects regression models (PROC MIXED in SAS) to account for the dependency between repeated measurements on the same child.

To examine the association of FWR and the corresponding water balance variables with solid-F&V ($F\&V_{solid}$) intakes, their distribution was grouped into sex-specific categories of consumption: quartiles of $F\&V_{solid}$ intakes ($g \cdot d^{-1} \cdot MJ^{-1}$) were assigned to low ($<25^{th}$ percentile), moderate ($\geq 25^{th}$ and $\leq 75^{th}$ percentile), and high ($>75^{th}$ percentile) categories. Differences between categories of consumers were again tested with unadjusted PROC MIXED models.

The impact of $F\&V_{solid}$ and F&V-juice ($F\&V_{juice}$) intakes on FWR was evaluated by using multivariable linear mixed-effects regression models (PROC MIXED), including both fixed and random effects. The random components of these models account for the nested nature of our data (children within families) and the lack of independence between repeated observations on the same person. Linear mixed-effects regression models consider all available measurements rather than using only participants with complete follow-up data [(119)]. The basic longitudinal regression model included FWR as the dependent continuous variable and $F\&V_{solid}$ and $F\&V_{juice}$ intakes, chronological age, sex, chronological study years (2000–2010), and energy partition (description below) as principal independent fixed effects. The following nondietary variables potentially affecting the association between $F\&V_{solid}$ or $F\&V_{juice}$ intakes and FWR were considered: BMI-SDS, 24-h creatinine excretion (mmol/d), physical activity, seasonality, and maternal overweight and maternal level of education. Only those variables that1) substantially modified the coefficient of $F\&V_{solid}$ or $F\&V_{juice}$ by 10%, 2) significantly predicted the FWR ($P < 0.05$), or 3) improved the fit statistic (Akaike's information criterion) were additionally included as fixed effects to our model. To allow for the distinction of within-person and between-person effects of dietary F&V intakes on FWR, the main predictors (F&Vs as solids and juices) were centered on each individual's mean of each individual predictor over time. Time-specific deviations from the person-specific means were used to test whether within-person changes in F&V intakes were associated with within-person changes on FWR. In addition, the person-specific, time-invariant means of the predictors were entered in the model to examine whether between-person differences in dietary F&V intakes are associated with differences in mean FWR between subjects [(134)].

The respective adjusted means of FWR in the categories of $F\&V_{solid}$ or $F\&V_{juice}$ consumers (low: < 25th percentile of person-specific means: moderate: ≥ 25th to ≤75th percentile; high: > 75th percentile) were the least-squares means predicted by the model when the other variables were held at their mean values.

To quantify the changes in FWR when "adding" solid F&Vs or F&V juices to the diet, the PROC MIXED model was additionally adjusted for all other relevant dietary predictors of FWR. The following dietary variables were considered: drinking water (mL/d), intakes of other food groups (milk and whey based milk products, cheese, meat/fish/eggs, cereals, potatoes, legumes, fat, and diverse listed in Table 5 (section 4.4) dietary fiber (g), and sodium excretion (as an estimate for salt intake). For energy adjustment of all PROC MIXED models, we used the energy partition model: ie, the dietary predictors (eg, $F\&V_{solid}$ and $F\&V_{juice}$) were adjusted for energy intake from all other foods. The estimates of the F&V food group reflect the effect of "adding" the respective food group, comprising both its energy and nonenergy effect [(164)]. Models for FWR contained a random statement for the family level and one for the person level with an unstructured covariance. The latter random statement considered individual differences in FWR at the first observation (intercept) and individual changes with increasing age (slope). On the basis of the known sex differences in urinary variables [(163)], the results of the models were presented stratified by sex.

Because the analyses indicated no interactions of age group with the relation of $F\&V_{solid}$ intakes and FWR (each P >0.8), data from both age groups were pooled for analysis.

Multivariate logistic regression was used to calculate ORs for the risk of hypohydration (FWR <0) by the defined categories of $F\&V_{solid}$ intakes ($g \cdot d^{-1} \cdot MJ^{-1}$) (low, moderate, and high) adjusted for age. Only the first observation of each child was included in this analysis.

All analyses were performed twice: 1) with mean dietary intakes calculated from the individual means of all 3 recording days and 2) only from the one day parallel to the urine collection (see the dietary assessment Chapter 4.4). Because calculated means of energy intake, TWI and intakes of F&Vs (solid and juice), drinking water, and milk did not differ significantly (tested with the Wilcoxon Mann-Whitney test) and the results of the PROC MIXED model were comparable (similar ß-values and identical significance levels; (data not shown), we decided to present only the results for 3-d mean dietary intakes as more stable values reflecting the children's usual dietary intakes.

5.2.4 Results

Descriptive characteristics for anthropometric, urinary, dietary and water variables of the study sample stratified by sex and age group are presented in **Table 9.** Median FWR values were positive in all age/sex groups; only in boys were negative values observed in the

25th percentile, denoting a risk of hypohydration. FWR was significantly lower in boys than in girls; accordingly, positive FWR values (euhydrated) were observed in 72% of the measurements in boys and in 84% of the measurements in girls. $F\&V_{solid}$ intake (g/d) increased significantly with age but showed no differences between boys and girls. When related to energy, $F\&V_{solid}$ intake ($g \cdot d^{-1} \cdot MJ^{-1}$) was significantly lower in 7- to 10-y-old boys than in the younger boys and also lower than in their female counterparts. $F\&V_{juice}$ intake contributed 10.7% to the TWI and 5.3% to total energy intake in comparison to the $F\&V_{solid}$ intake, which contributed 12.0% and 5.6%, respectively.

Medians (25th, 75th percentiles) for TWI were 1380 (1195, 1625) mL/d in 4- to 6-y-old children (n= 533 observations) and 1700 (1465, 1975) mL/d in 7- to 10-y-old children (n= 753 observations) ($P< 0.05$). Median (25th, 75th percentiles) water intake values (excluding metabolic water) stratified by age were 1215 (1040, 1450) mL/d for 4- to 6-y-old children and 1490 (1270, 1745) mL/d for 7- to 10-y-old children ($P< 0.0001$). The median ratio between TWI and energy was 1.0 mL/kcal for all groups.

FWR and the general variables for water balance by categories of $F\&V_{solid}$ intakes (low, moderate, and high) are presented in **Table 10.** Children with a high $F\&V_{solid}$ intake had a significantly higher FWR than children with a low intake. Accordingly, TWI from solid F&Vs was significantly higher in children with high $F\&V_{solid}$ intake; however, a significant decrease was observed in TWI from foods. Overall, TWI (from all foods and beverages) increased significantly with increasing $F\&V_{solid}$ intake. With regard to the urinary variables, we observed a significantly higher urine volume in the high-$F\&V_{solid}$ intake group, whereas the obligatory urine volume did not change [attributable to the constant solute load (mOsm/d)]. Only boys in the high-$F\&V_{solid}$ intake category had a significantly lower urine osmolality (mOsm/L).

Table 9. Anthropometric, urinary and dietary parameters of the study sample from Study II.

	Boys			Girls		
	4-6 y	**7-10 y**	***P*** [2]	**4-6 y**	**7-10 y**	***P*** [2]
Subjects (n)	146	163		147	163	
No. of measurements	269	364		264	389	
Age (y)	5.0 (4.8, 6.0)	8.1 (7.1, 9.2)		5.0 (4.1, 6.0)	8.1 (7.8, 9.1)	
BMI (kg/m²)	15.3 (14.7, 16.4)	16.1 (15.2, 17.4)	0.006	15.4 (14.6, 16.2)	15.9 (15.1, 17.6)	0.1
BMI-SDS	-0.23 (-0.67, 0.58)	-0.17 (-0.63, 0.40)	0.7	-0.10 (-0.68, 0.49)	-0.20 (-0.77, 0.46)	0.2
Urinary variables						
FWR (mL/d)	107 [3] (- 18, 270)	170 [3] (-20, 375)	0.003	190 (60, 340)	225 (70, 460)	0.0002
FWR, third percentile (mL/d)	-110	-140		-80	-100	
Urine volume (mL/d)	605 (450, 790)	805 (600, 1050)	<0.0001	610 (470, 785)	800 (600, 1050)	<0.0001
Urine osmolality (mOsm/L)	680 [3] (525, 866)	665 [3] (520, 854)	0.3	592 (466, 725)	570 (440, 744)	0.4
Urine solutes (mOsmo/d)	392 [3] (324, 444)	525 [3] (440, 605)	<0.0001	350 (310, 410)	460 (400, 536)	<0.0001
Dietary variables						
Energy intake (MJ/d)	5.7[3] (5.1, 6.3)	7.3[3] (6.6, 8.2)	<0.0001	5.4 (4.9, 6.0)	6.4 (5.7, 7.2)	<0.0001
F&V$_{solid}$ (g/d)	192 (120, 260)	200 (140,290)	0.003	200 (150,280)	220 (145,300)	0.001
F&V$_{solid}$ (g · d^{-1} · MJ $^{-1}$)	31.4 (20.4, 48.0)	28.2[3] (19.0, 40.0)	0.001	40.0 (27.0, 52.0)	33.1 (23.0, 46.0)	0.4
F&V$_{juice}$ (mL/d)	200 (95, 285)	200[3] (85, 350)	0.004	155 (65,260)	150 (40, 270)	0.9
F&V$_{juice}$ (mL· d^{-1} · MJ $^{-1}$)	33.0 (17.0, 52.5)	27.0 (12.0, 48.0)	0.1	29.0 (13.0, 45.2)	24.0 (7.2, 40.2)	0.005
TWI (mL/d) [4]	1420 (1200, 1640)	1785 [3] (1060, 2035)	<0.0001	1350 (1160, 1610)	1620 (1410, 1900)	<0.0001
water from beverages [5] (%)	45.5 (36.5, 52.3)	46.7 (40.1, 55.5)	0.001	45.6 (38.5, 52.1)	47.8 (41.5, 54.9)	0.006
water from foods [6] (%)	42.4 (36.2, 50.0)	40.4 (32.8, 47.1)	0.0003	43.4 (35.1, 48.9)	39.8 (33.6, 45.1)	0.006
metabolic water [7] (%)	12.4 (11.0, 13.8)	12.5 (11.0, 14.1)	0.8	12.2 (10.5, 14.2)	12.3 (10.6, 13.8)	0.1
TWI (mL/kcal)[8]	1.02 (0.91, 1.15)	1.00 (0.91, 1.15)	0.7	1.04 (0.90, 1.21)	1.03 (0.91, 1.20)	0.2
AI of total water[9] (mL/d)	1530	1925		1430	1720	

Foot note Table 9 (*next page)*

Foot note Table 9 (*Continued)*

[1] All values are medians; 25th, 75th percentiles in parentheses. Total of 1286 measurements, AI, Adequate Intake; BMI-SDS, BMI-SD score; F&V, fruit and vegetable; $F\&V_{juice}$, fruit and vegetable juice; $F\&V_{solid}$, solid fruit and vegetables; FWR, free water reserve; TWI, total water intake.
[2] Differences between age groups and sex were tested with linear mixed-effects regression models (PROC MIXED) to account for the dependency between repeated measurements on the same child.
[3] Significant differences compared to girls at the same age group.
[4] TWI, estimated as the sum of water from beverages (plain and mineral water and water content from beverages and F&V juices), water from foods (including F&V solids, milk, dairy products, and solid foods), and metabolic water (from oxidation).
[5] Water from beverages (%) = (water from beverages / TWI) X 100.
[6] Water from foods (%) = (water from foods / TWI) X 100.
[7] Metabolic water (%) = ([(0.41 x protein intake (g)) + (0.55 x carbohydrate intake (g)) + (1.07 x fat intake (g))]) / TWI X 100.
[8] Estimated as TWI/total energy consumed, according to the European Food Safety Authority recommendation of 1 mL/kcal for total available water intake /energy [(56)].
[9] AI= median TWI- FWR third percentil

Table 10. FWR and water balance by categories of solid F&V solid intake in children from Study II.

	$F\&V_{solid}$ categories[2]							
	Boys (n=210; 633 measurements)				Girls (n=214; 653 measurements)			
	Low	Moderate	High	*P*[3]	Low	Moderate	High	*P*[3]
$F\&V_{solid}$ (g/d)	98 (60, 120)	196 (160,240)	346 (290,415)	*	100 (80, 125)	210 (180,254)	348 (300,425)	*
FWR (mL/d)	40 (-60,240)	160 (0, 350)	185 (60, 460)	*	150 (25, 305)	200 (70, 400)	280 (125,530)	*
Age	7.0 [3] (5.3, 9.0)	7.0 (5.4, 9.0)	7.0 (5.0, 8.0)		7.2 (5.2, 9.1)	7.0 (5.1, 9.0)	7.0 (5.8, 9.0)	
BSA	0.97 (0.8, 1.1)	0.94 (0.8, 1.1)	0.95 (0.8, 1.1)	n.s.	0.93 (0.8, 1.1)	0.92 (0.8, 1.0)	0.92(0.8, 1.1)	n.s
Water intake (mL/d)								
TWI [4]	1600 (1300, 1870)	1610 (1345, 1870)	1720 (1440, 1985)	*	1420 (1205, 1610)	1520 (1300, 1750)	1640 (1350, 1970)	*
TWI from $F\&V_{solid}$	90 (55, 110)	180 (150, 220)	320 (270, 380)	*	90 (70, 115)	195 (160, 235)	320 (275, 390)	*
TWI from $F\&V_{juice}$	190 (95, 345)	180 (85, 300)	170 (75, 305)	*	150 (40, 240)	155 (60, 270)	115 (30, 220)	n.s
TWI from drinking water	500 (340, 720)	530 (380, 730)	560 (375, 800)	n.s.	525 (405, 685)	550 (395, 735)	570 (405, 770)	n.s
TWI from foods (except $F\&V_{solid}$)	700 (540, 860)	655 (545, 785)	610 (490, 730)	*	620 (490, 740)	565 (485, 680)	550 (460, 660)	*
Water Excretion (mL/d)								
Non renal water losses [5]	900 (695, 1105	840 (660, 1085)	880 (715, 1135)	n.s.	725 (520, 910)	750 (565, 930)	775 (615, 990)	n.s.
Urine volume	665 (460, 900)	710 (540,960)	745 (580, 1040)	*	680 (490, 890)	720 (530, 950)	795 (605, 1075)	*
Obligatory urine volume (mL/d)	565 (440, 695)	560 (460, 670)	535 (470, 670)	n.s	500 (415, 605)	485 (415, 590)	505 (410, 600)	n.s
Urine osmolality (mOsm/L)	782 (600, 945)	652 (515, 833)	620 (470, 750)	*	648 (502, 808)	580 (445, 720)	527 (400, 684)	n.s
Urine solutes (mOsm/d)	470 (366, 575)	464 (380, 554)	445 (390, 555)	n.s	416 (342, 501)	405 (342, 488)	420 (340, 500)	n.s

[1] All values are medians; 25th, 75th percentiles in parentheses. Total n=424 children (n=1286 observations). BSA, body surface area; F&V, fruit and vegetable; $F\&V_{juice}$, fruit and vegetable juice; $F\&V_{solid}$, solid fruit and vegetables; FWR, free water reserve; n.s., non-significant; TWI, total water intake.

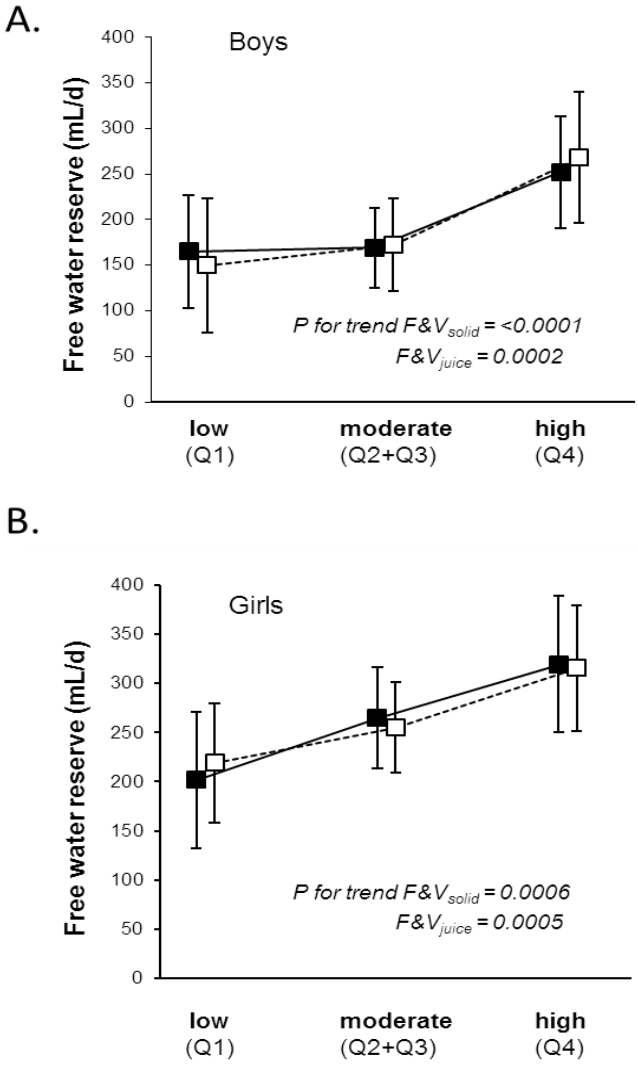
[2] Categories of solid F&V intake derived by quartiles of mean intakes per energy (MJ) combining the second and third quartile. Categories were defined as follows: lo2 (<25th percentile), moderate (≥n 25th to ≤ 75th percentiles), and high (> 75th percentile).

[3] Differences between categories of intake were tested with linear mixed-effects regression models (PROC MIXED) to account for the dependency between repeated measurements on the same child. **P* for significance for values < 0.05.

[4] Estimated as the total water from beverages (plain and mineral water and water content from beverages and F&V juices), water from foods (including F&V solids, milk, dairy products, and solid foods), and metabolic water (from oxidation).

[5] Estimated as the difference between TWI (mL/d) and 24-h urine volume (mL/d).

The results of the multivariable linear mixed-effects regression models (PROC MIXED) adjusted for age, energy partition; chronological study years, BMI-SDS, and 24-h creatinine excretion to investigate the impact of $F\&V_{solid}$ and $F\&V_{juice}$ intakeson FWR are presented in **Figure 5.** Boys and girls with consistently high $F\&_{solid}$ and $F\&V_{juice}$ intakes had significantly better FWR values; between-person differences and within-person changes (data on the latter not shown) were similar for $F\&V_{solid}$ and $F\&V_{juice}$ intakes.



[Taken from: Montenegro-Bethancourt et al Am J Clin Nutr. 2013; 98:1103-12].

Figure 5. Impact of solid F&Vs and F&V juices by categories of intake on FWR. Values are means (95% CIs) of quartiles of the respective F&V variables (between-person differences). Values were derived from the PROC MIXED model adjusted for energy partition (total energy minus the energy contribution from $F\&V_{solid}$ and $F\&V_{juice}$), age, study chronological years (2000-2010), BMI-SDS, 24-h creatinine excretion. *P* for continuous trend refers to the *P* values obtained in PROC MIXED models with F&V intakes as continuous independent variables. For boys, the respective ranges of F&V categories (built by age group) in low (Q1), moderate (Q2+Q3), and high (Q4) groups were as follows -$F\&V_{solid}$: 12-145, 140-275, and 275-675 g/d; $F\&V_{juice}$: 0-126, 120-310, 300-850 mL/d. The respective ranges of girls were as follows -$F\&V_{solid}$: 30-165, 160-285, 275-590 g/d; $F\&V_{juice}$, 0-80, 80-254, 250-930 mL/d.

The results from the PROC MIXED model, which was additionally adjusted for all other relevant dietary predictors of FWR (to quantify the changes in FWR when "adding" solid F&Vs or F&V juice to the diet), are presented in **Table 11**. The ß values for the between-person differences represent the difference in FWR in milliliters for 1-unit difference in the explanatory variable ($F\&V_{solid}$ or $F\&V_{juice}$) between the individuals across time. The ß values for within-person change indicate the intraindividual change in FWR (mL) for 1-unit within-person change in the explanatory variable. $F\&V_{solid}$ and $F\&V_{juice}$ intakes both were significantly positively associated with FWR at the inter-and intraindividual level. A difference of 100 g in $F\&V_{solid}$ intake between persons was related to a 56-mL between-person difference in FWR in boys and a 35-mL between-person difference in girls, whereas a within-person increase of 100 g in $F\&_{solid}$ intake predicted 39- and 40-mL within-person increases in FWR in boys and girls, respectively.

From the other food groups examined, as expected, drinking water and milk (milk and whey-based milk products) showed a significant effect on the FWR. According to our model, an intake of 100 mL of water from drinks increases the FWR by 44 mL in boys and 55 mL in girls. A lesser, but significant effect was observed for the milk group, which increased the FWR by 25 mL in boys and 33 mL in girls per 100 g of intake. The TWI from other food groups, i.e., food groups not included in the regression model: 1) cheese, 2) meats, poultry, seafood and eggs, 3) fats, 4) cereals, 5) potatoes, 6) legumes, 7) diverse (see Table 5, Chapter 4.4) had no significant effect on the FWR ($P>0.5$).

Multivariate-adjusted ORs for the risk of being hypohydrated (FWR < 0) showed that higher consumption of $F\&V_{solid}$ was significantly associated with a reduced risk of hypohydration. Boys in the low- and moderate-$F\&_{solid}$ intake categories showed a 2.6-fold (95% CI: 1.1-, 5.9-fold) and 1.1-fold (0.5-, 2.3-fold) higher risk, respectively, compared with the highest category (*P*-trend = 0.03). Girls had a 2.0-fold (0.8-, 4.8-fold) and 0.6-fold (0.3-, 1.5-fold) increased risk in the low and moderate categories compared with the highest category (*P*-trend = 0.02).

The fruit most consumed for this group of children were as follows: apples, bananas, and strawberries; in the vegetable category were these were cucumbers, carrots, and tomatoes. All F&Vs were consumed either raw or as part of preparations. With regard to fruit juices, the 3 most commonly consumed were apple, orange, and grape.

Table 11. Dietary predictors of FWR in the participants of Study II.

	Changes in Free Water Reserve (mL /d)[1]			
	Boys (n=210; 633 measurements)		Girls (n=214; 653 measurements)	
	ß (95 % CI)	*P*	ß (95 % CI)	*P*
F&V$_{solid}$ (g/d)				
Between-person difference	0.56 (0.33, 0.79)	<0.0001	0.35 (0.11, 0.58)	0.0042
Within-person change	0.39 (0.14, 0.64)	0.0025	0.40 (0.21, 0.59)	<0.0001
F&V$_{juice}$ (mL/d)				
Between-person difference	0.41 (0.27, 0.55)	<0.0001	0.45 (0.26, 0.64)	<0.0001
Within-person change	0.32 (0.17, 0.46)	<0.0001	0.37 (0.22, 0.52)	<0.0001
Drinking water (mL/d)[2]	0.44 (0.36, 0.51)	<0.0001	0.55 (0.48, 0.63)	<0.0001
Milk (g/d)[3]	0.25 (0.14, 0.36)	<0.0001	0.33 (0.21, 0.46)	<0.0001

[1] Values are the results of the longitudinal mixed model with a random intercept adjusted for age, study chronological years (2000-2010), BMI-SD score, 24-h creatinine excretion, total water intake from other foods (total water intake minus the total water intake from the food groups included in the model, i.e. solid F&Vs and F&V juices, drinking water and milk) and energy from other foods (total energy minus the energy contribution from the food groups included in the model). ß, estimated change of the dependent variable per unit change of independent variable.
[2] Comprises plain water and water component from other beverages.
[3] Milk and whey- based milk products.

5.2.5 Discussion

Although it is known that 24-h urinary osmolality and related FWR values are linked to the TWI from the diet [54,97,126,155], studies on the direct effect of specific dietary factors on HS are limited. To the best of our knowledge, **Study II** for the first time provides evidence and quantifies the association between F&V intake (solid and liquid) and HS in healthy children. Our analyses were based on the combination of dietary intakes, estimated from 3-d weighed records, and 24-h urine indexes to estimate the FWR; the latter to date is one of the recommended biomarkers to characterize 24-h hydration [8].

The findings from **Study II** showed significantly higher absolute FWR values after a higher consumption of solid F&Vs in both boys and girls. This, at first glance, contrasts with the hypothesis that a higher F&V intake might act as a compensatory mechanism for a diminishing of water intake from other sources. In the expected direction, the higher FWR was accompanied by a higher overall TWI in children with higher F&V intakes; however, in parallel a decrease in TWI intake from other foods was observed. This indicates at least a

partial compensatory effect of the TWI from solid F&Vs with regard to the TWI from other water sources. This also helps to explain why TWI from solid F&Vs increased by 230 mL from the low- to the high-$F\&V_{solid}$ intake category, whereas the observed net change in FWR was only 130–140 mL (still indicating a more favourable FWR with higher $F\&V_{solid}$ intakes). No differences were observed in the solute load (mOsm/d) from the diet; however, urine volume was significantly higher, which led to a significantly lower urine osmolality (mOsm/L) in the high-F&Vsolid intake group.

We chose to analyze total F&V intake and food groups in a conservative manner [i.e., total weight (g) including water and non-water content] rather than just by their moisture content to better represent the typical form in which food is generally consumed.

The lack of studies relating diet and urinary markers to assess HS was a disadvantage in comparing our results. To our knowledge, to date only 2 studies in children have associated dietary characteristics and FWR as an HS biomarker. In both studies it was suggested that FWR values were affected by the quality of the diet; however, neither of the studies systematically examined the dissociated food group effect on FWR [97,119]. Nonetheless, different authors have previously speculated that F&V content of the diet may be an important predictor of HS [126,163]. With the present study, we were able to confirm this assumption and quantify the isolated effect of the F&V food group on FWR.

Girls in this study showed a better HS than boys (especially in the younger age group). Interestingly, we could not confirm that the better HS observed in girls than in boys can be exclusively explained by a preference of girls for foods with a high water content such as F&Vs, as different authors have suggested [119,126,163,165,166]. $F\&V_{solid}$ (MJ/d) intakes were significantly higher only in the older age group, and TWI (related to energy) was the same in girls as in boys. Our data suggest that the lower nonrenal water losses in girls and the lower solute load (determining the obligatory urine volume) are 2 of the various explanations for the better HS observed in girls.

With regard to the second aim of **Study II**, the longitudinal mixed-effects regression model allowed a quantitative understanding of the relations of F&V intake ($F\&V_{solid}$ and $F\&V_{juice}$) and FWR when all other water-supplying sources were kept constant. The effects on FWR were comparable between F&V solids and juices. Intentionally, F&V juice was included separately as a drink in the model and controlled as another water source that predisposes children to better hydration. Yet, a specific effect of $F\&V_{solid}$ intake persisted after adjusting for $F\&V_{juice}$ and other water sources.

Not surprisingly, the only other significant dietary contributors to FWR were drinking water and milk (milk and whey-based milk products). In theory, our findings can be illustrated with a simple example (derived from the within-person changing ß-values): for a

boy with 1600 mL TWI/d (from drinking water, solid F&Vs, F&V juice, milk, and water from other foods), an extra intake of approximately half a glass of plain water (115 mL) would increase the FWR by 50 mL. The same effect would be observed when approximately three-quarters of a glass of orange juice (156 mL) or one medium-sized apple (~ 125 g) is additionally consumed. By contrast, drinking one glass of milk (200 mL) would be necessary to improve the FWR by the same volume.

Logistic regression analysis showed that the risk of hypohydration can be markedly influenced by $F\&V_{solid}$ intake. An ~250-g higher intake of solid F&Vs (~2 portions) could reduce the risk of having a negative FWR by 2.6-fold in boys and 2.0-fold in girls. However, these risk effects should be interpreted with caution because of the rather wide 95% CIs.

The median intake of F&Vs (solids and juices taken together) in this cohort of highly selected children was 390 g/d, nearly meeting the current dietary recommendations for children "to eat 400 g/d of combined items limiting the 100% F&V juice to one portion (150 mL)" [161,162,167]. Caution should be noted with regard to the definition of F&Vs, because we did not limit F&V juice portions and excluded foods such as legumes and potatoes, which are allowed in the recommendations. The data on the investigated children reflect the importance of solid F&Vs and F&V juice as dietary water contributors (23% of the TWI) and their relatively low energy contribution to the diet (~10%).

Current German recommendations for water intake in children, including metabolic water (4- to < 7-y-olds: 1600 mL/d; 7- to< 10-y-olds: 1800 mL/d) [110], are slightly higher than the median TWI values observed in our children (1380 and 1700 mL/d, respectively). Accordingly, we observed 22% measurements with negative FWR values. As described in our population, an additional water intake of 110–140 mL/24 h in boys and an extra 80–100 mL/24 h in girls would guarantee euhydration (i.e., minimizing a potential risk of hypohydration).

Strengths of this analysis were its longitudinal design, i.e., the availability of repeated measurements for.75% (n= 324) of the children, and the ability to quantify all foods consumed in 3 d by weighed dietary records encompassing a 24-h urine collection for assessing HS. A limitation of our analysis as well for other analyses of F&V intake quantification is the inconsistent universal definition and classification of F&Vs [167–170]. However, the advantages of having the data disaggregated into basic food items, as was applied in this analysis, helped us to reduce the chance of misclassification of foods (especially composite foods) and therefore to have a very accurate estimate of total F&V intake. A priori, we decided to group the F&Vs following a classification similar to the "5-A-Day" definition [167] but excluded potatoes and legumes. These are 2 culturally important food constituents with moderately high water content (~70%), but they showed no significant effect on the FWR in our sample.

Finally, the elaborate design of the DONALD Study results in a selected population with a relatively high socioeconomic status, which is not representative of the German population [114,115]. However, non-representativeness, in fact, is less relevant for the present analysis because we focused on the evaluation of physiologic associations. Furthermore, the relative homogeneity in socioeconomic status of the study participants should minimize the possibility that other unknown confounding factors distorted the primary association between F&V intake and HS.

In conclusion, findings of **Study II** suggest that regular consumption of F&Vs in children aged 4–10 y may lead to a better HS estimated by FWR values from 24-h urine samples. The hypothesis that a higher intake of F&Vs may lead to a compensatory reduction in water intake because of effective regulatory mechanisms proved to be only partially true. Our findings suggest that adding solid F&Vs to the diet could in fact improve the hydration status. On the basis of these findings, dietary water recommendations should focus not just on water from fluids to meet needs but should also consider the promotion and availability of F&Vs in schools.

5.3 Study III: 24-h iodine excretion and estimates of 24-h iodine from spot urines using a creatinine scaling method.[3]

5.3.1 Summary

Currently, the measurement of urinary iodine concentration (UIC, µg/L) is the recommended parameter to assess iodine status; yet UIC dependency on urine volume may limit its use as an accurate parameter for monitoring iodine status in populations.

We aimed to compare 2 approaches for the assessment of urinary iodine excretion in spot urine samples: UIC (µg/L) and a creatinine-scaled estimate of 24-h iodine excretion [est24h-UIE_{crea} (µg/d)] against actually measured 24-h urinary iodine excretion rates (24h-UIE, µg/d). Urinary iodine and creatinine were measured both in 24-h urine samples and parallel collected spot urines from 180 healthy participants of the DONALD Study, aged 6-18 y. 24h-UIE was used as quai-reference for actual iodine excretion. Published 24-h creatinine reference values served to calculate est24h-UIE_{crea}. Correlation analysis, cross-classifications, and Bland–Altman plots were used to evaluate agreement between the different assessment approaches. Correlation coefficients of 24h-UIE with UIC (r=0.12, r=0.22; *P* = n.s.) were substantially weaker than with est24h-UIE_{crea} (r= 0.41, r=0.47; *P*<0.001) in 6-12y olds and 13-18y olds, respectively. Cross-classification into opposite quartiles by UIC were 7% (6-12y olds) and 15% (13-18y olds) *versus* 5% and 3% by est24h-$UIE_{crea,}$ respectively. Bland-Altman plots indicated greater deviation from 24h-UIE for the UIC versus the est24h-UIE_{crea} approach.

Our findings in children and adolescents showed a clearly better comparability of real 24h-UIE with est24h-UIE_{crea} than with UIC. Whenever highest possible validity is required for iodine status assessment from spot urine sampling, the determination of est24h-UIE_{crea} appears to be the more accurate monitoring approach.

5.3.2 Introduction

Despite the increasing implementation of iodized salt fortification programs, iodine deficiency remains a common global problem [19–21]. Thus, parallel to the programs to eradicate iodine deficiency, low-cost and reliable indicators to monitor iodine status in populations are needed[13].

As described in Chapter 2.4, the common way to assess Iodine status of a population is by determining median urinary iodine excretion, usually obtained from spot urine samples. A population's median urinary iodine concentration (UIC) of >100 µg/L is suggested as an indicator of iodine sufficiency [22]. In spite of the relative simplicity and feasibility of using the UIC approach, the method presents a weakness as UIC (µg/L) can be affected by daily

[3] Resulting publication: Montenegro-Bethancourt G, Johner SA, Stehle P, Neubert A, Remer T. Iodine status assessment in children: spot urine iodine concentration reasonably reflects true 24-hour iodine excretion only when scaled to creatinine. Thyroid 2015; 25:688-97.

urine volume [12,13,74], and therefore it bears the risk of falsely under-or overestimate iodine deficiency prevalence. Meanwhile, it has been shown, that not even a large sample size is always sufficient to level out inter- and intraindividual variations in hydration status (HS) [74,81].

At the individual level, 24-h Urinary Iodine Excretion (24h-UIE, µg/d) is considered to be a quasi-reference standard [12,72], although it is clear that usually one 24-h urine is not enough to reliably assess individual iodine intake [171]. König et al. reported that even up to ten 24-h urine samples are needed to provide a valid estimate of usual individual iodine intake [172]. The major advantage of using 24h-UIE as an indicator is that it reduces the HS dependent variations in iodine excretion. However, the disadvantage of collecting 24-h urine samples is the elaborate procedure and therefore a risk of lower compliance, compromising data quality especially in field studies [12].

To overcome the dependency of urinary concentration measurements on HS, in practice, analytes have been frequently related to creatinine (CR) to minimize variation in urine volume, as CR is known to be excreted at a relatively constant rate in 24 h [76]. Accordingly, also the Iodine-creatinine ratio (I-CR ratio) has been used apart from UIC (µg/L) to characterize iodine status [12]. However, in children it turned out that this approach alone is inappropriate since the index, especially during the period of marked growth, shows a clear age-dependency that limits its applicability [77,78]. Therefore, the additional involvement of age- and sex- stratified 24-h CR reference values has been suggested as a more accurate approach to assess analyte excretions from analyte/creatinine ratios in spot samples. Remer et al [50] showed the successful applicability of the 24-h CR scaling method to estimate 24-h excretion rates of urinary analytes such as calcium, deoxypyridinoline and dehydroepiandrosterone sulfate quantified in spontaneous urine samples. Using predicted 24-h CR values for the estimation of 24-h Iodine has already been applied in adults from industrialized countries [44,72]. However, it is currently not certain whether the use of this methodological approach also provides a more accurate assessment of iodine status in children.

Thus, the goal of the present study was to evaluate and compare the applicability of two approaches to assess iodine status from spot urines in healthy children and adolescents: (i) UIC (µg/L) alone, the current WHO recommendation; and (ii) 24-h iodine excretion estimation (est24h-UIE_{crea}, µg/d), using a CR scaling method that involves published 24-h CR reference values for children and adolescents. Both approaches were validated against daily iodine excretion actually measured in parallel collected 24-h urines (24h-UIE, µg/d) of the same children and adolescents. By using urine osmolality as hydration index, we further tested our hypothesis that hydration has an independent relevant effect on iodine concentration measurements in spot urines.

5.3.3 Methods

Population of study III

For Study III, we analyzed data of 6–18 years old children and adolescents, participants of the DONALD Study between 1996 and 2002. The number of children included in this analysis was derived from a total of 1479 spot urines from the DONALD urine bank, available for the age range of interest and studied time period. Initially, all urine samples were selected if the following inclusion criteria were fulfilled: 1) availability of at least one spot urine sample in parallel with a 24-h urine sample collection [within ± 15 d of difference between collections (n=593)]; 2) no pathological finding according Dipstick analysis (protein, leucocytes, glucose, ketones, urobilinogen, bilirubin, blood, and nitrite; Combur[9] -Test; Roche, Mannheim, Germany (n=522); 3) complete 24-h urine sample, using as criteria a body weight-related 24-h creatinine excretion rate > 0.1 mmol/kg per d [(50)], as well as reported full collection compliance (n=512); 4) no reported intake of iodine containing medications (L-thyroxine, betadine, other povidone-iodine formulations) and/or nutrition products (multivitamins, high energy or protein supplements with added iodine, kelp, or other algae products) before or during urine collection (n=463). Exclusion periods due to ingestion of these iodine sources before urine collection, were up to one week for supplements and 6 months for medications. None of our children had ingested or incorporated amiodarone or iodinated contrast agents. The available sample pairs were further reduced from 463 to a final number of n=452, as 11 sample pairs were stemming from participants with more than one pair of urines per subject. From this subject pool, sample pairs of 120 (60 girls) 6-12 years olds and 60 (30 girls) 13-18 years olds were then randomly selected. Mean difference between spot and 24-h collections was ± 9 d.

For urine collection and analysis see the methods section (Chapter 4.5). Exclusion criterion for an incompletely collected urine sample was a body weight-related 24-h creatinine excretion rate < 0.1 mmol/kg per d [(50)]. Spot urine samples were collected from participating children at the examination centre on the day of the anthropometrical and medical assessments. Spot urine collection time has not been fixed in the protocol of the DONALD Study and therefore collections were distributed over the day between 8:00 a.m. and 5:30 p.m. However, the by far largest part (86 %) was collected randomly in afternoon (12:30 p.m. to 5:30 p.m.). Urinary analysis of 24-h urine sample and parallel spot urine sample included: Iodine, Creatinine, osmolality were determined following the analytical methods described in Table 5 (Chapter 4.5).

Anthropometrical measurements included weight, height, body surface area (BSA) (for methodological descriptions refer to Chapter 4.2).

Statistical analyses

SAS® statistical program (version 9.1.3, SAS Inc. Cary, NC) was used for data analysis. A *P*-value <0.05 was considered statistically significant. Descriptive statistics of the study sample are presented as medians (25^{th} and 75^{th} percentiles). Unpaired Wilcoxon's tests were used to compare variables between age groups and sex.

The 24h-UIE was measured in 24-h urine samples and was considered as the "reference standard" for determining iodine intake, i.e. iodine status. From the parallel collected spot urines, different parameters indicating iodine status were derived: 1) measured iodine concentration (UIC, µg/L), the current WHO recommendation; 2) Iodine-creatinine ratio (I-CR, µg/mmol), formerly recommended; 3) estimated 24h-UIE (est24h-UIE_{crea}, µg/d), based on UIC and 24-h CR reference excretion values [50]:

$$\textbf{Iodine excretion per 24-h [µg/d]} = \frac{\textbf{Iodine}\ [\mu g] \bullet [1/L]}{\textbf{Creatinine}\ [mmol]\ ] \bullet [1/L]} \times \frac{\textbf{Creatinine}\ [mmol]}{[24\text{-h}] \bullet [kg]} \times \textbf{Body weight}\ [kg]$$

Estimate | *Measured in spot urines* | *Reference values* | *Individual*

To evaluate the proportion of children in the present population at risk of inadequate iodine intake, we compared 24h-UIE with the estimated average requirements (EAR). Proportion of children below the EAR cut-off values [82] was estimated after considering a mean non-urinary iodine loss of 15% [84].

The urinary iodine variables of interest (24h-UIE, UIC, I-CR ratio, est24h-UIE_{crea}) were skewed (Shapiro-Wilcoxon <0.05), thus log transformed data was used for analysis of agreement between methods. Pearson's correlation coefficients and cross-classification were calculated for each indicator from the spot urine samples (UIC, I-CR ratio, and est24h-UIE_{crea}), in comparison with 24h-UIE (reference standard) across the different age-groups (6-12 y, 13-18 y). By means of cross-classification, the percentage of children who were classified into either the same or the adjacent quartile of urinary iodine excretion by UIC or est24h-UIE_{crea} respectively *vs* 24h-UIE as reference, or misclassified into the opposite quartile, were computed [50]. We used Bland-Altman plots to illustrate the differences between both UIC and est24h-UIE_{crea} *vs* directly measured 24h-UIE. For the Bland-Altman comparison that requires comparable units (here µg/d), each measured UIC (µg/L) was multiplied by age-characteristic average 24-h urine volumes (6-12 y, 1000 mL/d; 13-18 y, 1500 mL/d) as suggested by Zimmermann [13]. This "constant"-volume-derived estimate, calculated to obtain an assumed 24-h iodine excretion for the respective spot sample UIC is

termed est24h-UIE$_{assumedVOL}$. A quite constant, not systematically varying urine volume is generally assumed when using UIC for iodine status assessment.

To evaluate the practical consequences of the application of different I-assessment approaches, i.e. the conclusion that would be drawn from the different estimates, we tested for both estimated 24h-UIE (from the spot samples) and actually measured 24h-UIE whether mean daily renal iodine output fell below, reached or exceeded the respective age-group characteristic Recommended Dietary Allowance (RDA) [82] after considering 15% non-renal iodine losses. These findings were subsequently compared with the corresponding assessment results according to UIC measurements, which was related to the WHO reference level of 100 µg/L as criterion of sufficiency in two ways: first, comparison with the original measurement at the actual spot urine osmolality, and second, with the theoretical UIC at a desirable osmolality reflecting euhydration. Osmolality values of 585 mosm/kg in 6-12 y old [15] and 619 mosm/kg in 13-18 y old [60] were used as reference for desirable osmolality.

Finally, to test for the effect of HS on the distinct I-assessment approaches derived from spot urines (UIC and est24h-UIE$_{crea}$), we evaluated their deviation from the directly measured 24h-UIE, in relation to urine osmolality. For this, we grouped the distribution of urine osmolality (mosm/kg) into sex and age-group specific tertiles and calculated for each tertile the mean of the differences between the reference standard (24h-UIE) and either est24h-UIE$_{assumedVOL}$ (µg/d) or est24h-UIE$_{crea}$ (µg/d).

5.3.4 Results

Table 12 presents anthropometric and urinary characteristics of the study sample stratified by sex and age-group. Estimates of urinary iodine varied widely across the sex and age-categories depending on the kind of urine collection. Overall, children's and adolescents' median (25th, 75th percentiles) 24h-UIE was 69.4 µg/d (51.0, 91.5) and 98.0 µg/d (83.4, 130.1), respectively. For 118 of the 180 examined DONALD participants, two additional 24-h urine collections were available (with iodine measurements) within a 2-year time frame encompassing the actual parallel collection of the spot and the 24-h urine. One of these additional 24-h samples had been collected one year before and the other, one year after the combined spot and 24-h sampling. The corresponding overall 2-year-median of UIE (consisting of 3 separate 24-h collections per individual), was close to the median of the actually examined single 24h-UIE (see above) in both 6-12 years olds [67.3 µg/d (52.4, 86.4), n=73] and 13-18 years olds [103.0 µg/d (87.0, 125.0), n=45], indicating a rather comparable iodine intake over a longer time period. Correspondingly, the individuals' actual 24-h UIEs and their individual 2-year means were highly correlated (6-12 years: r=0.79, p <0.0001; 13-18 years: r=0.75, p<0.0001).

A relatively higher proportion (~30%) of children in the 6-12 y olds group had inadequate iodine intake (derived from comparison of 24h-UIE with EAR values) compared to the older group (~15%). UIC (µg/L) measured in spot urines was not significantly different

in any of the evaluated groups. 24-h urine osmolality (mosm/d) significantly increased by age in both groups, and boys had a significantly higher solute load compared to girls. However, sex differences in osmolality (mosm/kg) measured in spot urines was significant only in 6-12 y olds.

Table 12. General characteristics of the sample of Study III. Analysis of 24-h urines and parallel spontaneous urine samples from 180 children aged 6-18 years.

	6-12y (n=120)		13-18y (n=60)	
	Boys	**Girls**	**Boys**	**Girls**
Age (yr)	9.0 (7.0, 11.0)	8.5 (7.0, 10.0)	15.0 (13.2, 16.1)	14.0 (13.1,16.1)
Anthropometry				
Weight (kg)	31.3 (24.4,40.9)	28.7 (23.3, 37.7)	58.9[3] (46.2,62.8)	54.5[3] (49.2, 63.1)
Height (cm)	137.7 (128.6,149.0)	133.6 (122.9, 144.0)	170.4[3] (163.2, 175.6)	166.7 [3] (162.0,169.9)
BSA (m^2)	1.11 (0.95, 1.28)	1.05 (0.90, 1.24)	1.66 [3] (1.46, 1.81)	1.60 [3] (1.50,1.73)
24-h urine collections				
Volume (mL)	685 (535, 870)	600 (485, 855)	990[3] (725,1340)	1110[3] (825, 1595)
24h-UIE (µg/d)	77.9 (50.7, 97.2)	65.1 (50.2, 82.5)	101.1[3] (85.8,134.1)	95.6[3] (67.7,119.7)
UIC (µg/L)	108.2 (80.9,145.8)	106.7 (74.8, 143.0)	98.1 (82.0, 144.9)	74.9[3] (58.0, 115.8)
Creatinine (mmol/d)	5.13 (401, 6.44)	4.61 (3.63, 6.00)	11.40[3] (8.77, 13.66)	9.15[2,3] (8.27,10.69)
Osmolality (mosm/d)	532 (451, 606)	456[2] (363, 527)	718[3] (627, 873)	654[2,3] (485, 748)
% 24h-UIE below EAR[4]	30.0	33.3	13.3	16.7
Spot urines samples				
UIC (µg/L)	107.8 (74.0, 158.0)	107.3 (60.5, 140.0)	132.5 (90.0, 170.0)	99.3 (58.2, 139.1)
Creatinine (mmol/L)	9.30 (5.8, 11.8)	7.65 (5.1,11.6)	12.20[3] (10.0,17.2)	11.60[3] (7.2,16.9)
Osmolality (mosm/kg)[5]	948 (765,1075)	722[2] (405, 921)	933 (713, 1007)	783 (491, 986)
I-CR ratio (µg/g)	106.4 (85.5, 170.9)	126.5 (71.8, 162.5)	87.8 (74.3, 130.1)	77.9[3] (64.6, 92.3)
I-CR ratio (µg/mmol)	12.0 (9.6, 19.3)	14.3 (8.1, 18.4)	9.9 (8.4 14.7)	8.8[3] (7.3,10.4)

Abbreviations: BSA, body surface area; EAR, estimated average requirement; 24h-UIE, 24-h urinary iodine excretion; UIC, urinary iodine concentration; I-CR, iodine to creatinine ratio

[1]All values are medians; 25th, 75th percentiles in parentheses.

[2]Significant difference between boys and girls same age-group tested with unpaired Wilcoxon test.

[3] Significant differences between same sex different age-group tested with unpaired Wilcoxon test.

[4] Defined by using the daily iodine Estimated Average Requirement (EAR) for 4 < 10 y old, 65 µg /d; for 10 < 15 y old, 73 µg/d; for 15 <19 y old, 95 µg/d [(82)(82)] corrected for 15% of non-urinary losses.

[5] Osmolality measured in spot urines, data available for the study *n* 127 samples.

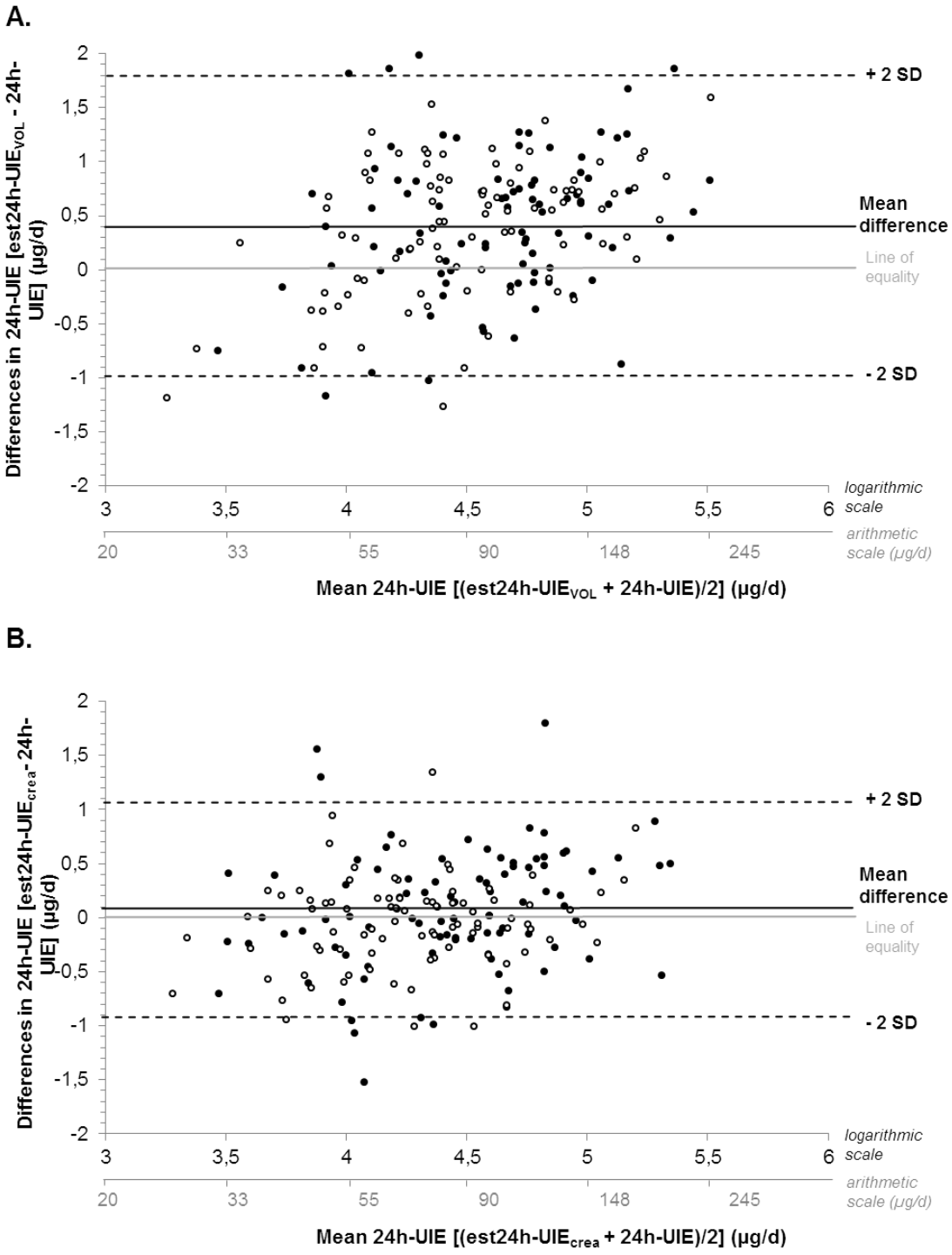
Table 13 presents the values for agreement (Pearson's correlation coefficients and cross-classification) between 24h-UIE (quasi-reference standard) and iodine assessment approaches measured in spot urines (UIC, est24h-UIE_{crea}, I-CR ratio). In all age groups est24h-UIE_{crea} was better correlated with 24h-UIE (r=0.41-0.47, P <0.05) than UIC alone (r = 0.12-0.22 P= n.s.). Misclassification into the opposite quartile occurred in 7 and 15% (6-12 and 13-18 y old) when UIC approach was applied, and in 5 and 3% (6-12 and 13-18 y old) when the 24-h creatinine scaling method (est24h-UIE_{crea}) was used.

The Bland-Altman plot for the total study sample indicated that differences in 24h-UIE vs. est24h-$UIE_{assumedVOL}$ were more scattered than vs. est24h-UIE_{crea}. Also, the interval within which 95% of differences between measurements by the two methods are expected to lie, were notably broader for est24h-$UIE_{assumedVOL}$ than for est24h-UIE_{crea} (**Figures 6A, 6B**).

Table 13. Simple correlation analysis and cross-classifications for agreement between differente iodine assessment approaches.
Measured daily UIE (from 24-h urines) and different approaches (UIC, est24h-$UIE_{crea,}$ I-CR ratio) to express iodine status from measurements in spot urine samples in children and adolescents.

	Iodine assessment approaches					
	UIC (µg/L)[1]		**est24h-UIE_{crea} (µg/d)[1,2]**		**I-CR ratio (µg/mmol)[1,3]**	
Pearson correlation coefficients	r	P	r	P	r	P
6-12 y old *(n=120)*	0.12	0.20	0.41	<0.0001	0.23	0.01
13-18 y old *(n=60)*	0.22	0.09	0.47	0.0002	0.36	0.005
Cross classification into quartiles						
6-12 y old *(n=120)*						
Same adjacent [n (%)]	85 (70.8)		96 (80.0)		91 (75.8)	
Opposite [n (%)]	8 (6.7)		6 (5.0)		11 (9.2)	
13-18 y old *(n=60)*						
Same adjacent, [n (%)]	42 (70.0)		48 (80.0)		41 (68.3)	
Opposite [n (%)]	9 (15.0)		2 (3.3)		2 (3.3)	

Abbreviations; UIC, urinary iodine concentration; est24h-UIE_{crea}, estimated 24 h urinary iodine excretion; I-CR, iodine to creatinine ratio
[1] Log transformed variable.
[2] Estimated with 24-h creatinine scaling method (description in the methods section).
[3] I-CR ratio has been formerly used as approach so it has been included only for illustrative purposes.



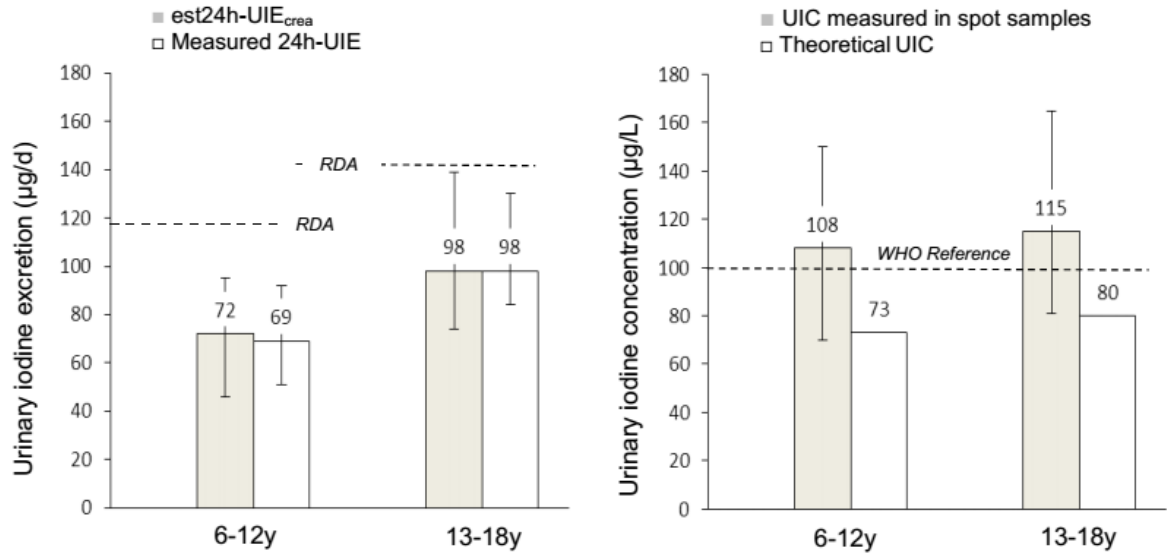
[Taken from: Montenegro-Bethancourt et al. Thyroid. 2015; 25:688-97].

Figure 6. Bland-altman plots of log-transformed data for the total study group of Study III. ● Boys ○ Girls. (A) The difference between log-transformed 24-h UIE (µg/d) estimated from UIC using age-characteristic average 24-h urine volumes (description in the methods section) [est24h-UIE$_{assumedVOL}$] (test method) and log-transformed 24h-UIE (reference standard) for each person (y-axis) is plotted against the mean of log-transformed UIE averaged from the two methods (x-axis). (B) The difference between log-transformed 24h-UIE (µg/d) calculated from iodine concentration measured in spot urines using the 24-h creatinine scaling method (est24h-UIE$_{crea}$) (test method) and log-transformed measured 24-h UIE (reference standard) for each person (y-axis) is plotted against the mean of log-transformed UIE averaged from the two methods (x-axis). Data are presented for the total study sample (n=180). The horizontal solid grey line (y=0) represents ideal agreement, where the differences between methods are zero; the horizontal black line indicates the mean of the differences;

the upper and lower dashed lines show the upper and lower 95% limit of agreement, respectively, presented as twofold standard deviation (±1.96 x SD). P value refers to the differences between medians of the two methods tested with paired t-test.

Median (25th, 75th percentile) values of the est24h-UIE$_{crea}$ and actual 24-h UIE were both compared with the age-corresponding RDAs showing comparable appraisal of iodine status with both approaches (**Figure 7A**). In case of UIC (as estimation parameter) a similar evaluation was only obtained when the desired hydration status was taken as a basis (**Figure 7B**).

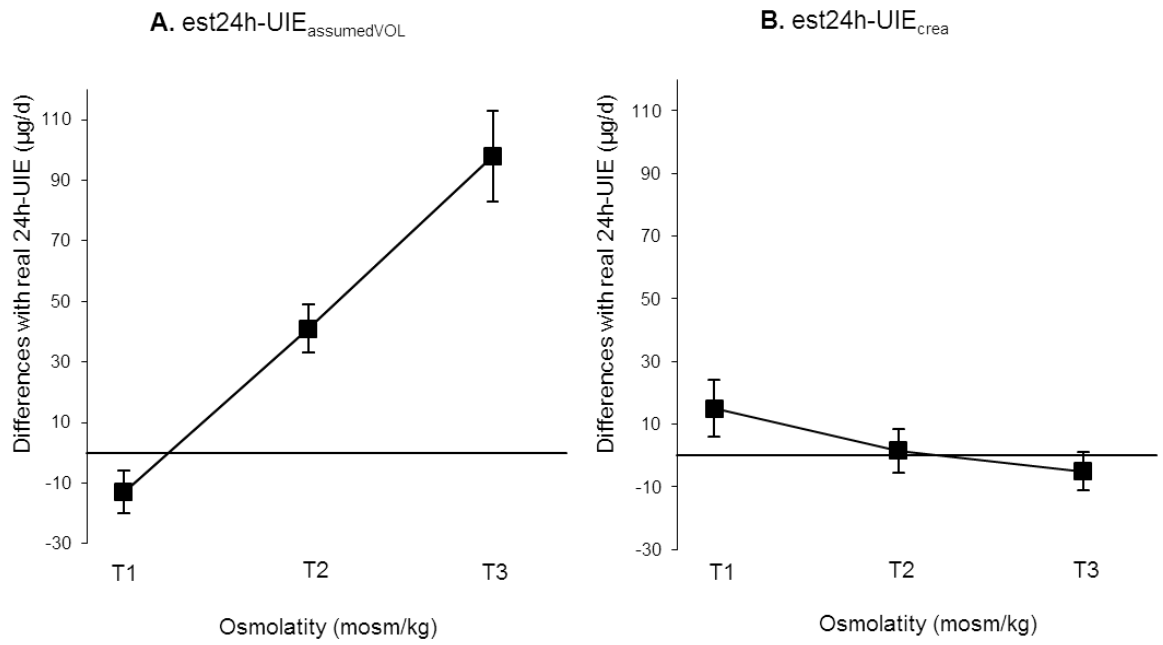
A. **B.**



[Taken from: Montenegro-Bethancourt et al. Thyroid. 2015; 25:688-97].

Figure 7. Urinary iodine excretion analysed in Study III: comparison to reference values Median(25th,75th percentiles) urinary iodine excretion analysed in 180 urine samples by age-group (A) est24h-UIE$_{crea}$ (µg/d) (■) estimated by means of the 24-h creatinine scaling method from spot urines (□) 24h-UIE measured in 24-h urines (gold standard). The dotted line refers to the RDA values for children according to age-group, after considering 15% non-renal iodine losses (i.e. 102 µg/d and 128 µg/d iodine for 6-12 and 13-18 y olds, respectively) [82]. (B) UIC (µg/L) (■) measured in spot urines (recommended standard) at 865 mosm/kg median osmolality in 6-12y and 884 mosm/kg in 13-18y. Theoretical UIC (µg/L) (□) at a desirable osmolality of 585 mosm/kg in 6-12y and 619 mosm/kg in 13-18y [60]. The dotted line refers to the WHO criteria of iodine sufficiency (>100 µg/L) [22].

Effect of osmolality on estimated iodine excretion for the two different approaches (est24h-UIE$_{assumedVOL}$ vs est24h-UIE$_{crea}$) is illustrated in **Figure 8**. The mean differences between est24h-UIE$_{assumedVOL}$ and actual measured 24h-UIE was significantly higher in children with a higher urine osmolality (P<0.0001). The mean deviation from real 24h-UIE increased by about 50 µg/d per tertile of osmolality. In comparison the mean differences between est24h-UIE$_{crea}$ and 24h-UIE were minimal and not significantly different (P>0.14).



[Taken from: Montenegro-Bethancourt et al. Thyroid. 2015; 25:688-97].

Figure 8. Dependency of urinary iodine concentration (µg/L) on urine osmolality evaluated in Study III. Dependency of urinary iodine concentration (UIC) multiplied by an assumed age-characteristic average 24-h urine volume (est24h-UIE$_{assumedVOL}$) (description in the methods section) (A) and est24h-UIE$_{crea}$ (B) on urine osmolality; analysed in the spot urine samples. The y-axis presents the difference between est24h-UIE$_{assumedVOL}$ (A) or est24h-UIE$_{crea}$ (B) and actual measured 24h-UIE. *P* values obtained in PROC MEANS analysis with the difference between methods as continuous variables. Medians (min-max) values for T1, T2 and T3 of urine osmolality were 416 (98-830), 865 (447-997), 1037 (893-1257) mosm/kg respectively.

5.3.5 Discussion

Currently, the recommended and most widely used method to estimate iodine status in populations is the measurement of urinary iodine concentration, which however, in individuals, is known to be highly influenced by HS [13]. With the present investigation we were able to demonstrate that HS can not only affect individual UIC but also average UIC of certain age groups during growth. As shown here in healthy children and adolescents, a HS-dependent shift of median UIC values is possible that no longer allows an appropriate assessment of their true iodine status. Therefore, the application of UIC without considering HS bears the risk of over-or underestimation of the actual iodine status. With the 24-h CR scaling method described here, we present a simple and reliable hydration status-independent approach to assess iodine status from spot urine samples in children and adolescents.

As recognized, the most reliable method of estimating iodine intake is the measurement of 24-h iodine excretion [12]. However, in epidemiological surveys, analysis of 24-h urines in general are rare because of the larger efforts and difficulties associated with this kind of urine collection [13,21]. Hence, a more convenient method to assess iodine status of

populations using spontaneous urine samples has been suggested, simply by measuring iodine concentration (UIC, µg/L). The underlying assumption for this approach is that usually there exists a "sufficient" number of spot urine samples to level out inter- and intraindividual variations in HS [13,21]. However, whether n=30 [22], or up to n=500 [75] subjects or spot samples are necessary to possibly overcome the confounding effect of day-to-day diurnal variation of dietary iodine intake or water (beverage) ingestion [171], has not been uniformly reported.

Because the concentration of iodine in urine depends on urine volume, alternative methods to estimate the daily urinary iodine excretion by additional measurements of CR concentration have been proposed. The I-CR ratio (µg iodine/g creatinine) had been applied as an assessment method decades ago. However, CR measurements were later on considered unnecessary [22], partly due to a putatively higher variation of I-CR compared to UIC measurements alone. Despite this, several authors proceeded to determine analytes in spot urine samples normalized to urinary CR. Mostly they used ratios of I-CR to estimate 24-h excretion rates of the trace element iodine, for example by multiplying the ratio with sex-and-age specific CR reference values. This has, in fact, been shown to yield more reliable assessments of real 24-h iodine excretion rates than simple UIC measurements in adults [44,171].

The obviously not appropriate evaluation of children´s iodine nutrition by the I-CR approach, was evidenced about two decades ago [77], because of the observed physiological, particularly strong-age-dependent increase in muscularity and hence CR production during growth. By establishing reference values for 24-h CR excretion for healthy children, it could be shown that their use allowed to reasonably estimate average 24-h excretion rates of certain analytes (calcium, deoxypyridinoline, dehydroepiandrosterone sulfate) from spot urine samples [50]. The results of the present study extend these findings also to the micronutrient iodine and support the application of this methodological approach when examining spot samples. In a relatively small sample of n=160 spot and parallel 24-h urine collections, the 24-h creatinine scaling method (est24h-UIE_{crea}), yielded satisfactorily comparable median excretion etimates with the measured actual 24h-UIE in the same individuals. Pearson correlations and cross-classifications confirmed the advantages of est24h-UIE_{crea} over simple concentration measurements (UIC).

By using the cut-off as proposed by the WHO (median of the population > 100 µg/L)[22] without further considering HS, a clear under- or (as in our study) overestimation can occur for iodine status in populations. In the present study, UIC findings inappropriately suggested iodine sufficiency in a group of 6-12 y old healthy DONALD participants, who in about 30% of cases (according to the actual 24h-UIE) showed iodine intakes below EAR. However, caution has to be taken here regarding the percentage of subjects below the respective EAR due to the intra-individual day-to-day variability, being present also with 24-h urine collections. As reported by Haldimann et al. [173] and also discussed by Zimmermann

and Andersson [13], appropriate adjustment for intra- and inter-individual variation of iodine excretion can result in a substantial attenuation of the proportion of individuals lying below the EAR.

In our sample of healthy 6-12 years old children, median UIC was >100 µg/L and the corresponding urinary osmolality ranged from 108 to 1257 mosm/kg. A similar osmolality range has recently been observed in 9-11 y old US children: 99-1259 mosm/kg [174]. Under the assumption that changes in HS and osmolality also affect UIC, we could show for children with such a relatively high osmolality that their median UIC at a more desirable osmolality will be markedly decreased towards values below 100 µg/L. In contrast with this, est24h-UIE_{crea} values were almost not influenced by HS and/or varying osmolality, and this is one of the main prerequisites for the use of spot urines to evaluate iodine nutrition. Accordingly, also in children a relevant confounding of HS on mere UIC measurements exists.

Theoretically, UIC and 24h-UIE can be used interchangeable, if the average daily urine volume produced by a certain population approximates 1 L, as it often (but not always) is the case in older schoolchildren [13,21]. We also tested this approach of a constant average 24-h urine volume (using est24h-$UIE_{assumedVOL}$) and observed no good agreement between est24h-$UIE_{assumedVOL}$ and the 24h-UIE (see Bland-Altman plots). On the other hand, we did observe a higher level of agreement between the est24h-UIE_{crea} method and real 24h-UIE in our children (Bland-Altman plots Fig 6B). Thus, even in a relatively homogeneous population as the one of the DONALD Study the approach of using UIC or "UIC interchangeable" to appropriately characterize real 24-h iodine status was not successful.

So far, few studies have investigated alternative approaches to estimate 24h-UIE from spot UIC values, and the existent literature only refers to studies conducted in adults [44,72,81,173]. Moreover, the IOM [82] has proposed one equation to simplify the calculation of daily iodine intake, using approximated values based on iodine bioavailability, observed urine volumes and weight; however, this approach represents only an approximation without considering the obviously common inter-and intra-individual variations in urine volume that can confound the IOM suggested method. We applied that formula and found an overestimation of iodine excess of about ~10 µg/d in 6-12 y old children (P=0.05), and ~ 50 µg/d in 13-18 y old (P <0.001) compared to real 24h-UIE (data no shown).

To our knowledge, the present study is the first to compare values of iodine excreted in 24-h urines against 24-h iodine estimated from spot urines in a sample of healthy children and adolescents. A particular strength of the present study lies on having 24-h urine samples parallel to spot urines from the same children, and measurement of the same urinary parameters (iodine, creatinine, and osmolality) in both samples for methods comparisons. Furthermore, the consideration of osmolality in the evaluated urinary parameters, allowed illustrating the effect of potential changes of HS on iodine excretion that until now were only observed by measuring urine volume [12,74].

In the present study, spot urines and 24-h urine collections were allowed to have a maximum time difference of 15 days. The fact that the single 24-h UIEs in our subjects (at

least in all in whom we could check it) were closely associated with their 2-year average iodine excretion (comprising 3 separate 24-h collections per individual) shows that the herein examined single 24-h UIEs, for the most part, reflect the subjects' longer-term iodine status. Thus, the 24-h urines represent a reasonable quasi-reference for the comparison with random spot samples, i.e., urines not collected at specifically defined time points, as is the normal practice in urinary iodine monitoring surveys.

Our finding of nearly the same median population estimates for spot-derived 24-h iodine and real 24-h iodine excretion both in 6-12 and 13-18 years olds suggest a reasonable applicability and suitability of the creatinine scaling approach also under more realistic field conditions with spot urine collections, for example in monitoring studies.

The challenge, in general, for adopting the 24-h creatinine scaling approach, might be the accustomed practice and familiarity of using UIC as a simple iodine biomarker, for which the cut-off values for sufficiency are worldwide in use. We considered UIC and est24h-UIE in some detail to provide the reader with an idea of the relative accuracies of the different in approaches (Fig. 6). The present creatinine scaling approach is based on UIC values measured in spot urine samples, thus the application is quite simple and practicable. Its advantage, as demonstrated, is that it provides a hydration status-independent 24-h iodine excretion level (comparable to 24-h iodine intake data) which makes it suitable to be compared to the official iodine intake recommendations. However for this, validated population thresholds for determining iodine sufficiency need to be developed. Currently such thresholds do not exist, apart from the concept to integrate the EAR values [111] into this threshold system. In this respect, important steps have been made by Zimmerman & Andersson and Haldimann et al. [13,173] who have convincingly suggested to use the EAR cut-point method for estimating the prevalence of iodine inadequacy.

Since mild to moderate iodine deficiency affects not just low-and middle- income countries, but also countries in the industrialized world [21]; the 24-h creatinine scaling method suggests itself as an alternative reliable approach to estimate iodine status from spot samples, at least in children from industrialized countries with similar dietary and physical developmental characteristics, as to be found in the population for which required CR reference values have been published. To further enhance the feasibility of applying this more accurate method for population's iodine assessment, in other children outside the above context too, the possible applicability of these already published CR reference values could be explored also for different populations (e.g. with substantially differing protein intakes) [175,176].

In conclusion, we found reasonable estimates of daily iodine excretion by applying the 24-h creatinine scaling approach (est24h-UIE_{crea}) when simply analyzing spot urines in healthy children and adolescents. The comparison with the conventional approach of UIC (µg/L) showed clear advantages, e.g. an almost entire HS independence. The successful application of est24h-UIE_{crea} also in larger epidemiological studies has just recently been

demonstrated even in adults (using different CR reference values) [81], and may thus constitute a fruitful and not too complicated way to assess iodine status more precisely in epidemiological surveys in children and young adults.

5.4 Study IV: Association of dietary ratio of animal to plant protein with 24-h urinary iodine excretion in healthy schoolchildren[4]

5.4.1 Summary

Adequate dietary iodine intake in children is essential for optimal physical and neurological development. Whether lower dietary animal food and salt intake may adversely affect iodine status is under discussion. We examined the association between dietary animal-to-plant protein (A/P) ratio with 24-h urinary iodine excretion (24-h UI, µg/d), and if this is modified by salt intake. 24-h UI was measured in 1959 24-h urine samples from 516 6-12 years-old participants of the DONALD Study. Parallel 3-d weighed food records were used to estimate dietary intakes. Protein sources were classified as: dairy, animal (A) and plant (P). A repeated-measures regression model (PROC MIXED) was used to analyse the effect of A/P ratios on 24-h UI. A/P ratios ranged from 0.5 (95%CI 0.4-0.6) to 1.6 (1.4-1.9) (lowest and highest quartile). After adjustment for total energy intake, main dietary iodine sources (dairy and salt intake), and further covariates, the inter-individual variation in A/P ratio was significantly associated with variation in 24-h UI. One unit higher A/P ratio predicted 6 µg/d (P=0.002) in boys, and 5 µg/d (P=0.03) in girls. This relationship was partially mediated by a higher salt intake at higher A/P ratios. These results suggest that lower consumption of animal protein is associated with a small decline in iodine excretion, partially mediated by decreased salt intake. Since limited salt and increased intake of plant-based foods are part of a preferable healthy food pattern, effective nutrition political strategies will be required in the future to ensure appropriate iodine nutrition in adherent populations.

5.4.2 Introduction

As discussed in Chapter 2.4, the essential role of iodine in thyroid hormone production and in the regulation of metabolism, growth and development has been extensively documented [61–63]. In most industrialized countries, the main dietary sources of iodine are iodized salt (~50%) and animal food products, of which dairy products are the largest contributors, accounting for up to 40% of total iodine intake as has been shown and discussed by different authors [83,84,88,177,178]. Despite the global WHO-Universal Salt Iodization Program [22], and regional strategies for iodine deficiency prevention and control, which in Europe included initiatives of legislation in the use of iodized salt in industrial food production [87], and more recently in bread [179,180], to mention some; iodine deficiencies in the European region still persist [19,20,69].

[4] Resulting publication: Montenegro-Bethancourt G, Johner SA, Stehle P, Remer T.Dietary ratio of animal to plant protein is associated with 24-h urinary iodine excretion in healthy schoolchildren. Br J Nutr. 2015; 114:24-33

Evidence from studies on the effect of diets containing little to no animal food products on iodine status is limited, but overall, the literature on this topic suggests that lower intakes of animal food can contribute to inadequate iodine intakes [99–101]. Although there is still controversy about the impact of reducing dietary protein intake in children and potential health outcomes [102], current dietary guidelines for healthy eating, for example, the "New American Plate" (NAP) from the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR), advise the limitation in the intake of animal protein and to increase the plant-based foods to prevent chronic diseases [103,104]. In addition, reducing sodium intake in children is also part of the global health initiatives in the prevention of chronic diseases[106–109]. The current typical Western diets, in contrast, are characterized by high intakes of processed foods, animal food products and salt [181]. As studies suggest an association between processed foods as well as meat and increased salt intake [40,182,183], the question arises whether, diets with lower animal foods (e.g. meats) and thereby possibly also decreased iodized salt intake may contribute to depleted iodine status as reflected in lower iodine excretion. To our knowledge, to date no studies have assessed the association of animal and plant-based food sources with urinary iodine status in children consuming typical Western diets. To specifically examine the long-term effect of diets predominantly animal or plant based on iodine status, we studied the 24-h urinary iodine excretion (24-h UI) in a sample of German schoolchildren providing multiple collections of both 24-h urine samples (with available sodium excretion measurements) and parallel 3-d weighed food records, during the years 1993-2010. In Germany, a particularly improved iodine status in the population has been observed since 1993 parallel to legislation amendment, facilitating the use of iodized salt (sodium chloride) in all processed foods [74,87]. However, the iodization of salt used in households or for food production is still on a voluntary basis. During the last years (starting in 2004), the use of iodized salt by the food industry began to decrease in Germany, and by now encompasses only < 30% of total added salt, probably contributing to a negative trend in iodine status [84,88].

We used the "animal-to-plant protein ratio (A/P ratio)" of the children's habitual diet as an approach to characterize predominantly animal or plant-based diets. We hypothesized that those children with healthier food patterns (low A/P ratio) have lower iodine excretion than children with high A/P ratio; the individual change in dietary sources of protein (animal related to plant-based) and 24-h UI was evaluated using repeatedly collected dietary records and 24-h urines. As a second aspect, we aimed to investigate whether the potential association between A/P ratio and urinary iodine excretion may be mediated by an increase in salt consumption (namely iodized salt) with high animal protein diets.

5.4.3 Methods

Population of study IV

For the purpose of this analysis we used data from children aged 6-12 years participating in the DONALD Study from January 1993 to December 2010 (n=675, n=2781 measurements). To be eligible for the present analysis, children had to be within the age-range, and providing at least 2 complete 24-h urine samples, each with concurrent 3-d weighed food record and anthropometrical data during the observational period. The individual number of 24-h urine samples and corresponding dietary records ranged from two to a maximum of seven possible measurements during the 17-year time period (for a 6-12 y age-range).

We excluded those observations that met one or more of the following criteria: 1) incompletely collected urine sample, i.e. when body weight-related- 24-h creatinine excretion rate was below the cut-off value of 0.1 mmol/kg body weight per day [(175)]; 2) implausible dietary records, i.e. when the total recorded energy intake was inadequate in relation to the basal metabolic rate [(184)]; 3) estimated total protein intake <0.66 g/kg of body weight, corresponding to the minimum maintenance requirement of protein [(185)]; or 4) use or intake of medication or supplements containing iodine.

Hence, the final study sample for **Study IV** consisted of 516 children (n=159 excluded), 51.4% male and 48.6% female, providing 1959 collected 24-hour urines and related dietary and anthropometrical records (mean 3.8 measurements per subject over the total study period).

Urinary assessment

24-h urine collection, urine storage and analysis were performed according to the standard operating procedures described in the method section (Chapter 4.5). Iodine concentration and 24-h sodium excretion was determined in the 24-h urine samples following the analytical method described in Table 5 (Chapter 4.5).

Nutritional assessment

The energy and nutrient intakes for **Study IV**, was as described in the methods section (Chapter 4.4). For the present analysis, the data were derived for each participant from the mean of the 3 dietary recording days. Foods were analysed according to the food group category described in Table 5 (Chapter 4.4).

The calculated protein intake was divided into 1) *dairy protein*: including protein content from milk and whey-based milk products (yoghurt, buttermilk, cream, condensed milk, quark) and all varieties of cheese; 2) *animal protein (A)*: including protein content from eggs; all type of meats and chicken; fish and seafood, and, 3) *plant protein (P)*: including protein content from wheat and all wheat products, cereals (breakfast cereals, oats, sprouts);

rice, potatoes, fruits, vegetables, mushrooms, soy products (only solid fraction of soymilk), legumes, nuts and pulses. Total protein was estimated as the sum of protein from all sources (dairy +A+P). Based on this division, dietary animal to plant protein ratios (A/P ratio) were calculated.

A priori, we decided to exclude *dairy protein* from the ratios for the following reasons: Dairy products are often important constituents of the vegetarian food pattern, not just in Germany but also in other industrialized countries such as the US, as literature suggests [186,187]. Milk- has a relatively high iodine content [66,90–92], and recent studies in German children suggest milk to be an important iodine source in the children's diet [84,88]. Due to these reasons, the inclusion of dairy in the A/P protein ratio may have introduced a correlation bias i.e. led to an overestimation of the association between the A/P ratio and 24-h UI. Milk and milk products whatsoever were included as separate covariate in the PROC MIXED model (see statistical analyses).

The regular use of iodized salt was reported in dietary records or parental interviews by nearly 100% of the participating children. Sodium (mg) and salt intake (g) were estimated from the urinary sodium excretion values (1g NaCl= 400 mg Na).

Anthropometry and parental data

Anthropometric measurements of this analysis, included weight, height, BMI, BMI-SDS, and Body Surface Area (BSA) calculated as described in Chapter 4.2. Maternal education and maternal overweight (BMI≥25 kg/m^2) were tested additionally as parameters that can potentially influence the dietary preferences in children [188].

Statistical analyses

Descriptive data for anthropometric, dietary and urinary parameters are presented as medians and interquartile ranges stratified by 1) sex, and 2) quartiles of A/P ratio. Quartiles of A/P ratio (excluding dairy protein) were grouped as low (lowest quartile), medium (middle 2 quartiles), and high (highest quartile), with the low category used as the reference group. All observations (i.e. also repeated samples from the same individual) were included for the calculations of medians. Sex differences and differences between A/P ratio quartiles were tested by repeated measures regression models (PROC MIXED, also linear mixed regression model in SAS) to account for the lack of independence that exists between repeated observations on the same person (comparable to a cross-sectional analysis).

PROC MIXED including both fixed and random effects, was also used for testing the longitudinal association of A/P ratio with 24-h UI (µg/d). An advantage of the MIXED procedure is that it does not exclude children from the analysis if they have missing data for a

particular time point, but it analyses all variables on the assumption that any missing data are missing at random. The random component of the PROC MIXED model accounts among other for the lack of independence that exists between repeated observations on the same person.

The basic longitudinally regression model included A/P ratio, age, time and time x time as fixed effects. The variable "time" (continuous, 1993-2010) was included as time, and time x time in the model because of the suggested variation of the 24-h UI through the time period [74,84]. Potentially confounding factors influencing 24-h UI were considered as covariates. Only variables that substantially modified the coefficient of A/P ratio by >10% in the basic models, or significantly predicted the outcome variable (24-h UI) or improved the fit statistic (Akaike´s information criterion; AIC), were included in the subsequent multivariate analyses. All final multivariable models (Model 1) included A/P ratio, age (years), time (chronological time of study years 1993-2010 and time x time), energy intake (MJ/d), 24-h urine volume (mL/d) and milk and milk products (g/d) as independent fixed effects. Diets with high animal protein intake may also be characterized by higher salt intake [119] which is one of the most important iodine sources. Because salt intake might mediate the association between A/P ratio and iodine excretion, 24-h urinary sodium excretion (as marker for salt intake) was tested in final multivariable models (Model 2). To allow for the distinction between within-person and between-person effects of A/P ratio on 24-h UI, the data were first centred on the respective person-specific means of the A/P ratio over time (i.e. individual's mean), and these means were used to predict the between-person effects on 24-h UI (i.e. the associations of between-person differences in protein consumption with differences in mean 24-h UI between the subjects). After that, time-specific deviations from the person-specific means were calculated (i.e. individual value minus the individual's mean) and used to test whether within-person changes on protein intakes were associated with within-person changes in 24-h UI [133,134].

To estimate the prevalence of children at risk of inadequate iodine intake, 24-h UI excretion levels (µg/d) were compared to the EAR (estimated average requirements, i.e. 55 µg/d for 4-8 y old; 62 µg/d for 9-13 y old) [82], after considering a mean non-urinary iodine loss of 15% [84].

5.4.4 Results

A general description of the study sample with respect to anthropometry, dietary intakes and 24-h urine parameters is shown in **Table 14**. Overall median (25^{th}, 75^{th} interquartile range) of urinary iodine excretion (24-h UI) among these children was 75 µg/d (56, 98). Both, iodine excretion (µg/d) and iodine concentration (µg/l) were significantly higher in boys than in girls, but no significance remained when corrected by individual energy

intake. Regarding dietary intakes, median estimated protein intake was 1.7 g/kg (1.4, 2.0) for all children. Animal protein, including dairy, contributed 65.7 % to the total protein intake, ranging from 17.3 - 88.8%.

Table 14. Anthropometric, nutritional and urinary characteristics of participants of Study IV.

	Boys	**Girls**	**P** [1]
Subjects (n)	265	251	
No. of measurements	985	974	
Age (y)	9.0 (7.02, 10.83) [2]	9.0 (7.05, 10.88)	
Anthropometric variables			
BMI	16.4 (15.2, 17.8)	16.4 (15.1, 18.0)	0.27
BMI-SDS	-0.18 (-0.76, 0.44)	-0.20 (-0.80, 0.48)	0.033
Urinary variables			
Iodine concentration (µg/l)	109 (81, 143)	94 (67, 129)	<0.0001
24-h Iodine excretion (µg/d)	80 (61, 103)	70 (53, 91)	<0.0001
24-h iodine excretion (µg/MJ/d)	10.9 (8.4, 13.8)	10.6 (8.2, 13.7)	0.22
Urine volume (ml/d)	730 (554, 966)	735 (547, 981)	0.86
Urine volume (ml/d/MJ)	98 (76, 128)	110 (83, 146)	<0.0001
Sodium excretion (mg/d)	2025 (1522, 2610)	1762 (1360 ,2610)	<0.0001
Sodium excretion (mg/d/MJ)	280 (210, 350)	270 (0.21, 0.34)	0.94
Dietary variables			
Estimated salt intake (NaCL, g/d)[3]	5.1 (3.8, 6.5)	4.4 (3.4, 5.8)	<0.0001
Total energy (MJ/d)	7.4 (6.6, 8.3)	6.6 (5.8,7.5)	<0.0001
Protein (% of energy)	12.7 (11.4, 13.9)	12.5 (11.2, 13.8)	0.21
Carbohydrates (% of energy)	52 (47.9, 55.6)	51.9 (48.3, 55.9)	0.25
Fat (% of energy)	35.1 (31.9, 38.8)	35.3 (32.0, 38.9)	0.38
Protein per body weight (g/kg)	1.8 (1.52, 2.11)	1.6 (1.36, 1.88)	<0.0001
Animal protein (g/d) [4]	15.8 (11.3, 21.4)	14.4 (10.3, 19.1)	<0.0001
Dairy protein (g/d) [5]	17.2 (12.6, 22.6)	14.1 (10.4, 19.2)	<0.0001
Plant protein(g/d)	17.6 (14.3, 21.0)	16.0 (13.0, 19.2)	<0.0001

BMI-SDS, sex-and age-independent BMI standard deviation scores.
[1] Sex differences were tested with a linear mixed-effects regression model (PROC MIXED). PROC MIXED was used to account for the dependence of multiple measurements within the same child.
[2]Values are medians (25th and 75th percentile in parentheses).
[3] Estimated from urinary sodium (1g of NaCl =0.4 g Na)
[4]Animal protein includes protein from fish, poultry and all meat products.
[5]Dairy protein includes protein from milk and all milk products

Cross-sectional comparison of anthropometrical, urinary and dietary variables by quartiles of A/P ratio is shown in **Table 15**. Both, urinary iodine excretion (µg/d) and iodine concentration (µg/l) were lower in the low animal protein diet than in the protein-rich diet (high ratio) in boys and girls, however not significant. Overall, iodine excretion values below the EAR were observed in 14.3 % (n=280) of the measurements. The proportion of observations below the EAR was higher in the lower animal protein eaters (12.8 % in boys,

19.2% in girls) than in the higher animal protein consumers (10.3 % in boys, 14.5% in girls) but statistically not significant. Children in the high ratio category consumed more protein than children in the lower ratio category (differing by ~ 6 g/d). In the expected direction, sodium excretion was higher when more animal protein was consumed (high ratio) but differed significantly only in boys. Proportion of mothers with lower education level was significantly higher in the high-A/P ratio in boys and girls ($p<0.0001$). Proportion of maternal overweight was higher in the high-ratio group, but was significant only in girls ($p=0.0002$) (data no shown).

Results of the repeated-measures regression analyses (PROC MIXED) with urinary iodine excretion (24-h UI, µg/d) as dependent variable are shown in **Table 16**. ß values for the individual time invariant mean values of A/P ratios represent the difference of 24-h UI (µg/d) when the dietary A/P ratio differs by one unit between the individuals across time. ß-values for intra-individual change indicate the within-person changes in the 24-h UI (µg/d) for a unit of change in A/P ratio across time. A higher A/P ratio was significantly associated with higher iodine excretion, before (model 1), and after adjustment for sodium excretion (model 2), however ß values diminished after adjustment for sodium excretion. Overall, the effect strength was greater between individuals than within individuals (different ß values and significance). **Figure 9** graphically illustrates the proportions of protein sources by A/P ratio category (low, middle, high) and its effect on 24-h UI, obtained from adjusted means of the 24-h UI from the PROC MIXED model.

Additionally, a separate analysis was conducted using the same PROC MIXED model to evaluate the strength of association of the A/P ratio and 24-UI, including dairy protein in the A/P ratios (dairy+animal/plant-based protein). The results of this analysis were similar to those presented in Table 16, the effect estimates were only slightly enhanced. For instance, in boys at the between-person level, the effect estimate increased from 8.7 µg/d (ratios excluding dairy) to 8.9 µg/d (including dairy) (model 1) and 6.3 µg/d to 9.1 µg/d (model 2). In girls, the corresponding values were 7.4µg/d and 7.4 µg/d (same values) (model 1) and 4.5 µg/d to 7.4 (model 2) respectively.

Table 15. Comparison of anthropometric, nutritional and urinary characteristics between categories of A/P protein ratios of participants of Study IV.

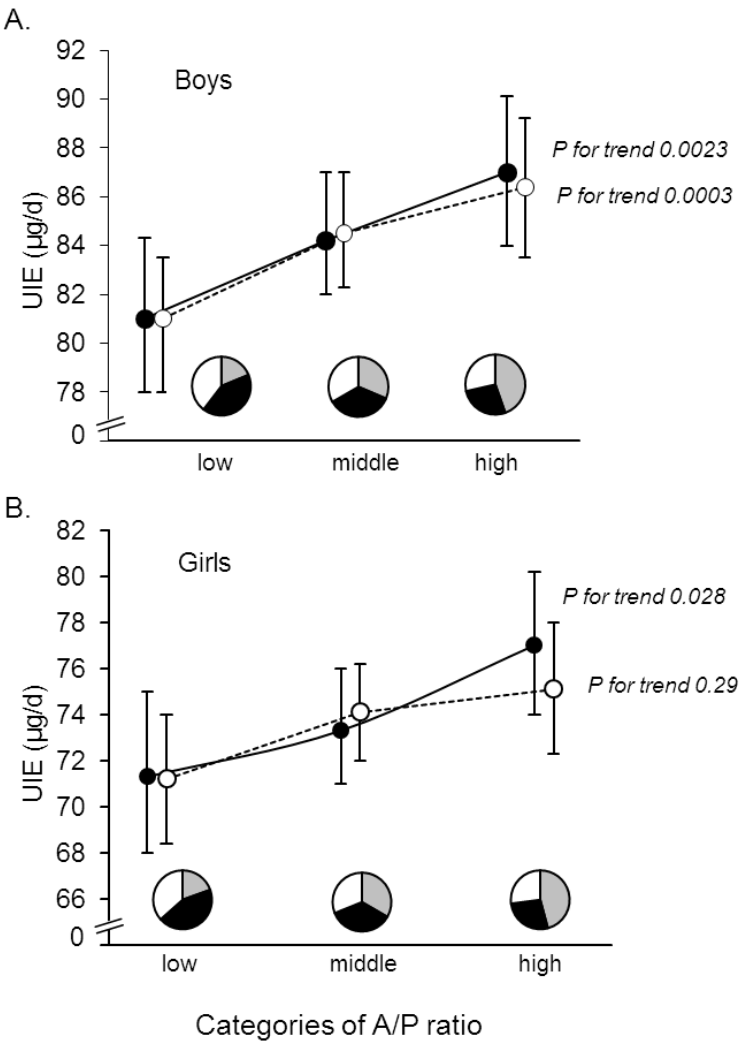
	Ratio of A/P protein					
	Boys (n= 265; 985 measurements)			Girls (n=251; 653 measurements)		
	Low	High	P_{trend}[1]	Low	High	P_{trend}[1]
Ratio of animal to plant protein [2]	0.49 [3](0.35,0.55)	1.61 (1.43, 1.94)	<0.0001	0.48 (0.36,0.57	1.54 (1.39, 1.86)	<0.0001
Age	9.0 (7.0, 10.6)	9.0 (7.0, 10.9)		9.0 (7.0, 10.8)	9.0 (7.0, 10.8)	
Anthropometric variables						
Body weight (kg)	30.8 (25.1,37.2)	30.5 (25.7, 38.7)	0.85	29.6 (24.5,36.4)	31.0 (24.7,38.8)	0.32
BSA (m^2)	1.09 (0.95,1.25)	1.08 (0.96, 1.28)	0.93	1.07 (0.92,1.23)	1.10 (0.94,1.26)	0.29
Urinary excretion variables						
Iodine concentration(µg/L)	107 (81, 139)	110 (85, 144)	0.23	89 (68, 127)	100 (70, 134)	0.54
Iodine per day (µg/d)	76 (57, 102)	84 (64, 108)	0.50	65 (51, 85)	73 (54, 97)	0.13
% 24-h UI below EAR[4]	12.8	10.3	0.55	19.2	14.5	0.57
Sodium per day (mg/d)	1875 (1420,2470)	2130 (1600, 2820)	0.027	1600 (1200,2110)	1870 (1490,2480)	0.053
Volume (mL/d)	730 (550, 960)	725 (550, 980)	0.52	720 (560, 980)	710 (545, 980)	0.52
Dietary variables						
Total energy (MJ/d)	7.4 (6.5,8.4)	7.4 (6.4,8.3)	0.76	6.6 (5.6,7.5)	6.6 (5.8,7.5)	0.61
Total protein (g/d)	50.2 (41.8, 59.4)	56.3 (48.2, 66.0)	<0.0001	42.0 (35.2, 50.2)	49.6 (43.1, 58.3)	<0.0001
Animal protein (g/d) [5]	9.0 (6.5, 11.5)	24.6 (20.0, 29.7)	<0.0001	8.1 (5.5, 10.4)	21.9 (17.4, 26.2)	<0.0001
Dairy protein (g/d)[6]	19.2 (14.3, 25.8)	15.7 (10.8, 20.8)	0.0002	14.8 (10.4, 20.2)	13.3 (9.7, 17.2)	0.0005
Plant protein (g/d)	20.5 (17.4, 24.2)	14.5 (12.2, 17.3)	<0.0001	17.9 (15.0, 21.3)	13.3 (10.8, 16.2)	<0.0001
Dietary fiber (g/d)	20.1 (16.7, 24.0)	15.2 (13.0, 18.1)	<.0001	18.1 (15.5, 22.0)	14.1 (11.5, 17.3)	<0.0001

[1] P_{trend} was tested with PROC MIXED using the data of the total sample of 516 subjects with 1959 measurements. PROC MIXED was used to account for the dependence of multiple measurements within the same child.
[2] Ratios estimated as protein animal/plant (excluding dairy).
[3] Values are medians (25th and 75th percentile in parentheses).
[4] Defined by using the Estimated Average Requirements (EAR) [(46)] corrected for 15% non-urinary losses. Differences were tested with chi square test.
[5] Animal protein, include fish, poultry and all meat products.
[6] Dairy protein, include milk and all milk products

Table 16. Association between ratios of animal to plant protein intake and 24-h urinary iodine excretion in participants of Study IV.

	Urinary iodine excretion (µg/d)							
	Boys (n= 265; 985 measurements)				Girls (n= 251; 974 measurements)			
	between-person level[1]		within-person level[2]		between-person level[1]		within-person level[2]	
	ß (95% CI)	*P*	*ß* (95%CI)	*P*	*ß* (95% CI)	*P*	*ß* (95% CI)	*P*
A/P protein ratio								
Model 1[3]	8.7 (4, 13)	0.0002	6.7 (3, 11)	0.004	7,4 (3, 12)	0.001	2.4 (-1, 6)	0.18
Model 2[4]	6.3 (2, 10)	0.002	6.6 (1, 8)	0.0003	4.5 (1, 9)	0.028	1.8 (-2, 5)	0.29

[1]ß- Values of the between-person level indicate a difference in 24-h urinary iodine excretion in µg/d for a change of 1 unit of the ratio of animal to plant protein intake between the individuals across time.
[2]ß-Values of the within-person level indicate an intra-individual change in 24-h urinary iodine excretion (in µg/d)) with a 1 unit change in the animal to plant protein ratio within one child.
[3]Model adjusted for the variables: age (years); time period (1993-2010), time x time; total energy (MJ/d); 24-h urine volume (mL/d); milk and all milk products (g/d).
[4]Model additionally adjusted as for model 1 plus 24-h sodium excretion (mg/d).



[Taken from: Montenegro-Bethancourt et al. Br J Nutr. 2015; 114:24-33].

Figure 9. Least square means (95% CIs) of 24-h UI (µg/d) by category of animal to plant protein ratio in (A) boys and (B) girls

Model 2 (Table 16) of the multivariable adjusted linear mixed models was used for prediction. P for continuous trend refers to the P values obtained in linear regression models with 24-h UI as continuous variable. ●— Between- person changes, ○··· Within- person changes. Medians of A/P ratio protein in low (Q1), middle (Q2-Q3), and high (Q4) categories were in boys: 0.48, 0.90, and 1.61; in girls: 0.47, 0.92, and 1.54 respectively. The pie graph presents the different proportions of protein sources in the categories of A/P protein ratios: animal (grey), dairy (white) and plant-based (black). Animal protein contribution by ratios category: was (A) in boys: low: 19 %, middle 31 %, high 45%; (B) in girls: 20%, 33%, high 46% respectively.

5.4.5 Discussion

In the present longitudinal analysis of children consuming a diet characterized by relatively high protein intake (typical Western diet), we examined the importance of changes in the proportion of animal to plant protein for changes in iodine status, and the corresponding role of salt intake. Our findings suggest that a high ratio of animal to plant protein (A/P) is associated with greater iodine excretion (µg/d), even if other relevant dietary sources of iodine (e.g. salt intake) are controlled for.

Suggestive evidence of a higher risk of sub-optimal iodine status has already been proposed when diets lack animal food products. This notion builds on studies mainly conducted in adults, who are vegetarians or vegans, which may lead to deficits in iodine as well in other micronutrients [99,100,189], whereas in children this information is scarce. Most previous cross-sectional studies in children characterizing iodine status reported urinary iodine concentration (µg/L), but usually lack of data on the dietary habitual intake [177,178,180,190,191]. In our longitudinally analysis, we were able to simulate different dietary protein patterns, and quantify the effect of lower animal food intake, resembling almost a vegetarian-diet, on iodine excretion. One example to illustrate the apparent effect of increasing the A/P ratio on the 24-h UI, can be deduced from our model. Assuming a 50 g total protein intake (~ average protein intake for boys in the low-A/P ratio), made up by 20 g from dairy, 10 g from animal and 20 g from plant protein, the estimated A/P ratio is 0.5. Increasing the A/P ratio by 1 unit, would modify the proportion of the sources, i.e. an intake of 18 g from animal protein, and 12 g of plant-protein, keeping the dairy protein in 20 g. Theoretically, according to our adjusted model (model 2), an about 6 µg/d higher 24-h UI would be expected between boys with otherwise similar dietary intakes (including sodium intake) and within the same boy, with one unit increase in A/P ratio. In girls, no association was found between an increase in the ratio and changes at the within person level, possibly due to smaller individual changes that we were not able to fully identify.

One possible mechanism thought to explain the positive association of A/P ratios with iodine excretion, is the suggested association between sodium intake and consumption of animal foods. The main dietary sources of sodium ingested by children in Europe and Northern America are processed foods, often of animal origin [40,192,193]. Longitudinal analysis suggests that meat intake (especially cold cuts) increases 24-h sodium excretion as earlier reported in a group of DONALD children [182]. These findings were confirmed by our analysis as we observed higher sodium intake in the high A/P ratio category (Table 2). However, the positive association of A/P ratio with 24-h UI in the longitudinal PROC MIXED model remained significant after adjustment for sodium excretion, albeit with somewhat reduced effect estimates (ß-values of the A/P ratio). Therefore, the association between A/P ratio and 24-h UI in children seems to be only partially mediated by the amount of sodium intake. It is interesting that when including dairy protein in the animal protein fraction from the A/P ratios predicted a quite similar increase of 24-h UI, compared to the ß values when the dairy is excluded from the ratios. It is possible that the relatively high but constant contribution from the dairy group observed over the whole range of A/P categories (Fig 9, pie graphs) explains the observed results.

Traditionally, the iodine status of populations is measured in terms of urinary iodine concentration (UIC, µg/L), generally obtained from spot urine samples [13,70]. More recently, it has been acknowledged that whenever feasible, 24-h UI (µg/d) should be the "reference standard" for measuring iodine intake, as it incorporates urine volume, which can strongly

affect dilution of the sample and therefore iodine concentration [12,73,84]. With our analysis, which comprises a large number of 24-h urine samples (~2000), we were able to confirm the importance of using 24-h UI (µg/d) instead of concentration (µg/l) to define the iodine status. Based on the WHO 100 µg/L cut-off for iodine adequacy, our observed median concentration in boys (109µg/l) suggests sufficiency, and in girls (94µg/L) modest insufficiency. Due to an often better hydration status in females compared to males [163,194], such a difference is not unexpected; accordingly, energy corrected 24-h excretion rates of iodine (Table 14) denoted that no sex differences exist.

As trends towards alternative nutrition practices, i.e. preferences to consume organic foods, use of no iodized salt, and vegetarian diets are becoming more popular in European children [195,196], the issue of what constitutes an optimum diet and micronutrient composition has important public health implications. In an effort to achieve healthier diets, even slight changes in food sources, especially in animal products (a decline in dairy, eventually, increasing grain intakes), may potentially at the same time negatively affect micronutrients such as iodine as recently observed by Perrine et al. in a group of USA schoolchildren [177]. Accordingly, when the subjects of the present analysis were divided into low vs high A/P ratios, the proportion of children with iodine status below EAR was higher in the lower animal foods consumers, although the differences were not significant. Also the observed effect estimates for a change of A/P ratio for 24-h UI were small. A change in the A/P ratio of about 1 unit predicted a difference in 24-h UI of ~ 6 µg/d, equalling to about 10% of the EAR. Nonetheless this dietary factor should not be overlooked as a long-term etiological risk factor that might contribute to cause iodine deficiency disorders in children, such as cognitive performance as literature suggests [67,197]. Especially with regard to the planned salt reduction measures that will also reduce the intake of iodized salt (the main iodine source for children), the achievement of an adequate intake in nearly all children is an important goal.

Discrepancies on the adequate amounts and sources of protein intake in children exist. It has been shown in epidemiological and clinical studies that the amounts of dietary protein intake as well as its sources are important in the prevention or risk increase of chronic diseases, as reviewed in the report by Hörnell et al [102]. In our study population, the median protein intake (1.7 g/kg) was higher than the 0.9 g/kg recommended for German children [198], however the overall contribution was within the range of representative German schoolchildren's dietary protein intake (10-15% of energy) [199].

Strengths of Study IV included the longitudinal design, with two to seven repeated measurements throughout childhood for each individual and the available data in a relatively large number of children, which better allowed us to use models more suitable for the evaluation of the association of diet with urinary biomarkers (24-h UI) than models based on cross-sectional designs. Even though single 24-h UI collections do not reflect habitual iodine intake [12], the repeated measures of dietary intakes concomitant with analysis of urine samples in the same subject, allows both assessment of the gross average iodine status over

years and the influence thereupon of the children's changes in dietary components (from year to year).

As it is recognized, the participants of the DONALD Study are characterized by a higher socioeconomic status than the overall German population [115], and do not represent subjects with rather extreme dietary characteristics, e.g., a very high intake of sodium, as present in other population groups [199]. However, despite the homogeneity of our sample, we could still observe significant associations regarding the maternal education with the children's preferred protein sources. The higher proportion of mothers with a higher level of education in the low A/P ratio category, suggest that homes with better educated mothers probably are more likely to adopt more healthy food patterns, which potentially may in turn lead to a moderate deficiency in iodine supply.

In conclusion, **Study IV** shows a positive association between higher ratio of animal to plant protein and iodine status in children. Accordingly, children with healthier and therefore more desirable food patterns (lower intake of animal protein) demonstrate somewhat lower iodine excretion. As many children in Western populations are probably already in the highest A/P ratio, increasing animal protein intake would not be advisable. To nonetheless ensure adequate iodine nutrition, alternative prophylactic measures to prevent iodine deficiency are needed. One solution could be the mandatory use of iodized salt in processed foods and for bread [180,200,201] (on a voluntary basis- which currently in Germany is only < 30 % [88]), additionally to the use of iodized salt at home. Furthermore, substituting some of the meat protein for animal foods with higher iodine content (e.g. fish) may also benefit the iodine nutrition in those consuming western type diets. Altogether, the current dietary recommendations that advise the limitation in the intake of animal protein and to increase the plant-based foods to prevent chronic diseases do not contradict with adequate iodine nutrition, however, potentially they may do so if no further actions towards general iodine prophylaxis measures are taken.

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6. General Discussion

Nutritional biomarkers are essentially needed to provide objective measures of nutrient status, as missing objectivity is one of the limitations of the commonly applied dietary assessment methods such as food frequency questionnaires, multiple-day food records and 24-h dietary recalls [1,3,4]. The aim of the present thesis was to exemplarily examine the application of urinary biomarkers for the assessment of hydration and iodine status and their interaction with dietary patterns in children. Additionally, to provide information on potential analytical measurement errors, a methodological analysis of the long-term stability of chemical urinary analytes was conducted for the present and for future biomarker analysis.

The results of the present thesis substantially underline the potential of the application of existent biomarkers, however also uncovered pitfalls that still need to be addressed to accomplish promising non-invasive, and more valid and reproducible tools that can be widely used in epidemiological research in children.

In the following general methodological issues related to the DONALD Study –the data basis for all performed studies- are discussed, as well as the main findings from **Study I to IV** in context of the related interpretational and practical implications. A more specific discussion with regard to the single studies has been provided in the discussion sections of Chapter 5.1-5.4.

6.1 Methodology strengths and limitations

All analyses of the present thesis, **Studies I-IV**, were based on sub-samples of the DONALD Study. The strengths of the DONALD Study lie, first, in its prospective-longitudinal design, covering a period from birth until young adulthood. This design with annually concomitant assessments of the children's medical, anthropometrical, dietary and urinary characteristics build the alomost perfect scenario to investigate the subject-related biological associations between exposure and outcome. The data structure of the DONALD Study allows not only associating short-term dietary intake as a measure of nutritional exposure, but also the separation of between-person and within-person changes in a longitudinal perspective providing information on different sources of variation of the outcome. This means that both inter and intra-individual variation can be concomitantly analysed, as shown in **Studies II** and **IV.**

Perhaps the most important and valuable characteristic of the design of the DONALD Study, besides its longitudinal character, relies on its large urine biobank. To date the DONALD urine biobank stores > 8000 aliquotes of 24-h urine samples and a number of related spot urines from the 3-4 to > 18 yr old participants. In this respect, the long-term urine

biobanking allowed addressing specifically methodological questions on stability of urine biomarkers (**Study I**), and, in **Study III**, the direct comparison of iodine intake estimates from 24-h urine samples with parallel spot-urines for the evaluation of the reliability of the assessment of iodine status in populations. 24-h urine collections- although not comprehensible applicable in large studies- are usually seen as "gold standard" in clinical and epidemiological settings because they reflect the total renal production of urine during this time [11,32–35]. A collection period of 24-hours may be especially important for assessing daily levels of metabolites or urine parameters that are affected by circadian rhythm, by diurnal variation, or by total urine volume (e.g. sodium, iodine, osmolality) as it has been described for the urinary analytes examined in the DONALD Study. Despite the careful DONALD protocol for urine collection, 24-hour excretion rates are also prone to bias, as they depend on the completeness of 24-h urine samples. Completeness of a 24-h urine implies that no relevant amounts of urine is lost, and that the collection time period is within a certain range (i.e. not markedly longer or shorter than 24-hours) [202]. Thus, to verify completeness of 24-hour urine samples, an external marker such as p-aminobenzoic acid has been suggested as the most appropriate method [37,202]. However, one of the characteristics of the DONALD Study design, regarding urine collection is its non- invasive nature, and applying this technic would require the additional administration of tablets containing p-aminobenzoic acid, thus increasing participant burden of the DONALD children. Creatinine is usually excreted at a relative constant rate throughout the day [37] thus body-weight related creatinine reference values are useful to detect marked errors in the urine collection of healthy children and adolescents [50]. Furthermore, completeness of the urine collections is checked based on the records in which the time of every micturition on the collection day is noted. The latter is checked for plausibility by the dietician collecting the urine containers in the families [115].

Additionally, the urine data in the DONALD Study is analysed using highly standardised procedures [115,175] by trained and quality-monitored personnel that has been working for the DONALD Study for many years. A high degree of continuity and reduced inter-and-intra-observer variability can thus be guaranteed, which is of particular concern for laboratory measurements. Reliability on using the same analytical equipment was achieved, due to stringent and systematic maintenance, repair and overhaul by technical company, but also to the highly mechanically and electronically specialized in-house technician. In this laboratory context, the methodological pre-analysis (**Study I**) assessed how well the measurements can be performed to reduce the error associated with analytic measurement of the respective metabolites, what is essential to consider when choosing an appropriate nutritional biomarker over a long observation time.

The general idea behind the application of nutritional biomarkers is to reduce the error that dietary intake methods may induce [3,203]. In this respect, collecting dietary intake data is accompanied by limitations, because there is always the possibility of under-(and over-)

reporting and in accuracy in the reporting, especially in children [203,204]. Weighed dietary records, as they are part of the DONALD protocol, are often considered as one of the best available protocol-based methods for nutritional assessment [41]. For nutrients such as protein, previous analyses from the DONALD data have confirmed that weighed dietary records provide solid estimates of total protein intake in children and adolescents [9]. Additionally, the precise assessment and structure of the DONALD dietary data allows classifying specific food-groups at the ingredient level, therefore reducing the chance of misclassification of foods especially in composite foods. This disaggregation of foods to the basic components is especially valuable when accurate estimates of specific foods groups are needed such as it was the case in **Study II** for Fruit and Vegetables; and in **Study IV** for the protein ratios of animal to plant-based foods.

The elaborate design however, and the high number of follow-up visits, also result in a non-representative study sample, displaying higher education and socioeconomic status compared to the general German population, which is also more interested in nutrition and health-related topics [115]. Consequently, the DONALD sample is relatively homogeneous, and extremes of dietary behaviour may thus be underrepresented [115]. This may somehow decrease statistical power to detect associations of those lifestyle factors not considered in detail. However, it is important to emphasize that representativeness is of minor importance when subject-related biological exposure-outcome associations are investigated, as it does not necessarily affect physiological relationships [115,205]. With respect to generalization of the results to other populations, there would be limitations only if the DONALD participants were biologically different from other paediatric age groups [206], or if the distribution of characteristics in the DONALD where compared with external data dependent on environmental factors such as socioeconomic status [115]. However, this is not assumed in this context.

6.2 Interpretation and implication of study results

The application of new biomarkers, as well as the evaluation of the known biomarkers for nutritional intakes and status, is an essential part for the progress and perspectives in continuing nutritional epidemiology [4]. The currently expressed need for the development of biomarkers that are clearly related to dietary intakes is based on the requirement of validations of dietary instruments and methodological-and measurement-error research, as it has been suggested by different authors [2–4]. In this thesis we have demonstrated also for long-term observations in children the applicability of accepted urinary biomarkers for hydration and iodine status, and have stressed the pitfalls that have to be addressed for a successful rather unbiased use of urinary biomarkers.

Potential use of biochemical analytes: long-term stability -under moderate freezing conditions and without use of preservatives

In **Study I** (methodological pre-analysis) an appropriate stability and validity was shown for a number of relevant renal excretion parameters if urine samples are collected without added preservatives and stored at -22 ºC for long-term periods over 10 yr. As it was mentioned in Chapter 5.1.5, one strength of this study was that it combined two important urine storage conditions (temperature and use/non-use of preservative) that until now had not been examined together in one study, yet. With regard to statistical purposes, our findings suggested that at least for the evaluated metabolites, no statistical adjustment for varying storage duration is required in the respective data analysis.

Under an epidemiological perspective, these findings can provide valuable information for observational and intervention studies in which urine collections are performed and samples are stored for longer time period. The analytes under study represent selected basic nutritional and /or physiological variables of which some (e.g., approved dietary biomarkers like iodine, urea, or the mineral anions or cations) are probably analyzed directly after completion of respective study-specific urine collections; whereas others, such as acid-base, stone-risk or hydration parameters, may become important not until crucial research questions turn out at a later time point. For example, the role that acid-base balance may play in blood pressure development in healthy children, was just recently analysed in children from the DONALD Study [144,145], however the acid-base specific NAE tritrations or related potential renal acid load measurements were not routinely performed in the first analysis of the collected urine, as it might be the case in other epidemiological studies using urines as biological fluids.

Since biological sample collection is an integral part of various epidemiological studies, but may also represent the most costly and challenging element in field-studies, the here proposed urine-collection and storage condition may help reducing the costs, especially in contexts with limited resources. It is possible that urine aliquots are stored in a freezer (~ -20 to -25 ºC) for fairly long periods of time, eliminating or minimizing the need for cold ultra-cold freezer storage facilities (temperatures ranging from up to -30 to > -80ºC), which may require more expensive and sophisticated equipment.

For the potential applicability and the correct interpretation of the examined metabolites representing nutritional biomarkers (either alone, or combined with other components), it is important to anticipate the laboratory measurement error (e.g. intra-assay, inter-assay coefficients of variance). This error should be included in the reporting of the results to help researchers to interpret findings and to allow comparisons with other studies using the same biomarker metabolites. Estimation of analytical precision and the use of standard quality control procedures, as it for example is done in the DONALD Study (Chapter

4.5; and Chapter 5.1.3) can help diminishing laboratory analytical error and warrants confidence that the analytes are accurately measured for further use. Thus, determining and quantifying the laboratory error for the examined analytes here (e.g. creatinine, sodium, osmolality, iodine), and combining them with other already established nutritional biomarkers (such as it has been done in this methodological pre-analytical study) will therefore be an important step ensuring validity in the area of non-invasive investigations in nutritional epidemiology.

Assessment of hydration status

For the assessment of hydration status, changes in urine parameters such as 24-h volume and osmolality are considered as markers for short- term water intake exposure [8]. Assessment of dietary water intake through semi-quantitative and more quantitative methods of food recording has serious limitations and to some extent only reflects the water availability and dietary patterns [207], and varying water losses (through sweat and faeces) are not considered. Because of the importance of an adequate hydration status for health [17,53,155], assessment of hydration status and furthermore the maintenance of an adequate hydration status, especially in children, is of relevance. With comparatively simple interventions (habit modifications), water intake can be increased in children as it was done for example in an intervention in German schoolchildren [208]. Children were educated on the value of water and provided with special filtered drinking fountains and water bottles in school. At the end of one year the children increased their water intake by 1.1 glasses /d, in addition to a reduction of their risk being overweight [208].

By using the Free Water Reserve (FWR) as a marker of hydration status, in **Study II**, the potential applicability for assessing 24-h hydration status at both the population and individual levels, and related to intakes of specific food groups, has been further explored. In healthy individuals, *negative FWR* values, reflect high osmolality values, due to a higher solute load which may be caused by low water intake from fluids but also by diets with low water content foods e.g. lower intake of F&V as it has been earlier suggested [97,163], but never been shown directly in a research study. The consequence of inadequate water intake may compromise health of children. The immediately consequences of poor hydration status include an increased risk for dehydration, decrease in physical and cognitive performance. In the longer term, adequate hydration status is associated with a reduction in urinary tract infections, hypertension, functional decrements including impaired intellectual performance in school children or kidney disease as some evidence suggests [17,53,209]. The recent application of the physiological measurement FWR is a specific tool to assess hydration status at least to define populations' adequate intakes [15,16,18]. In a more complex manner, the FWR is an indicator of hydration that can be interpreted at both individual and population level, because it represents the individual 24-h hydration status (*euhydration, risk of*

hypohydration), in combination with population data of "*risk of hypohydration*" without necessarily presenting clinical signs of dehydration and therefore may reveal important starting points for the preventive medical field [16] (see also Chapter 2.3).

In **Study II**, one practical application was exemplified that allowed the quantification of the effect of consuming an important specific food group (F&V) on hydration status; an association that has only been speculated and never been proven. The longitudinal mixed-effects regression models allowed a quantitative understanding of the relationship of F&V intake, as solid and as juice, with the FWR when all other dietary water sources were kept constant. As described in Chapter 5.2.5, in theory for a boy with a total water intake of 1600 ml /d from all dietary sources (i.e. plain water and water from beverages, solid F&Vs, F&V juice, milk and whey based milk products, and water from other foods), an extra intake of approximately half a glass of plain water (115 mL) would increase the FWR by 50 mL. The same effect would be observed when approximately three-quarters of a glass of orange juice (156 mL) or one medium-sized apple (~125 g) is additionally consumed. By contrast, drinking one glass of milk (200 ml) would be necessary to improve the FWR by the same volume. This has important implications regarding the improvement of individual hydration status and for the public health promotion of F&V consumption.

In our study, children consuming regularly F&Vsolid showed a more favourable hydration status (FWR) than those children consuming less. The median intake of F&V for this sample population was 390 g/d, nearly the globally recommended F&V intake (400 g/d) [157,159], reflecting a relatively healthy food pattern. However, no negative effects have to be expected by the resulting water surplus of higher intakes of F&V, even in individuals with a more favourable hydration status, as water balance is well regulated in a wide range of water needs and intakes (Chapter 2.3). Furthermore, F&V, especially in solid form have a relatively low energy density compared to other foods (or beverages) and therefore prevent adding extra calories to the children´s diet.

Water is a nutrient understudied and no general consensus exists on the reference values. Therefore, the adequate intakes (AI) can be used as comparison parameters [56,107], as described in chapter 2.4. The important feature of the presented concept of FWR and calculation of AI is that it is not only based on median total water intake and maximum concentration capacity of the kidneys (as described in Chapter 2.3), but considers individual 24-h hydration status and adds a safety margin to ensure adequate intakes in nearly all (97%) healthy persons of a population. The parameter FWR is a biomarker for hydration that may also have utility in predicting future risk of disease or long-term functional outcomes if the less healthy "negative FWR values" are present in a population. Results from our study

suggest that even in healthy populations a large number of children (around 22%) can have negative FWR values, thus denoting long-term "risks of hypohydration".

The Current German recommendations for water intake in children are 1600 mL/d for 4- to <7 y olds, and 1800 mL/d for 7 to < 10-y-olds (including the metabolic water) [110]. Based on the total water intake calculated from the food records, DONALD children were only moderately below the recommended intakes (Table 9). The importance of using the FWR concept as marker of hydration status, is that it provides data on "how much" in terms of water intake would be needed to reach an adequate hydration. According to our findings, to reach adequate intakes (values of the 3rd percentile, observed in Table 9) for this exemplarily population, an additional water intake of 110-140 mL/ 24 h in boys and an extra 80-100 mL/24 h in girls would guarantee euhydration.

One limitation of the applicability of the FWR as a marker of hydration status, may be the limited availability of high quality data on water intake in most of the countries [17], and parameters such as osmolality and renal concentration capacity are even less available [155]. However, the latter is a general limitation of the assessment methods for hydration status. To date most of the recommendations for water are based only on reported dietary intake [56], this has led to the concerns about possible ways to overcome the so far missing data on urinary osmolality in different populations. Incorporating measurements of urine osmolality and population-specific maximum renal concentration capacity is not an easy task, but efforts on research should be directed to gather this information to diminish the existent gap on these important components, and then FWR can serve as a suitable measure to derive specific water recommendations in distinct populations. Furthermore it could be used as tool for developing a deeper understanding of the relationship between dietary patterns (i.e. preferences for foods with higher vs. lower water content, high energy density, etc) and metabolism of water.

A practical interpretation that can be derived from the application of the FWR as marker of hydration status at population level is that for populations in which 24-h urine samples show a high percentage of subjects in the range of *risk of hypohydration*, preventive measures to increase the common level of water intake have to be considered. In a broader context, to monitor the progress of programs aiming to promote healthy food patterns in children to prevent chronic diseases by the *increase of water intake,* for example interventions providing free access to water in schools [208], or effective initiatives to promote F&Vs in schoolchildren [210,211] could be a useful strategy and would complement the interpretation of the FWR as a biomarker for hydration status.

Assessment of iodine status

The "gold standard" biomarker for assessment of iodine status is the measurement of

urinary iodine (as it was described in Chapter 2.4) [13,21]. The assessment of iodine intake by dietary records is limited due to the lack of reliable databases of iodine concentrations for various foods, and the difficulty to assess the amount of iodine that comes from iodised salt used in industrial food production as well as at home [21]. Because about 90% of the iodine intake is excreted in urine (only about 10- 15% are non-renal iodine losses) [212] urinary iodine excretion is considered the most important indicator of iodine intake. We have focused on two different aspects of the potential use of urinary iodine as marker of iodine status (**Studies III-IV).**

Urinary iodine concentration vs estimates of 24-h iodine excretion

Urinary iodine concentration (measured in spot urine samples) is the most widely used and recommended parameter to estimate iodine status of a population [22]. However, with regard to this recommendation our results of **Study III** confirmed the hypothesized pitfalls that can emerge when using just UIC measured in spot urines as biomarker for iodine status at population level. **Study III** revealed a high dependency of UIC on hydration status, which has previously suggested by other authors [12,72] too. Two main issues have emerged from the results of **Study III**. Firstly, the application of the proposed methodological approach, namely the "creatinine scaling method", using published 24-h creatinine reference values for children and adolescents, can successfully eliminate most of the influence of hydration status on the biomarker iodine excretion. As it was shown in the results (section 5.3.5), this method has the advantage that still only measurements of urinary iodine and creatinine in spot samples are needed; however when the UIC/creatinine concentration ratio is corrected by the corresponding creatinine reference values (in this example: Remer et al. 2002 [50]), the effect of hydration status is attenuated and the derived estimates of 24-h iodine excretion are similar to the real measured 24-h urinary iodine excretion in 24-h urine samples, i.e., to the reference standard for iodine status assessment [12]

As suggested by the results of **Study III,** considering only iodine concentration instead of an absolute 24-h UIE estimate would have masked a non-satisfying iodine nutritional status in our sample population. As 24-h urine collections are normally not feasible for large population studies, the estimation of 24-h iodine excretion from spot urine concentration values by age-and sex-adjusted creatinine reference values, shows to be a reasonable approach to minimize the confounder urine volume. Also the simple iodine/creatinine ratio (as it was formerly recommended by the WHO) has been investigated in comparison to real 24-h iodine excretion (Table 13), however the approach that showed the best results was the 24-h creatinine scaling method. While a similar approach has been applied to validate data on iodine excretion among adults [44,72,171], this is the first study examining this assessment methodology in a population of healthy children and adolescents. If correctly applied and interpreted, our findings may provide a valuable contribution to the

field of iodine assessment methods. As it was demonstrated in this particular example, the proposed estimated 24-h scaling represents a simple and widely applicable way to accurately and reliably monitor iodine status in populations by using spot urine samples.

Secondly, **Study III** shows how the 24-h iodine excretion values, estimated from spot urine samples can be applied to define the iodine status of populations. As it was described in Chapter 2.5, inadequate iodine intake in individuals is defined as an iodine intake lying below the estimated average requirement (EAR), whereas inadequate intake of populations is defined as median intakes below the recommended dietary allowance(RDA) [111]. To define the nutrient inadequacy of habitual dietary intakes of populations, the EAR cut-point method has been suggested [13], and just recently been successfully applied in a Swiss adult population [173]. With this method, estimated 24-h iodine intake values are compared to the IOM's EAR reference values [82]. Individuals with iodine intakes below the EAR are suggested to be at risk of iodine deficiency and the nutrient intake of a population is regarded as satisfactory when most (97-98%) of the individuals within the population meet their respective EAR, i.e. acceptable prevalence of inadequate intakes is 2-3% [111]. This EAR cut-point method therefore can be easily applied for spot urine measurements of large epidemiological studies, using the estimated 24-h creatinine scaling approach as suggested in **Study III**.

The results of **Study III** suggest the 24-h creatinine scaling method to be a useful approach to estimate iodine status of populations from spot urine samples, at least in children from industrialized countries with similar dietary and physical developmental characteristics, like the population for which the required creatinine reference standard has been published [50] For application in other contexts, in principle there are some considerations that need to be taken into account. First, the use of appropriate and reliable reference values for urinary 24-h creatinine excretion is essential, and second, due to the known day-to-day variation in iodine measured [171] and the frequently described day-to-day variation in 24-h creatinine [37], the obtained output values do indeed characterize excretion values of a groups, but not for an individual [50]. Thus, for children with other lifestyle background or from countries outside central Europe (e.g. with different sources and intakes of protein [50,176], the applicability of the creatinine reference values used in **Study III** [50] has to be verified.

Preference for a more plant-based food pattern and iodine status

In **Study IV**, we addressed for the first time in children, consuming a typical western diet, the effects of changes in the proportion of animal to plant protein on iodine status by using 24-h urinary iodine excretion as marker of iodine status. The analyses pointed out important issues related to diet and iodine excretion. First, our results suggest that a high ratio of animal to plant protein (e.g. higher intake of animal foods and lower intake of plant foods) is associated with greater iodine excretion, even if other relevant dietary sources of iodine

(e.g. salt intake) are allowed for. Second, a possible mediational role of salt intake, explaining the higher 24-h iodine excretion in children with diets higher in animal food products has been observed. This is in agreement with the literature showing that in children the main dietary sources of sodium (salt) are foods of animal origin [119,183]. However, as it was shown in our longitudinal analysis (Chapter 5.4.4), the association of A/P protein with iodine excretion still remained significant (although diminished) when including sodium excretion (as marker of salt intake) in the model therefore confirming that the effect of a diet high in animal protein cannot only be explained by a higher (iodized) salt intake.

As described in Chapter 2.4 and 2.5, the relationship between dietary intakes and iodine status is affected by the intakes of animal sources (milk, meat, fish, eggs), iodine uptake inhibitors (goitrogens), and carrier substances (iodized salt). Measures of habitual iodine intake should consider the overall dietary pattern, including assessment of intakes of carriers within the meal or shortly thereafter [21]. Most foods are low in iodine in relation to physiological needs [83,213]. Only marine foods and milk products from animals reared with iodine-enriched feeds or pastured on iodine-rich soils contain high amounts of iodine. It is for this reason that universal salt iodization programs call for iodizing salt for all uses, including household (cooking and table) and food industry, and therefore is the main fortification vehicle to provide iodine. In industrialized countries and very probably in countries with economies in transition, the main proportion of salt consumed comes from commercially processed foods (about 80%), only 20% comes from household salt [61,64,84]. The current WHO-UNICEFF-ICCIDD guideline recommends salt iodization of 20-40 mg of iodine per kg salt [22]. Hypothetically, at a mean of 25 µg of iodine per g salt, 4-8 y old and 9-13 y old children would satisfy their iodine requirements (EAR, 65 µg/d and 73 µg/d), if consuming 2.6 g and 2.9 g fully iodized salt per day respectively. In Germany, per legislation, iodized salt contains 15-25 µg per g salt, therefore the mean lies at 20 µg per g salt. If 100% of salt would have been iodized, the observed median of 4.2 g/d salt intake of those children with lower intakes of animal protein from **Study IV**, some what would have been enough to reach their required iodine intake (EAR) (4.2 x 20= 84 µg/d). However, this is not the reality in Germany, where the iodization of salt used in households or for food production is still on voluntarily basis and is estimated to only cover <30% of processed foods. Therefore, as it was confirmed in **Study IV**, even with higher intakes of salt (that is not desirable) still, there was a proportion of children at risk of inadequate iodine intake of nearly 15% (overall proportion of children below EAR 14.3%).

A raising concern on the current salt reduction recommendations to help to prevent hypertension and its complications and its interaction with iodine status has emerged [106,214,215]. Generally, a consensus accepted, is that salt reduction and iodine deficiency prophylaxis measures are not mutually exclusive if adequate iodisation programs are

performed [214,216,217]. However, this is not the only concern regarding current developments in dietary patterns and iodine status. As described in Chapter 2.5 and in **Study IV** (section 5.4.2), diets containing little to no animal food products might contribute to inadequate iodine intakes, as studies suggest [99–101]. In this respect, also the current guidelines for healthy eating (e.g. New American Plate from the World Cancer Research Fund) [104], that advice to limit the animal protein and to increase the plant-based foods to prevent chronic diseases have to be critically investigated with regard to their effects on iodine status.

One limitation of our study on iodine status and the effects of a plant based diet in children, could be the fact that we did not investigate the parallel intake of specific dietary goitrogens. They are suggested to inhibit the iodine uptake and therefore negatively affect iodine status. *Goitrogens* are natural compounds of plant foods such as broccoli, brussels sprotus, cabbage, cauliflower, cassava [105]. Therefore, it could even be hypothesized that a higher goitrogen intake in children preferring a more plant based diet compared to those with a mixed diet, additionally impairs thyroid's iodine status – an effect that has not investigated in our study.

The trends towards alternative nutrition practices, i.e. preferences to consume organic foods, use of no iodized salt, and vegetarian diets are becoming more popular in European children [195,196].The issue of what constitutes an optimum diet and micronutrient composition has important public health implications. As described in the results (section 5.4.5) a change in the A/P protein ratio of about 1 unit predicted a difference in 24-h UI of ~6 µg/d, equalling to about 10% of the EAR. This factor should not be overlooked as a long-term etiological risk factor that might contribute to cause iodine deficiency disorders in children, such as cognitive performance as the literature suggests [67,197].

What was intended to exemplify with **Study IV**, more than a promotion of iodized salt or higher intake of animal food products to assure adequate iodine intake, was to raise a general awareness, for a further in depth examination of the existent prophylactic measures in iodine. A preference for plant-based foods and plant-based protein sources to be in agreement to food guidelines for healthy eating is undoubtedly advisable. However, it should be considered that limiting animal protein sources could lower iodine intake, and this, be counterproductive for achieving adequate intakes, especially if dairy products are included in the limitations. Therefore, to ensure adequate iodine intake in populations at risk of iodine deficiency, alternative prophylactic measures to prevent worsening of the iodine status situation are advisable. For populations that rely more heavily in commercially produced foods, the coordination and cooperation with the food industry and areas of the health sector responsible for the legislation on iodization of salt are needed. Parallel, substituting some of the meat protein for animal foods with higher iodine content (e.g. fish) may also benefit the iodine nutrition in those already consuming western type diets. A more graduate approach that

could also be achievable is to search for and evaluate the use of alternative vehicles for iodine fortification, such as it has been done regarding bread [200,201] or, for example, regarding the biofortification of vegetables with iodine [218].

7. Conclusions

Much of the evidence relating food and nutrient intake to chronic disease risk relies on information gathered by various dietary assessment techniques. Dietary assessment techniques, especially in children and adolescents, are prone to reporting measurement errors, which might bias the interpretation of links between dietary intake and health. Therefore, there is a need to develop, check and establish reliable and valid nutritional biomarkers for clinical assessment and public health practice as well as for research purposes.

With three application examples, we could underpin the effectiveness of using urinary biomarkers as non-invasive methods for nutritional research in children, and presented reasonably valid methods for defining nutritional status of two important public health nutrients: *water* and *iodine*.

The selection and use of non-invasive biomarkers in nutrition has changed considerably as new technologies are available. "Urine" represents one of the most appealing biological fluids for further exploration in epidemiological research in children. In many contexts, providing urine samples may be preferred over other fluids (e.g. blood). Culturally, giving urine is a practice more common and accepted in diverse settings, also urine collection is non-invasive, and especially children may show less resistance than when providing blood samples, which in fact influences the ability to collect samples and increase participation. The difficulties of obtaining high-quality urine samples in field-studies could be diminished by the application of standard procedures of specimen collection and processing. Lack of adequate infrastructure and facilities for collecting, handling, transporting and storage of samples, may be a limitation in context with limited resources. However, these issues can also be anticipated and addressed by planning and implementing optimal logistical procedures.

For long-term prospective studies, consideration of long-term quality control is important. Long-term laboratory quality control, involves methodological accuracy and precision, stability of analytes (including effects of changes over time, freeze-thaw effects, temperature of storage), among others. The observed stability of the urine metabolites examined in this thesis -stored at a temperature level of about -20 ºC for a period up to 15 y and without addition of urine preservatives- strongly suggest the measurement validity and potential application of a number of urinary biochemical analytes for later analysis in clinical or epidemiological long-term studies.

While currently most nutrition and health programs tend to focus on improving nutrition in early infancy, still nutrition in childhood and adolescence remains important. Children 4-18 y old, are in need to meet their maximum growth potential, optimal cognitive function, activity level, immune function and school performance. Although deaths from non-communicable diseases mainly occur in adulthood, exposure to risk factors begins in childhood and builds up throughout life, thus adequacy of diets and health promotion interventions in this age-group can help to ensure adequate nutrition and prevent non-communicable diseases in later life. Knowledge on the most important dietary influences on

nutritional status of children has enabled the development of urinary biomarkers for nutrition, and the success of their application as exemplarily examined in this thesis.

In this thesis important issues that have been recognized as pitfalls in the application of biomarkers in nutrition were outlined [2,3]. Furthermore, the studies in this thesis support the research needs and recommendations suggested a) in the literature review on "*Water hydration and health*", regarding the understanding of hydration measurement and requirements [17]; and b) in the Guideline: Fortification of Food-Grade Salt with Iodine from the WHO [219].

The specific points addressed in the present thesis are briefly summarized as follows:

- Water is an essential nutrient linked to daily performance and both short-and long -term health. The physiological concept *Free Water Reserve* is based on urinary measurements and, when properly used, may be a promising tool for the assessment of individual and population hydration status. Healthy eating patterns recommend higher intake of *Fruit and Vegetables* (*F&V)* for the beneficial effect on prevention of related chronic non-communicable diseases (e.g. obesity, cancer, and hypertension) especially in children. However, higher F&V intake appear also to have a positive effect on children´s hydration status. Thus, by increasing consumption of F&V in solid form, at least in relatively well-nourished western populations, a general increase in total body water (measured by free water reserve as marker of hydration) was observed. This increase in FWR was even similar to the one, regularly observed from other important fluid dietary sources: plain water, water from beverages, juice, milk; therefore the regular intake of F&V does also help to reach *adequate intakes* of water.
- The assessment of Iodine status in populations is of increasing public health importance, since insufficient iodine intake is frequent among children in developing and developed countries. Currently, programs of iodine prophylaxis (the most common is the promotion of iodized salt), are targeted at the population level. Thus, such programs require periodic measurements of population iodine status for monitoring. Urinary iodine is an objective biomarker of exposure, as it is an excellent indicator of recent iodine intake. The commonly used biomarker to characterize iodine sufficiency in populations is the urinary iodine concentration (UIC, µg/L), usually measured in spot urine samples. The correct interpretation of the UIC cut-off as defined by the current reference standards of the WHO (UIC > 100 µg/L) is crucial, as UIC is recognized to be highly dependent on hydration status. It is suggested here, that UIC data can be corrected by specific age- and sex- adjusted

creatinine reference values (using the estimated 24-h creatinine scaling approach) to eliminate the dependency on hydration status. These corrected values, which reflect 24-h urinary iodine excretion (i.e., the reference comparison standard), can then be compared to the EAR to characterize iodine status in populations. Ideally, in a near future, iodine status monitoring programs may improve with the inclusion of ”biomarkers for iodine status” that could provide reliable information on the prevalence of iodine deficiency by using approaches such as the *estimated 24-h creatinine scaling method* beyond the usual UIC assessment methods.

- The current worldwide iodine status of populations is fragile as it depends on prophylactic measurements, mainly on the use of iodized salt. With respect to animal/plant-protein intake, a preference for plant-based foods and plant-protein sources to be in agreement to food guidelines for healthy eating, may be advisable. However, it might be considered that limiting animal protein sources could lower iodine intake, and this, be counterproductive for achieving adequate intake of this brain nutrient.

The present thesis shall contribute to the understanding of the mutual relationships between exposure and status of two important nutrients: *water* and *iodine*, defined by the measurement of urinary biomarkers for potential use in epidemiological research in children. There is a need of further exploration of the methodological aspects from the here presented examples for the transfer into other contexts, especially were application of biomarkers has been limited so far. For a generalised application at large-scale, before they become the preferred biomarkers for *hydration* and *iodine*, additional carefully performed confirmative studies are needed.

8. References

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List of publications

Publications

This thesis aimed to examine the association between different dietary patterns on the urinary biomarkers for hydration and iodine status in Children. It resulted in the following publications (chronological order):

- Montenegro-Bethancourt G, Johner SA, Remer T. Contribution of fruit and vegetable intake to hydration status in schoolchildren. Am J Clin Nutr. 2013; 98:1103-1112.
- Remer T, Montenegro-Bethancourt G, Shi L. Long-term urine biobanking: Storage stability of clinical chemical parameters under moderate freezing conditions without use of preservatives. Clin Biochem. 2014; 47:307-311.
- Montenegro-Bethancourt G, Johner SA, Stehle P, Neubert A, Remer T. Iodine status assessment in children: spot urine iodine concentration reasonably reflects true 24-hour iodine excretion only when scaled to creatinine. Thyroid. 2015; 25:688-97.
- Montenegro-Bethancourt G, Johner SA, Stehle P, Remer T. Dietary ratio of animal to plant protein is associated with 24-h urinary iodine excretion in healthy schoolchildren. Br J Nutr. 2015 ;114:24-33.

Presentations

- Montenegro-Bethancourt G, Johner SA, Remer T. Fruit and vegetable intake and hydration status in German schoolchildren. 50. Wissenchaftlicher Kongress der Deutschen Gesellschaft für Ernährung (DGE), 20-22.03.13 Bonn, Germany, Proc Germ Nutr Soc 18:8.
- Montenegro-Bethancourt G, Johner SA, Remer T. Fruit and vegetable consumption and hydration status in schoolchildren. I International and III National Hydration Congress, 4-6.12.13 Madrid, Spain, Nutr Hosp 28 (supl):34.
- Montenegro-Bethancourt G, Johner SA, Remer T. Dietary animal to plant protein ratio and 24-h Urinary Iodine Excretion in healthy schoolchildren. 51.Wissenchaftlicher Kongress der Deutschen Gesellschaft für Ernährung (DGE), 12-14.03.14 Paderborn, Germany, Proc Germ Nutr Soc 19:8
- Montenegro-Bethancourt G, Johner SA, Remer T. An apple and a glass of water. Competitive or complementary for hydration status in children? Answers from "DONALD". Symposium: Kinderernähruhg und Prävention: von der Forshung in die Anwendug. 110. Jahrestangung der Deutschen Gesellschaft für Kinder-und Jugendmedizin e.V. (DGKV). Leipzing, Germany 14.09.2014.

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