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MASTER THESIS

Sina-Isabel Warz

Stable isotope ratios of carbon and nitrogen after weight loss
A secondary analysis of the CarbFunc study

Department of Clinical Medicine

Centre for Nutrition

Thesis supervisor: Jutta Dierkes

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LIST OF ABBREVIATIONS

AA	=	Amino Acids
AMPR	=	Animal And Marine Protein Ratio
APR	=	Animal Protein Ratio
AP+USPR	=	Animal And Unspecific Protein Ratio
ASI	=	Added Sugar Intake
BIA	=	Bioelectrical Impedance Analysis
BMI	=	Body Mass Index
CIR	=	Carbon Isotope Ratio
CF-IRMS	=	Continuous Flow- Isotope Ratio Mass Spectrometry
CIR	=	Carbon Isotope Ratio
CO_2	=	Carbon Dioxide
DXA	=	Dual-Energy X-Ray Absorptiometry
EA	=	Elemental analyzer
FM	=	Fat Mass
FFM	=	Fat Free Mass
HCLF	=	High Carbohydrate-Low Fat diet
IRMS	=	Isotope Ratio Mass Spectrometry
LCHF	=	Low Carbohydrate-High Fat Diet
NIR	=	Nitrogen Isotope ratio
RBC	=	Red Blood Cells
RCT	=	Random Controlled Trial
S-CIR	=	Serum Carbon Isotope Ratio
S-NIR	=	Serum Nitrogen Isotope Ratio
SIR	=	Stable Isotope Ratio
U-CIR	=	Urine Carbon Isotope Ratio
U-NIR	=	Urine Nitrogen Isotope Ratio
WC	=	Waist Circumference
VPDB	=	Vienna Pee-Dee Belemnite

ABSTRACT

Background The increasing health problem of overweight and obesity has ignited a renewed focus on weight loss, while at the same time, unintended weight loss related to either age or disease has become a clinical problem. Weight loss can be challenging to assess, especially in clinical practice, as earlier weight and time frames are critical factors in evaluating weight loss. Today, only absolute weight change and BMI are used to estimate weight loss, neither distinguishing whether fat mass or fat-free mass is lost. Therefore, identifying a biomarker that could be used to assess the amount and type of weight loss would be desirable and clinically important. A stable isotope ratio could be such a potential biomarker.

The stable isotope ratios of light elements of carbon (CIR) or nitrogen (NIR) have been used as a dietary biomarker of sugar intake and meat or fish intake. This thesis investigates their use of biomarkers of weight change exploiting the tissue-diet discrimination of stable isotopes in different tissues compared to stable isotope ratios of foods. Weight loss implicates the use of energy from stored tissues, which have a different stable isotope ratio than foods, and thus, we hypothesize changes in CIR and NIR upon weight loss.

Aim Investigate whether CIR and NIR are suitable biomarkers for recent weight loss in obesity and measurements of obesity.

Method CarbFunc is a weight-loss study in otherwise healthy people with obesity, consisting of three dietary interventions lasting 12 months. Dietary intake was regularly recorded, and anthropometric measurements and blood and urine samples were collected at baseline and then every three months for 12 months. In this explorative sub-study, 120 participants from the CarbFunc study were included. Food records from baseline and three months were used for assessing nutritional intake, and anthropometric measurements at baseline and three months were used to calculate weight loss and changes in body composition. Serum and spot urine samples from baseline and three months were used to analyze carbon and nitrogen isotope ratios.

Results At baseline, CIR in serum and urine was weakly to moderately and inversely associated with weight, waist circumference, and fat-free mass. Associations were stronger in males than in females. NIR, neither in serum nor in urine, was not associated with any anthropometric measurement in all participants and was weakly and inversely associated in men.

The median weight loss in all participants between baseline and three months was 5kg (interquartile range 3-8 kg). There were only subtle changes in CIR and NIR from baseline to three months. We observed significant differences between men and women in serum and urine NIR but not in CIR. CIR in serum and urine and serum NIR decreased from baseline to three months in females while increasing in males. Urine NIR increased in both sexes. At three months, serum CIR correlated with changes in weight, waist circumference, and fat-free mass, while urine CIR correlated with fat-mass changes in all participants and urine NIR with waist circumference change. CIR had a weak but significant association with weight loss and fat-mass change in females and with waist circumference in both sexes. Urine NIR correlated significantly with changes in weight and waist circumference in females and changes in waist circumference, fat-mass, and fat-free mass in males. Associations with urine NIR were only found in men with waist circumference and fat-mass. We also observed associations of CIR and NIR with dietary intake of carbohydrates, animal and marine protein.

Conclusion CIR and NIR after weight loss were not significantly different from baseline in obese, otherwise healthy participants. At three months, associations with changes in weight, fat mass, or fat-free mass were only weak and different in men and women. More research should also focus on the association of obesity with stable isotope ratios.

Even though this was an explorative study and more research should be done before firm conclusions are drawn, the suitability of CIR and NIR as biomarkers of weight loss in obese persons may be limited. Further research is needed, including any association between stable isotope ratios and unintended weight loss.

SAMMENDRAG

Bakgrunn: Det økende helseproblemet med overvekt og fedme har ført til et fornyet fokus på vekttap, samtidig som utilsiktet vekttap knyttet til enten alder eller sykdom har blitt et klinisk problem. Vekttap kan være utfordrende å vurdere, spesielt i klinisk praksis, ettersom tidligere vekt- og tidsrammer er kritiske faktorer for å evaluere vekttap. I dag er det kun absolutt vektendring og BMI som brukes til å estimere vekttap, uten å diffferensiere om fettmasse eller fettfri masse har blitt tapt. Derfor vil det være interessant å identifisere en biomarkør som kan brukes til å vurdere mengden og typen vekttap. Et stabilt isotop forhold kan være en slik potensiell biomarkør.

De stabile isotop forholdene mellom lette elementer av karbon eller nitrogen har blitt brukt som en diett biomarkør for sukkerinntak og kjøtt- eller fiskinntak. Denne oppgaven undersøker deres bruk av biomarkører for vektendring ved å utnytte vev-diett-diskrimineringen av stabile isotoper i forskjellige vev sammenlignet med stabile isotopforhold i matvarer. Vekttap innebærer bruk av energi fra lagret vev, som har et annet stabilt isotop forhold enn matvarer, og derfor antar vi endringer i CIR og NIR ved vekttap.

Mål Undersøke om CIR og NIR er en passende biomarkør for nylig vekttap

Metode CarbFunc er en vekttapstudie hos ellers friske personer med fedme, bestående av tre kosttilskudd som varer i 12 måneder. Kostinntak ble regelmessig registrert, og antropometriske målinger og blod- og urinprøver ble tatt ved baseline og deretter hver tredje måned i 12 måneder. I denne eksplorative delstudien ble 120 deltakere fra CarbFunc-studien inkludert. Matregistreringer fra baseline og tre måneder ble brukt for å vurdere næringsinntak, og antropometriske målinger ved baseline og tre måneder ble brukt til å beregne vekttap og endringer i kroppssammensetning. Serum- og punkturinprøver fra baseline og tre måneder ble brukt til å analysere karbon- og nitrogenisotopforhold.

Resultat Ved baseline var CIR i serum og urin svakt til moderat og omvendt assosiert med vekt, midjeomkrets og fettfri masse. Assosiasjonene var sterkere hos menn enn hos kvinner. NIR, verken i serum eller i urin, var assosiert med noen antropometrisk måling hos alle deltakerne og svakt og omvendt hos menn.

Det median vekttap for alle deltakere mellom baseline og tre måneder var 5kg (3-8). Det var bare subtile endringer i CIR og NIR fra baseline til tre måneder. Vi observerte signifikante forskjeller mellom menn og kvinner i serum og urin NIR, men ikke i CIR. CIR i serum og urin og serum NIR sank fra baseline til tre måneder hos kvinner mens de økte hos menn. Urin

NIR økte hos begge kjønn. Etter tre måneder korrelerte serum-CIR med endringer i vekt, midjemål og fettfri masse, mens urin-CIR korrelerte med fettmasse hos alle deltakerne og urin-NIR med midjemål-endring. CIR hadde en svak, men signifikant sammenheng med vekttap og fettmasse hos kvinner og med midjemål hos begge kjønn. Urin NIR korrelerte signifikant med endringer i vekt og midjeomkrets hos kvinner og endringer i midjeomkrets, fettmasse og fettfri masse hos menn. Assosiasjoner med urin-NIR ble kun funnet hos menn med midjeomkrets og fettmasse. Vi observerte også assosiasjoner mellom CIR og NIR med diettinntak av karbohydrater, animalsk og marint protein.

Konklusjon CIR og NIR etter vekttap var ikke signifikant forskjellig fra baseline hos overvektige, ellers friske deltakere. Etter tre måneder var assosiasjonene til endringer i vekt, fettmasse eller fettfri masse bare svake. Endringene og assosiasjonene ser ut til å være kjønnsavhengige, også for kostinntaket.

Videre er det uklart om fedme er assosiert med stabile isotop forhold.

Selv om dette var en utforskende studie og mer forskning bør gjøres før sikre konklusjoner trekkes, kan egnetheten til CIR og NIR som biomarkører for vekttap hos overvektige personer være begrenset. Ytterligere forskning er nødvendig.

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1 Introduction

1.2 Intended and unindented weight loss

Since 1975 the global prevalence of obesity has tripled. The World Health Organization estimates that 2.8 million people die each year due to overweight or obesity (1). The increasing incidence of obesity has started a renewed focus on a healthy lifestyle and weight-loss strategy as the primary prevention and treatment measure for overweight or obesity. It is proven that even moderate weight loss of 5% body mass in overweight or obese people can delay diabetes or prevent progression from prediabetic to diabetes type 2 (2, 3), reduce hypertension, dyslipidemia, and reduce the risk for cardiovascular diseases (4, 5).

At the same time, unintended weight loss is a common clinical problem, especially in elderly or cancer patients and overweight/obese patients (6). Unintentional weight loss can result from disease, cachexia, or a poor diet. Many diseases lead to metabolic, hormonal, and dietary changes, also due to medication which may also affect appetite, leading to weight loss (7, 8). Unintentional weight loss is associated with increased risk of morbidity and mortality, in-hospital complications, higher admissions rates to institutions, and poorer quality of life (6, 9). In obese patients, unintended weight loss can be harder to detect and be at risk of not being taken seriously (10)

Whether weight loss is intended or unintended, weight loss requires a negative energy balance by reduced energy intake, increased energy expenditure, or both. Thus, the body will then use its energy stores, primarily adipose tissue but also fat-free mass (FFM) (11, 12). Weight loss in obesity aims to reduce the adipose tissue and have the least possible decrease in FFM. However, all weight loss will also lead to FFM loss, whether intended or not. FFM loss may account for up to a quarter of total body weight lost (13). Unintentional weight loss is associated with an even more significant proportional loss of FFM (14). Maintaining FFM is essential as reduced muscle mass increases the risk for osteoporosis and sarcopenia, which are associated with decreased physical function and increased risk of injury and mortality (15, 16).

For overweight or obese people, the loss of FFM can impede further weight loss. This could be due to a down-regulation of metabolic processes, such as protein turnover and basal metabolic rate (13, 17, 18). With weight loss, the oxidation from fatty acids to gain ATP will decline, and this lowering of fat oxidation limits the ability for sustained intentional physical activity. A weight loss of 10kg will reduce fat oxidation by 20g or 180kcal/day (19, 20). Skeletal muscle performance will also change with weight loss, and the skeletal muscle efficiency will increase; thus, fewer calories per unit of work will be required (19, 21, 22). It is thought that this change in muscle efficiency stands for 35% of the change in non-resting daily energy expenditure (22).

The average energy expenditure in males and females will decrease by 21kcal/day and 15kcal/day for each 1kg loss, respectively (11). This reduction will gradually diminish the energy imbalance and stabilize the weight (11, 12, 23). Therefore, weight loss will always be most prominent at the start of a weight-loss period and then normally plateau after approximately six months (24).

1.1.2 Effect of different diets on weight loss low carbohydrate vs. low-fat diet effect on weight loss

Today, there are many popular weight-loss diets. Low-carbohydrate and low-fat diets are two diets that may be most popular and have gotten a lot of attention in the media and by scientists (25-28). There are many different variants of low-carbohydrate and low-fat diets, differing in types and amounts of nutrients or foods. It is typical for a low-carbohydrate diet to contain less carbohydrate than recommended in the macronutrient distribution of 45-60% (29) and that the diet is high in fat and protein (30). A low-fat diet typically contains <30% energy from fat and is higher in carbohydrates and protein.

Both diets have significantly reduced weight and improved metabolic risk factors (27). However, most participants in weight loss trials do not achieve more than 5-10% weight loss and, depending on the starting weight, do not achieve normal weight status. Some studies found that low-carbohydrate diets result in more significant weight loss than low-fat diets, despite similar energy intake (31-33). A meta-analysis done by Johnston in 2014 compared 48 random controlled trials (RCTs) and found that both diets were associated with similar weight loss (25).

Few studies regarding low-carbohydrate vs. low-fat diets researched different effects on body composition. One study has observed a higher body fat loss with low-carbohydrate than low-fat diets (34). This can be related to changes in insulin concentrations, as lower insulin

secretion and serum concentrations promote the mobilization of fatty acids from body fat (34, 35). Also, a greater loss in FFM was observed in low-carbohydrate diets than in low-fat diets (30); this has also been observed in two other studies (31, 36). This can be due to additional body water loss, as ketosis may cause water extraction (37) or lower insulin concentrations since insulin inhibits proteolysis (30, 38).

Today, there is no consensus on if one of the two diets has a greater effect on weight loss. Important to remember is that these studies are often also limited in comparison because there is no clear definition of the two diets and their macronutrient content, and there can be differences in overall energy intake during the study time.

1.1.3 Weight loss assessment

In clinical practice weight loss is expressed as percentage weight loss:

((Usual or previous weight (kg) – Actual weight (kg))/ Usual or previous weight (kg)) x 100 (39, p.49).

Typically, weight loss is estimated and followed up with numerical weight changes, and body mass index (BMI) is assessed. BMI is often evaluated to know if a person has crossed a BMI category, for example, from average weight (18.5-24.9kg/m^2) to underweight (>18.5kg/m^2) or obese (>30kg/m^2) to overweight (25.0-29.9kg/m^2). The disadvantages of numerical weight changes and BMI are that it does not provide information about body composition (15) and that the starting weight is often difficult to assess if no earlier record of weight exists.

While weighing is usually not an issue in weight loss studies with obese participants, it can be challenging in disease-related weight loss. Even though there are many different weight options, such as chair weight, lift weight, and bed weight, these options are not available for everyone (private homes, various institutions) or are too time-consuming for some institutions, e.g., bedridden patients. Alternatively, weight can be estimated either by self-report or others (39, p.49). A proxy for weight loss can also be estimated by looking at how clothes fit or clothing size changes. However, this does not often consider the time frame of weight loss and amount of weight loss, which are the two key factors when assessing weight loss in clinical practice.

Assessing body composition is essential in evaluating weight and weight loss, as risk factors associated with weight gain/loss and health benefits are related to changes in fat mass (FM) and FFM (15, 40). For the measurement of body composition, there are several complex methods available, including bioelectrical impedance analysis (BIA), dual-energy X-ray absorptiometry (DXA), body density, and total body water estimates, and all of these predict body composition from body properties (41).

All these methods have advantages and disadvantages, including radiation in DXA, availability in clinical practice, or feasibility (under water weighing). Due to its practicability and non-invasiveness, BIA has become the method of choice in many studies. BIA measures the body's resistance to a small electric current and estimates total body water, FM, and FFM. The estimated total body water is used to calculate FFM, and then from the bodyweight difference, FM is estimated (41, 42). BIA results can be influenced by hydration state, prediction equation, electrode placement, and device (43). All the multiple prediction

equations for BIA are population-specific, making it challenging to apply these on an individual level (41, 42). However, several BIA equations have also been developed for use in obesity (44). This limits its clinical application, and the value of BIA is primarily epidemiological, as it is the only predictive method for estimating FFM (41).

The difficulties associated with the determination of weight loss and the tissue-specific weight loss could be solved if there was a biomarker that mirrors the amount and the type of weight loss and that could be measured or estimated in body tissues or fluids.

The current thesis investigates whether stable isotope ratios (SIR) of carbon or nitrogen could be such biomarkers for weight loss. This could possibly give information about body composition but maybe also be a faster and easier way in clinical practice to assess weight.

1.2 Stable isotope ratio

An isotope is an atom of the same element but differs in the number of neutrons in the nucleus, therefore also in the atomic mass (45). The term «isotope» means same («iso») place («topos») and refers to that isotopes of the same element occupy the same position in the periodic table (46). There are radioactive and stable isotopes, and the stable isotopes do not undergo radioactive decay (45). By convention, isotopes with a lower atomic mass are termed «light,» and isotopes with higher atomic mass are termed «heavy.» There are multiple naturally occurring isotopes for most elements, and it is common for one isotope to be more abundant than others. In nature, each element always appears as a mixture of its isotopic form. The lightest isotope is the most abundant for stable isotopes of the common elements in biological molecules (Table 1). The SIR refers to the ratio of atomic abundance of two stable isotopes of the same element. Animal ecology, geochemistry, and archaeology use SIRs as a widely used, well-established tool. (47, 48).

Table 1: Stable isotopes of common atoms in biological molecules (45)

Element	Stable Isotope	Abundance (%)*
Hydrogen	^1H (H)	99.985
	^1H(D)	0.015
Carbon	^{12}C	98.892
	^{13}C	1.108
Nitrogen	^{14}N	99.635
	^{15}N	0.365
Oxygen	^{16}O	99.759
	^{17}O	0.037
	^{18}O	0.204
Sulphur	^{32}S	95.0
	^{33}S	0.75
	^{34}S	4.21
	^{35}S	0.014

∗ *Abundance does not always add to 100% because radioactiv isotopes are left out of this table*

1.2.1 Delta value and standards

Stable isotopes of the same element may have different reaction rates because of their atomic mass difference. These reaction rate differences can cause isotopic fractionation in the environment and are manifested by the difference in the ratio of heavy to light isotopes. Therefore the SIR differs naturally in, for example, plants or foods. These natural variations in the SIRs usually occur at the fourth, fifth, or sixth decimal place (49). Thus, measurement accuracy and reporting are most important, and SIRs are reported in units of relative abundance rather than as absolute ratios (45). SIRs are reported as delta (δ) values and expressed in units of permil (‰) (50). This is because the difference in SIRs at natural abundance is slight and makes the scale more tractable (45).

$$\delta X = ((R_{sample} - R_{Standard})/R_{standard}) \times 1,000‰ \qquad (46)$$

Delta values are reported relative to universal international standards defined by the International Atomic Energy Agency (Table 2) (51). These standards are usually in geological materials; for example, the standard for carbon is limestone. Other standard materials used are water and air for hydrogen and nitrogen reference standards. If the stable isotope has a lower abundance in the studied material, the delta values will always be negative. An example of this is $\delta^{13}C$ from living organisms (45).

Table 2: International standards of stable isotope ratios (51)

Element	International Standard	Isotope ratio of the standard
Hydrogen	Vienna Standard Mean Ocean	$^2H{:}^1H = 0.00015576$
Carbon	Vienna Pee-Dee Belemnite	$^{13}C{:}^{12}C = 0.0112372$
Nitrogen	Atmospheric N_2 (Air)	$^{15}N{:}^{14}N = 0.0036765$
Oxygen	Vienna Standard Mean Ocean	$^{18}O{:}^{16}O = 0.0020052$
Sulphur	Vienna Canon Diablo Troilite	$^{34}S{:}^{32}S = 0.0450045$

1.2.2 Measuring stable isotope ratios

Isotope ratio mass spectrometry (IRMS) is used for measuring naturally occurring differences in SIR (50). There are two types of IRMS, dual intel, and continuous flow. Dual intel-IRMS is a measurement of SIRs from pure gases, where a sample gas and standard gas are alternately introduced into an IRMS with the help of a system of valves. With continuous flow, it is a sample preparation device, samples are converted to gases, and mass spectrometer in one where analysis is conducted in a continuous flow of helium carrier gas. An elemental analyzer (EA) is the most commonly used for converting solid samples to gases in dietary analysis. EA combusts solid organic samples into Carbon Dioxide (CO_2), nitrogen gas, water, and Sulphur Dioxide. Water is then removed, gases are carried in a stream of helium through a separation column, and distinct peaks for each gas are generated. The gas enters the IRMS at the ion source and is then bombarded with high-energy electrons. Electrons from the molecules are dislodged because of the collisions and convert the molecules into positively charged ions. These ions are then aimed down a curved flight tube, and a magnetic field will bend their flight path to a degree that depends on atomic mass. Molecules with a higher mass will bend less than molecules with a lower. This leads to striking different ion detectors at the end of the flight tube (45).

1.2.3 Metabolic isotope effects

The SIR of carbon and nitrogen in biological tissue is primarily determined by the SIR of the dietary source and physiological and metabolic processes (52). This is termed consumer tissue-diet discrimination (53). The carbon consumer tissue-diet discrimination is typically about 0-1% (54). Lipids are generally ^{13}C depleted; ^{13}C values of lipids are 5-7‰ lower than tissue protein or carbohydrate because of enzymatic preference for ^{12}C early in lipid synthesis (45). Decarboxylation discriminates between ^{12}C and ^{13}C; therefore, evolved CO_2 is ^{13}C depleted (55). Amino acid (AA) composition also affects the tissue's CIR. The CIR of essential AA tracks only dietary protein. The CIR of non-essential AA is a mix that reflects the CIR of dietary protein and non-essential AA synthesized using carbon from sources such as carbohydrates or fat (56).

It is estimated that the consumer tissue-diet discrimination of NIR is about 3-4%, and ecologists have long used this difference to assess the trophic positions of animals (57). The reason that ^{15}N is higher is likely a result of a strong preference for ^{14}N in the process of excreting waste nitrogen (52, 55). The preference could be due to fractionation during

transamination and deamination of AA (58). Because of this, body proteins are more enriched with ^{15}N than exogenous dietary proteins (55).

1.3 Stable isotope ratio as a biomarker

In ecology, CIR is used to reflect the dietary variation of carbon sources as indicated by the variation of CIR (59, 60). NIR is in biology used to characterize and determine the trophic position in the food web (61, 62). In archeology, SIRs are used to describe ancient diets extensively (59). This use of SIRs in ecology, biology, and archaeology has led to an interest in SIR as a dietary biomarker. Today their use in dietary assessment studies is limited but growing. There are also interests in investigating SIR and its association with nutritional status and human diseases.

An ample reason for the interest in SIR as a dietary biomarker is the need for an objective assessment of dietary intake, especially when the association of diet with non-communicable diseases is investigated (63-66). Current methods for reporting dietary intake are primarily subjective because they involve self-report and rely strongly on memory and are thus influenced by cognition, memory, and social desirability. People may also lack consciousness of food eaten and lack of ability to estimate portion size or amount eaten (67, p.17). Therefore, are diet assessment measures subject to random and systematic error and elective bias and can be burdensome to administer (65, 66, 68-70).

1.3.1 Advantages and disadvantages of SIR as a biomarker

An advantage of using SIR as a biomarker is that the measure of interest is at the atomic level and not the molecular level. Therefore, SIRs are not affected by dramatic alterations of the molecular structure, such as autoclaving or short-term cryostorage. The SIRs of a sample cannot change unless atoms of the elements of interest have been added or lost. Blood additives have been thought to alter SIRs, but the effect is most likely minimal. Some studies have shown that ethylenediaminetetraacectic acid, polymerized acrylamide resin, or sodium fluoride additives do not affect the CIR and NIR of blood samples (45, 71, 72).

The most significant disadvantage of using SIR as a dietary biomarker is the influence of confounding factors. One crucial confounding factor is substantial differences in diet composition between countries; therefore, delta values are not always comparable (73). This implies that relative values are preferable to absolute delta values (55). One other important factor is that cohorts must be formed with care since isotopic differences can also be caused by common medical treatments, local nutritional habits, and sex (72). Further complicating

the use as a biomarker is that SIRs differ depending on which tissue or body fluid is used, this has been investigated for NIR in detail by Huelsemann and is presented in Figure 1.1. The reason for the differences is that different biological specimens may reflect different periods or varying nutrient turnover and different diet-tissue discrimination.
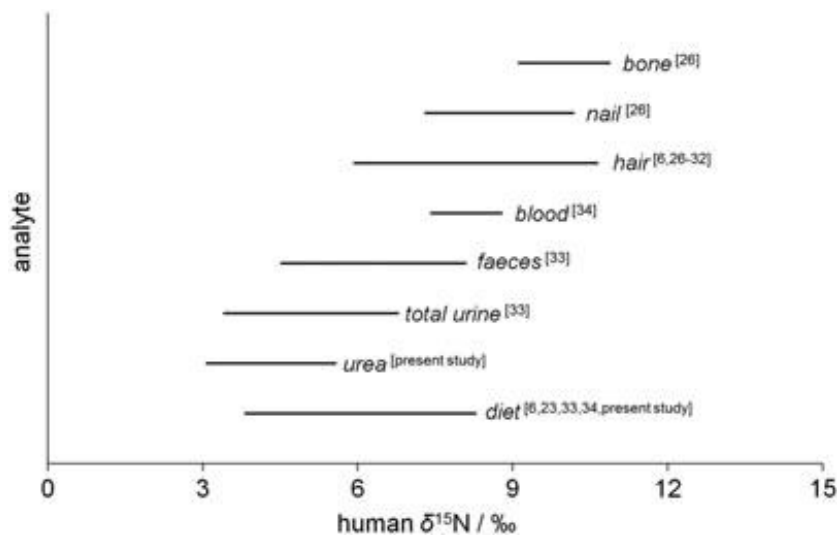
Figure 1 Delta 15N in different human body tissues/fluid. *The figure is taken from reference (74)*

1.3.2 Stable isotope ratio variations in plants and animals

When carbon is transferred from one part of the ecosystem to another, isotope fraction occurs (75). All carbon in plants and animals is derived from atmospheric CO_2 (45). From atmospheric CO_2, carbon is incorporated into vegetation during photosynthesis. The photosynthesis process prefers the lighter isotope, and ^{12}C is more absorbed than ^{13}C. This preference is due to the binding and fixing enzyme Rubisco. There are three main photosynthesis pathways. The C_3 pathway results in an intermediate product that is a molecule with three carbon atoms (75). The plants with this photosynthesis pathway are called C3 plants, wheat, rice, beans, and most fruits and vegetables (45, 76). The second photosynthesis pathway is the C_4 pathway, leading to a four-carbon molecule (75). The plants of this pathway are called C4 plants; the main representatives are corn, sugar cane, and sorghum (45, 76). These two pathways are different because C4 plants have different photosynthetic physiology. C4 plants are native to a dryer environment and have evolved a mechanism that reduces water loss. This has diminished Rubisco's ability to discriminate against ^{13}C (45, 77). The marine pathway of photosynthesis derives carbon from bicarbonates, which are richer in ^{13}C because of their preference in the conversion of atmospheric CO_2 to

bicarbonate (45, 78). Marine plants have the highest value of CIR, with -17‰ to -20‰ (45, 68). C4 plants have CIR values 12-13‰ higher than C3 plants (45, 76, 79).

NIR is of interest as a potential biomarker for protein sources and seafood intake. Tissue nitrogen primarily derives from dietary protein, and in animals, NIR is elevated over NIR from plants (45). Because of the preference for the lightest isotope in generating and excreting waste nitrogen, the NIR values tend to be 3‰-4‰ higher than diet (45, 61, 80). Seafood's NIR values (10‰-20‰) are elevated over plants and other terrestrial animals (45, 81). This increase in NIR happens at each step of the food web (45).

1.3.3 Human studies on SIRs

Like other animals, humans will incorporate the carbon atoms from their diet into their tissue. Studies have shown that animal CIR values reflect their diet (61), with an average increase of 1‰ of tissue ^{13}C compared to the feed (80). Studies from the USA have investigated whether CIR could potentially be used as a biomarker for added sugar intake (ASI) as it is elevated in corn and sugar cane, making 70% of caloric sweeteners and meat from corn-fed livestock (69, 82). Several studies have found an association between CIR, ASI, or red meat/animal protein (63, 68, 69, 83, 84). Important to note is that most of these studies used only women as a study subject. A major limitation of CIR as a biomarker for ASI is that red meat and animal protein also correlate with CIR (63, 68, 69, 83, 85), and corn-based foods can confound the associations (63).

Multiple studies have researched NIR as a biomarker for meat, animal protein, seafood, and fish intake. Cohort and controlled feeding studies from the United States (US) and Europe have found associations between NIR and fish intake (63, 68, 81, 86-88). In addition, several studies have found an association between NIR, meat, and animal protein intake (85-87, 89).

Although limited, some studies have looked at CIR and NIR in human diets, such as vegan, vegetarian, and omnivores. These studies have been limited because of the use of different body tissues such as fingernails, hair, blood, or serum, a small number of vegans and vegetarians within the studies, and adherence to diet has been poorly defined in most studies. However, these studies confirmed that CIR and NIR were different in the three diets and that typically CIR and NIR were lowest in vegans and highest in people with an omnivore's diet (75, 86, 90-92).

Several studies today show evidence that pathologies directly related to metabolism, such as nutritional stress, malnutrition, diabetes, obesity, and metabolic syndrome, have an impact on SIR (93-98). A study in children with chronic malnutrition or stunting from Bangladesh found that the children's hairs were both CIR and NIR depleted. This could be because the diet was poor in nutritional value, and thereby the diet contributed little CIR and NIR, leading to hair being depleted of CIR and NIR (95). Another study has found an association between CIR increase in hair with an increase in BMI; this can be because of the utilization of diet proteins (93). In patients with metabolic syndrome, it was shown that NIR was higher with an increased number of components of metabolic syndrome. The same study showed a lower CIR when glycemia increased, or HDL cholesterol concentration was low (96). In adolescents and children, it has been found that CIR in a fingerstick blood sample or erythrocytes increases at high sugar intake or high calory diet. This seemed unrelated to HbA1c or C4 plants sugar consumption (97, 98).

In pre-clinical studies with rats subjected to caloric restriction, normal or high-fat diet regimes, caloric restriction causes a general decline in peripheric protein content. Still, the impact on isotope compositions varies between organs (99). Liver and plasma proteins were ^{15}N-enriched, while skeletal muscles were ^{15}N-depleted. In addition, the δ ^{13}C value in proteins decreased in all tissues showing a general CIR depletion (55, 100, 101).

To our knowledge, there are very limited to no studies about SIR and weight changes in humans. Studies in animals have shown changes in SIR due to nutritional stress. One human study by Fuller has investigated SIR changes due to nutritional stress during pregnancy. The study found no differences in hair δ^{13}C, but hair NIR increased. This finding can indicate that NIR is not only influenced by dietary habits but also by the nitrogen balance in the body. This study is very limited, as it was conducted in pregnant women with hyperemesis gravidarum and very few participants (94).

2 Hypothesis and aim

2.1 Hypothesis

We hypothesize that CIR and NIR are biomarkers of recent weight loss:

During weight loss, adipose tissue will be used as a source of energy, and stored fatty acids will become available. As these are depleted of ^{13}C, the CIR in serum and urine should decrease.

NIR appears to be influenced by the nitrogen balance, therefore, will a catabolic state increase NIR values. This is because weight loss leads to changes in AA homeostasis, the liver oxidizes more AA, and this process discriminates against ^{15}N. This leads to that AA left behind for protein synthesis are ^{15}N-enriched.

2.2 Aims

The overall aim is to investigate whether CIR and NIR are suitable biomarkers for recent weight loss. To date, there is no biomarker for weight loss available. Weight loss is intended for obese persons, but we do not have biomarkers that indicate prior weight loss or can indicate the type of tissue lost. As CIR and NIR reflect different metabolic pathways, these may be useful indicators of weight loss. Therefore, we want to measure NIR and CIR in serum and urine of obese people before and after weight loss. We also want to investigate whether CIR and NIR in serum or spot urine are associated with body composition or measures of obesity in obese, otherwise healthy adults.

3 Method

3.1 Study Design And Population

This is a secondary analysis of the CarbFunc study. This study uses blood and urine samples from CarbFunc to investigate a possible association between weight loss and CIR and NIR. Participants were recruited in Bergen, Norway, and 192 male and female subjects with abdominal obesity were included in the RCT. The recruitment process is described elsewhere (102).

These participants were randomized into these isocaloric dietary groups differing in carbohydrate content and quality; 1) a low-fat high-carbohydrate diet primarily based on acellular carbohydrate sources, 2) a very-high-fat low-carbohydrate diet, 3) a low-fat high-carbohydrate diet based on cellular carbohydrate sources. The diets were isocaloric and provided 2500 kcal for men and 2000 kcal/d for women. Protein content was 17 E% for all diets. The study is registered at clinicaltrials.gov with the identifier NCT03401970. As results on body weight loss were similar for the three diets, this exploratory analysis is for all participants and not groupwise.

3.1.1 Inclusion and exclusion criteria

Inclusion criteria were age (20–55 years) and < 5% change in body weight within the last two months. Exclusion criteria included smoking, known food allergies, alcohol consumption of > 2 alcohol units per day, recent surgical or antibiotics treatment during the past two months, statins and diabetes medication, severe diseases, chronic inflammatory bowel disease, pregnancy, breastfeeding, and post-menopause. Only participants who attended the three-month study visit were included in this secondary analysis.
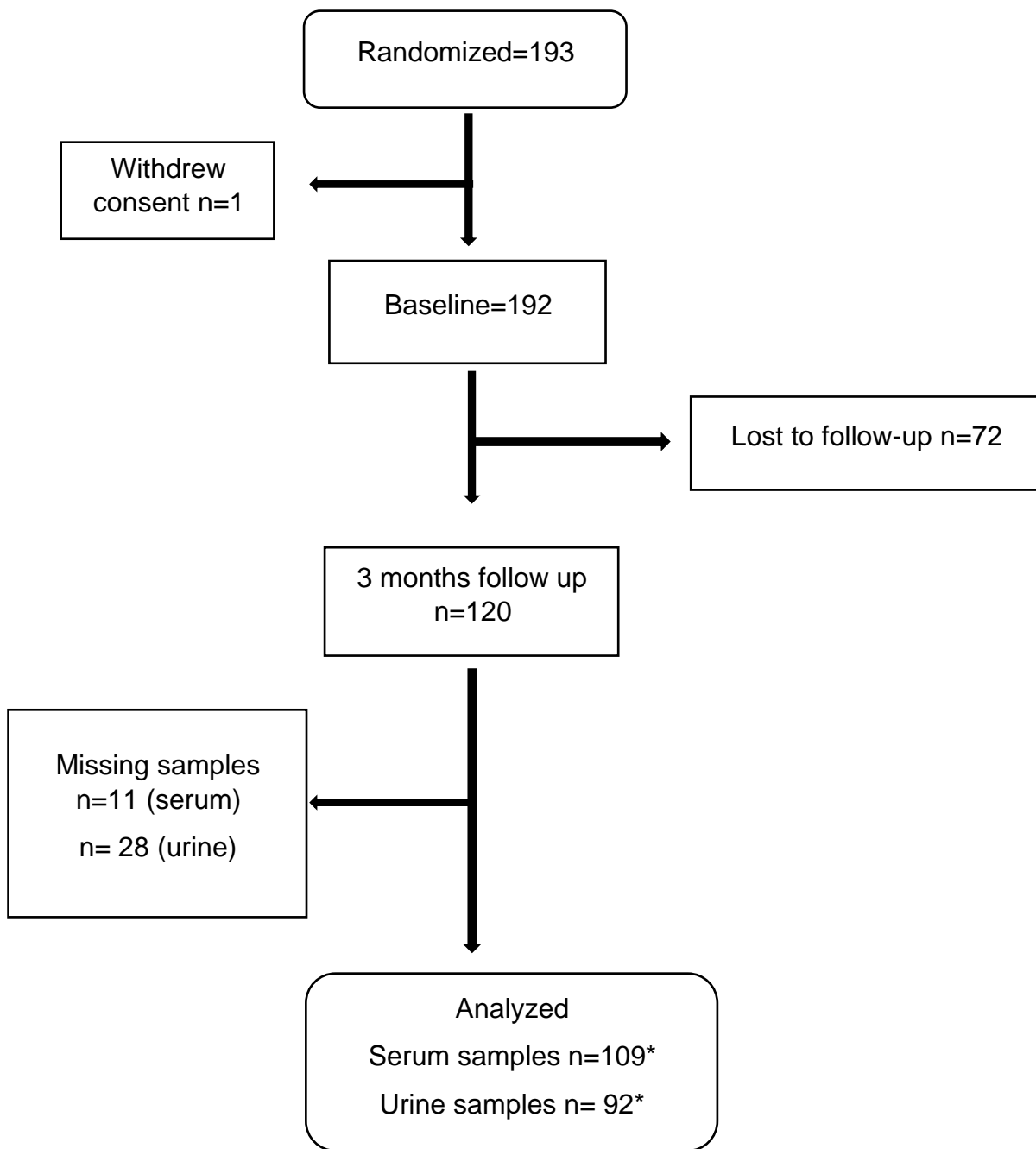
Figure 2 Inclusion and exclusion flow chart over participants included in the SIR analysis.

3.2 Dietary intake data

The participants conducted a six-day weighed food record at baseline and regular intervals during the study. These six days included four weekdays and two weekend days. In most cases, 5 of 6 or 6 of 6 days were consecutive days. Before starting food recording, the study participants received training in using the dietary recording system (www.diett.no; operated by Dietika AS, Slemmestad, Norway) and a personal user ID for the system for submitting daily food consumption. In addition, the participants were handed out kitchen scales. At the baseline of dietary data collection, the participants were asked not to change their diet in any form. Participants were asked to record the consumption of food and beverages, including all meals and snacks, the weight and amount of all consumed ingredients and products, the time of intake, and any additional comments on their dietary intake.

Nutritional intake from the dietary recording data was primarily calculated based on the latest update of the official Norwegian Food Composition Table or the nutrient declarations provided by the producer/retailer. When Norwegian data were unavailable, values from international databases were used (Danish or US food composition tables, three and eight food items, respectively). Added sugar was used as provided by the Norwegian Food composition table (Matvaretabellen.no). Added sugar was defined by the Norwegian Food composition table as " Refined or industrially processed sugar in the form of glucose, fructose, lactose, maltose, sucrose, glucose syrup and other hydrolysed starch products as well as honey that is added during industrial production or during home preparation" (103)

After baseline, the participants selected and planned meals according to their given dietary group, with the application of a booklet with recipes designed for the study. Further, the participants were instructed to record their food intake for three days every 14 days, including any deviations from the assigned diets.

We calculated the source of protein as animal, plant, marine, and unspecified protein. Animal sources included meat, processed meat, egg, and dairy, plants included cereals, bread, legumes, nuts, and other plant foods, marine included fish and other seafood, and unspecified included composite foods (pizza, cakes, etc.).

3.3 Anthropometric measurements

Anthropometric measurements from the CarbFunc study used in this thesis are weight, height, calculated BMI, waist circumference (WC), and calculated body composition by BIA. All the anthropometric measurements were measured at baseline and repeated at three, six, nine, 12, and 24 months. This thesis uses only data from baseline and after three months.

Weight (kg) and height (m) were measured by a Class III approved calibrated scale and measured to the nearest 0.1 kg and a portable stadiometer (Seca, Hamburg, Germany) in the upright position with Frankfort plane horizontal. BMI was calculated by dividing weight in kilograms with square height in meters (kg/m^2).

WC (cm) was measured by using a non-elastic measuring tape. The tape was placed horizontally around the waist at the midpoint between the bottom of the ribs and the top of the hip bone. When the participant breathed out, the measurement was read. WC was measured three times, and the mean of the last two measurements was recorded.

A segmental multifrequency BIA (Seca mBCA 514, Seca GmbH & Co. KG, Hamburg, Germany) was used to measure resistance and reactance in the body. The measurements were conducted after the manufacturer's instructions. The participant stood upright and barefoot on the integrated electrodes and the hands holding the same pair of hand electrodes with space between the middle and ring finger on both sides (44, p.33). Resistance and reactance were used to calculate FM and FFM with a validated formula provided by the device (44).

3.4 Biochemical measurements

Blood and urine samples were taken at every study visit. A peripheral venous catheter was inserted following the standard procedure for the blood samples, and blood samples were taken at baseline (0min) and then at 30min, 60min, 90min, 120min, and 240min for a postprandial test (which is not relevant here). In this study, we only used the baseline sample. 5ml was taken for serum samples and mixed eight times in the serum gel tube. Samples were centrifuged at x g after 30 min clotting time and frozen at -80 degrees.

Participants delivered urine samples of around 30ml of morning urine on the same day as the study visit. After providing the urine samples, they were marked and sent to the laboratory for pipetting. Each vial contained 0.6 ml and was then frozen at -80°C.

Serum glucose and lipids were measured at the central laboratory of the Haukeland University Hospital. Serum glucose was measured by a photometric method on an

autoanalyzer. Serum lipids were measured using standard methods on a Cobas c702 autoanalyzer (Roche Diagnostics, Mannheim, Germany). Serum triacylglycerides and total cholesterol were measured with an enzymatic colorimetric method, respectively. LDL cholesterol was measured photometrically, and HDL cholesterol was measured by a homogeneous enzymatic colorimetric method.

3.5 CIR and NIR analyses

SIRs of carbon and nitrogen were measured at the Stable Isotope Laboratory at the University of Oslo (UIO: CLIPT). Stable isotope analysis of carbon and nitrogen has been done as previously described (104). Briefly, 8μL Serum and 15μL urine were pipetted into tin capsules and air-dried. EA IRMS system, consisting of a flash EA and a DeltaV IRMS (Thermo Scientific, Germany), was used to measure CIR and NIR simultaneously. The CIR and NIR values were normalized to the Vienna Pee Dee Belemnite (VPDB) and AIR scales, respectively, using two different internal reference materials incorporated into each analytical run: JGLUT (L-glutamic acid; CIR = -13.43 ‰; NIR-4.34‰) and POPPGLY (glycine; CIR= -36.58 ‰; NIR 11.25 ‰) (both from Fisher Scientific). An additional quality control material, JALA (alanine, CIR= -20.62 ‰; NIR–3.20 ‰) (Fisher Scientific), was incorporated into every run. CIR of both reference materials and quality control sample were calibrated to the VPDB scale using LSVEC (lithium carbonate, CIR = -46.6 ‰) and NBS-19 (calcium carbonate, CIR = 1.95‰) (both obtained from the International Atomic Energy Agency, Austria). The NIR values were calibrated to the AIR scale using USGS40 (L-glutamic acid, NIR -4.52‰) and USGS41 (L-glutamic acid, NIR = 47.57‰) (both obtained from the United States Geological Survey). Analytical precision was based on repeated analyses of quality assurance material JALA (Fisher Scientific).

3.6 Data analysis

IBM SPSS Statistics Software version 27 was used for all data analysis conducted. Descriptive statistics were used to describe participants' characteristics at baseline and three months for the entire cohort, sex, and the three diet groups.

Before any other statistical analyses were conducted, different variables were calculated. Weight change, WC change, FM change, and FFM change were calculated by subtracting the 3month value from the baseline value (thus, positive values mean a reduction!). To calculate participants' animal protein ratio (APR), total animal protein intake per gram per day (g/d) was divided by total protein intake g/d. The animal and unspecific protein ratio (AP+USPR) was calculated by adding animal and unspecific protein g/d together and was then divided by total protein intake (g/d). For the animal and marine protein ratio (AMPR), the same calculation as AP+USPR was done.

Descriptive data are presented as median with interquartile range. Spearman correlation was used to investigate if there were correlations between CIR/NIR (baseline or three months) and anthropometric variables at baseline or three months and between CIR or NIR at three months with weight changes or body composition changes. This was done in the entire cohort and for both sexes, respectively.

The normal distribution of the variables of interest (CIR, NIR) was tested with the Shapiro Wilk test. The assumption of normal distribution was violated for urine CIR (U-CIR) at three months, serum, and urine nitrogen at baseline. No violation of the assumption of normal distribution of these variables was found for the other variables of interest. (Table S1 in appendix).

Before linear regression, the data set was split by sex. Linear regression investigated the association between the response variable and explanatory variables. The linear regression was done in five different models, starting with the unadjusted model, including only the explanatory variable (weight change, WC, FM, or FFM). The following models 2, 3, and 4 contained each one additional variable: BMI, Carbohydrate E%, and AMPR separately. The final model 5 contained BMI, Carbohydrate E%, and AMPR.

4 Results

4.1 Descriptive statistics

In total, 120 participants from the CarbFunc study attended the three month visit and could have been included in this exploratory study. Of these, 109 serum samples and 92 urine samples were analyzed for CIR and NIR. 94 participants were recruited in 2017, and 26 were recruited in 2019. The distribution of women and men was 48% (n=58), and 51% (n=62), and the participants' distribution for the different diet groups was 28% (n=34) for high carbohydrate-low fat Acellular diet (HCLF), 44% (n=53) for low-carb and high fat diet (LCHF) and 27% (n=33) for HCLF Cellular diet. The median age was 44 years, and at baseline, the median BMI was 37 kg/m^2. Tables 3a and 3b give an overview of the basic characteristics of the included participants at baseline and after three months.

Table 3a: Descriptive data for participants at baseline, according to sex and diet group, Median (interquartile range)

	Total n=120	Female n=58	Male n=62	HCLF Acellular n=34	LCHF n=53	HCLF Cellular n=33
Age years	44 (37-48)	43 (35-47)	44 (39-49)	44 (36-49)	43 (36-48)	44 (38-48)
Female/Male	58/62			17/17	25/28	16/17
BMI kg/m²	37 (32-40)	37 (34-40)	36 (33-39)	37 (33-38)	36 (32-40)	38 (34-42)
Weight kg	110 (100-125)	106 (92-116)	117 (105-131)	108 (100-123)	107 (96-118)	122 (108-130)
WC cm	118 (109-126)	112 (104-120)	122 (114-130)	115 (106-125)	117 (110-124)	124 (112-132)
FM kg	48 (38-56)	50 (41-57)	44 (35-54)	49 (39-54)	45 (36-57)	49 (43-60)
FFM kg	63 (55-74)	55 (51-59)	73 (67-80)	66 (54-74)	62 (54-71)	69 (57-77)
S-Glucose mmol/L	5.3 (5.0-5.6)	5.0 (4.8-5.3)	5.5 (5.2-5.9)	5.3 (5.0-5.7)	5.2 (5.0-5.5)	5.3 (4.9-5.8)
S-TG mmol/L	1.3 (1.0-1.9)	1.1 (1.0-1.5)	1.6 (1.3-2.2)	1.2 (1.0-1.7)	1.3 (1.0-2.0)	1.5 (1.1-1.8)
S-TC mmol/L	5.0 (4.3-5.5)	4.9 (4.1-5.4)	5.0 (4.6-5.8)	4.9 (3.8-5.2)	5.0 (4.3-5.7)	5.0 (4.6-5.9)
S-HDL mmol/L	1.2 (1.0-1.3)	1.3 (1.1-5.1)	1.1 (1.0-1.2)	1.1 (1.0-1.3)	1.2 (1.0-1.4)	1.2 (1.0-1.4)
s-LDL mmol/L	3.3 (2.7-3.7)	3.1 (2.6-3.6)	3.4 (2.9-3.9)	3.3 (2.7-3.6)	3.3 (2.7-4.0)	3.3 (2.9-3.7)

Note: Measurements of 2 participants were missing for FM. Measurements of 3 participants for FFM were missing. Abbreviations; BMI=Body Mass Index, FM=Fat Mass, FFM=Fat Free Mass, HCLF=High Carbohydrate-Low Fat diet, kg/m²=kilogram divided by height in meters, LCHF=Low Carbohydrate-High Fat Diet, S-Glucose=Serum Glucose, S-TG Serum Triglyceride, S-TC=Serum Total Cholesterol, S-HDL=Serum HDL cholesterol, S-LDL=Serum LDL cholesterol, WC=Waist Circumference

Table 3b: Descriptive data of participants at three months, according to sex and diet group, Median (interquartile range)

	Total n=120	Female n=58	Male n=62	Acellular HCLF n=34	LCHF n=53	Cellular HCLF n=33
BMI kg/m²	34 (31-38)	35 (32-38)	34 (31-37)	35 (32-37)	33 (30-37)	36 (33-40)
Weight kg	106 (93-116)	101 (88-109)	110 (100-123)	106 (96-114)	103 (91-113)	115 (101-125)
WC cm	112 (106-121)	110 (101-117)	118 (108-125)	109 (104-121)	111 (105-117)	119 (111-126)
FM kg	42 (32-51)	46 (37-54)	38 (30-47	43 (35-50)	38 (30-49)	46 (38-55)
FFM kg	64 (54-73)	54 (50-58)	72 (67-78)	66 (55-76)	61 (53-67)	67 (56-76)
S-Glucose mmol/L	5.2 (4.9-5.6)	5.0 (4.8-5.3)	5.4 (5.1-5.8)	5.2 (4.8-5.7)	5.2 (4.9-5.6)	5.2 (4.9-5.5)
S-TG mmol/L	1.1 (1.0-1.5)	1.0 (1.0-1.3)	1.2 (0-1.5)	1.2 (1.0-1.6)	1.0 (1.0-1.3)	1.2 (1.0-1.6)
S-TC mmol/L	4.6 (4.0-5.2)	4.5 (4.0-5.1)	4.7 (4.4-5.4)	4.5 (3.8-5.2)	4.8 (4.3-5.5)	4.5 (4.0-5.1)
S-HDL mmol/L	1.1 (1.0-1.3)	1.2 (1.1-1.3)	1.1 (1.0-1.2)	1.1 (1.0-1.2)	1.2 (1.0-1.4)	1.1 (1.0-1.3)
S-LDL mmol/L	3.0 (2.7-3.5)	2.8 (2.5-3.5)	3.1 (2.8-3.7)	2.8 (2.5-3.6)	3.1 (2.7-3.7)	3.0 (2.5-3.5)

Note: Measurements of 2 participants were missing for BMI, Weight, and WC. Measurements of 13 and 14 participants were missing for FM and FFM. Abbreviations; BMI=Body Mass Index, FM=Fat Mass, FFM=Fat Free Mass, kg/m²=kilogram divided by height in meters, S-Glucose=Serum Glucose, S-TG Serum Triglyceride, S-TC=Serum Total Cholesterol, S-HDL=Serum HDL cholesterol, S-LDL=Serum LDL cholesterol, WC=Waist Circumference

For the anthropometric measurements, two and three measurements of participants were missing for FM and FFM at baseline, two measurements of participants were missing for BMI, weight, and WC, and 13 and 14 measurements were missing for FM and FFM at three months. All anthropometric measurements decreased at three months, except for FFM.

Table 4a: Dietary intake data for participants at baseline, according to sex and diet group, Median (Interquartile range)

	Total n=120	Female n=58	Male n=62	Acellular HCLF n=34	LCHF n=53	Cellular HCLF n=33
Energy kcal/d	2428 (2033-2758)	2250 (1903-2529)	2613 (2276-2966)	2420 (2100-2696)	2374 (1869-2746)	2597 (2235-2921)
Carbohydrate g/d	237 (196-282)	222 (195-264)	245 (199-294)	246 (204-299)	220 (195-270)	245 (189-294)
Carbohydrate E%	40 (35-45)	39 (36-42)	40 (35-46)	40 (35-44)	40 (36-45)	39 (33-45)
Protein g/d	104 (87-121)	93 (80-106)	116 (102-127)	101 (85-118)	102 (81-124)	110 (94-122)
Plant Protein g/d	26 (21-33)	26 (19-31)	26 (22-35)	28 (22-36)	25 (20-30)	28 (21-37)
Animal Protein g/d	56 (43-67)	47 (39-59)	62 (51-76)	56 (40-66)	50 (41-67)	61 (52-70)
Marine Protein g/d	7 (2-12)	5 (1-13)	7 (3-12)	7 (2-10)	6 (1-12)	9 (4-14)
Unspecific Protein g/d	9 (4-16)	6 (4-12)	13 (5-25)	8 (5-15)	13 (5-22)	5 (3-12)
APR %	54 (45-63)	53 (45-63)	55 (45-63)	52 (43-65)	53 (45-63)	57 (51-62)
APR+USPR %	66 (57-74)	62 (55-70)	68 (61-76)	65 (55-76)	67 (60-75)	65 (57-72)
AMPR %	62 (56-69)	62 (55-69)	62 (56-69)	63 (55-67)	60 (53-68)	64 (59-72)
Fat g/d	106 (87-129)	90 (81-121)	110 (101-131)	105 (88-121)	103 (86-25)	109 (85-129)
Fiber g/d	22 (17-26)	21 (16-24)	22 (19-27)	22 (19-27)	21 (17-25)	22 (17-27)
Added Sugar Intak g/d	29 (57-74)	30 (20-54)	29 (14-45)	28 (18-56)	27 (15-44)	31 (14-54)

Note: APR calculated by Animal protein intake / total protein intake. AP+USPR is calculated by AP+USP/ total protein intake AMPR is calculated by AP+MP/total protein intake.
Abbreviations; APR= Animal Protein Ration, APR+USPR=Animal Protein And Unspecific Protein Ratio, AMPR=Animal And Marine Protein Ratio, g/d=gram per day, HCLF= High Carbohydrate-Low Fat Diet, kcal/d=Calorie per day, LCHF= Low Carbohydrate-High Fat Diet.

Table 4b: Dietary intake data for participants at three months, according to sex and diet group, Median (Interquartile range)

	Total n=120	Female n=58	Male n=62	Acellular HCLF n=34	LCHF n=53	Cellular HCLF n=33
Energy kcal/d	2268 (2035-2588)	2049 (1960-2222)	2563 (2363-2774)	2280 (2025-2671)	2261 (2049-2597)	2207 (1935-2562)
Carbohydrate g/d	205 (62-263)	192 (53-231)	244 (67-287)	246 (216-289)	60 (50-91)	232 (196-266)
Carbohydrate E%	39 (11-44)	41 (16-44)	33 (9-44)	43 (30-45)	17 (6-41)	41 (34-44)
Protein g/d	96 (83-104)	83 (81-92)	104 (100-110)	98 (83-108)	95 (83-106)	95 (83-104)
Plant Protein g/d	23 (15-29)	21 (14-26)	25 (16-31)	30 (25-34)	21 (13-26)	17 (10-24)
Animal Protein g/d	53 (39-67)	50 (36-66)	57 (44-69)	46 (25-59)	62 (46-72)	53 (40-66)
Marine Protein g/d	2 (0-15)	2 (0-10)	2 (0-19)	0 (0-17)	0 (0-14)	9 (0-19)
Unspecific Protein g/d	12 (0-22)	7 (0-22)	16 (0-25)	19 (1-25)	7 (0-21)	5 (0-20)
APR %	53 (42-68)	57 (44-70)	52 (42-67)	42 (30-52)	59 (48-75)	57 (46-70)
APR+USPR %	70 (60-78)	71 (61-78)	70 (59-78)	63 (56-71)	75 (65-81)	72 (58-81)
AMPR %	63 (52-75)	64 (50-78)	62 (55-69)	52 (39-64)	65 (55-81)	68 (56-82)
Fat g/d	107 (87-166)	97 (82-163)	118 (102-192)	99 (81-111)	166 (143-198)	91 (83-102)
Fiber g/d	26 (17-41)	24 (15-41)	32 (18-42)	33 (24-40)	17 (14-22)	43 (31-54)
Added Sugar Intake g/d	0 (2-13)	4 (0-14)	2 (0-10)	9 (5-27)	1 (0-7)	0 (0-2)

Note: 24 nutritional intake measurements were missing. Note: APR calculated by Animal protein intake / total protein intake. AP+USPR is calculated by AP+USP/ total protein intake AMPR is calculated by AP+MP/total protein intake. Abbreviations; APR= Animal Protein Ration, APR+USPR=Animal Protein And Unspecific Protein Ratio, AMPR=Animal And Marine Protein Ratio, g/d=gram per day, HCLF= High Carbohydrate-Low Fat Diet, kcal/d=Calorie per day, LCHF= Low Carbohydrate-High Fat Diet.

Males had higher protein intake measured as gram per day (g/d) than females at baseline, mainly attributed to the higher intake of animal protein. Animal protein was the primary protein source overall, and marine protein contributed little to the overall protein intake. Fat intake in grams per day was also higher in males than females at baseline.

Measurements of dietary intake were missing from 24 participants at three months. As expected, total energy intake and intake of macronutrients were lower in females than in males at three months. Carbohydrate (g/d) decreased in females but was stable in males. Carbohydrate (E%) increased slightly in females. Although carbohydrate (g/d) was stable in males, the E % contributed from carbohydrates decreased (due to lower total energy intake). As expected, carbohydrate intake (both %E and g/d) was substantially different between the low-fat and low carbohydrate groups, with unchanged intake in the high carbohydrate groups and decreased intake in the high-fat group at three months.

The absolute protein intake (g/d) decreased from baseline to three months, mainly attributed to reduced animal protein intake. Animal protein (g/d) was still the primary source of protein (54%), and the intake of marine protein (g/d) decreased. AMPR (%) stayed stable from baseline to three months and was 64% in females and 62% in males at three months.

Fat intake (g/d) increased from baseline to three months in the high-fat group but was unchanged in the carbohydrate groups. Fiber (g/d) intake increased from baseline to three months, more in males than in females and the carbohydrate groups, while remaining low in the high-fat group. Added sugar intake (g/d) decreased substantially after three months and was higher in females than males. The acellular high carbohydrate group had, as expected, substantially a higher added sugar intake than the other groups

4.1.1 Stable isotope ratios

The stable isotope ratio of carbon was higher in serum than in urine (Figure 3). Differences between sexes did not achieve significance neither at baseline nor at three months, however, changed in different directions in women compared to men. Serum CIR (S-CIR) was higher in females than males at baseline, but the difference was not significant (p=0.213) and decreased from baseline to three months in women, while it was stable in males (p=0.614). In females, U-CIR was higher than in males at baseline and decreased in females but increased in males. U-CIR at baseline and three months did not differ significantly in sexes (p= 0.404 and 0.835, respectively).

Concerning NIR, serum NIR (S-NIR) was about double compared to urine NIR (U-NIR), and there was no significant difference between females and males in S-NIR at baseline (p=0.631) (Figure 4). In females, S-NIR decreased during three months, while S-NIR in males slightly increased. The difference between sexes in S-NIR at three months was significantly different (p=0.012). U-NIR was lower in women than in men at baseline and increased in both sexes from baseline to three months (p= 0.095 and 0.073, respectively)

We also investigated individual changes in CIR and NIR (Figure 5). There were no differences between men and women in changes of S-CIR, S-NIR (p=1.00 and p=0.423, respectively), and U-CIR and U-NIR (p=0.343 and p=0.528, respectively).
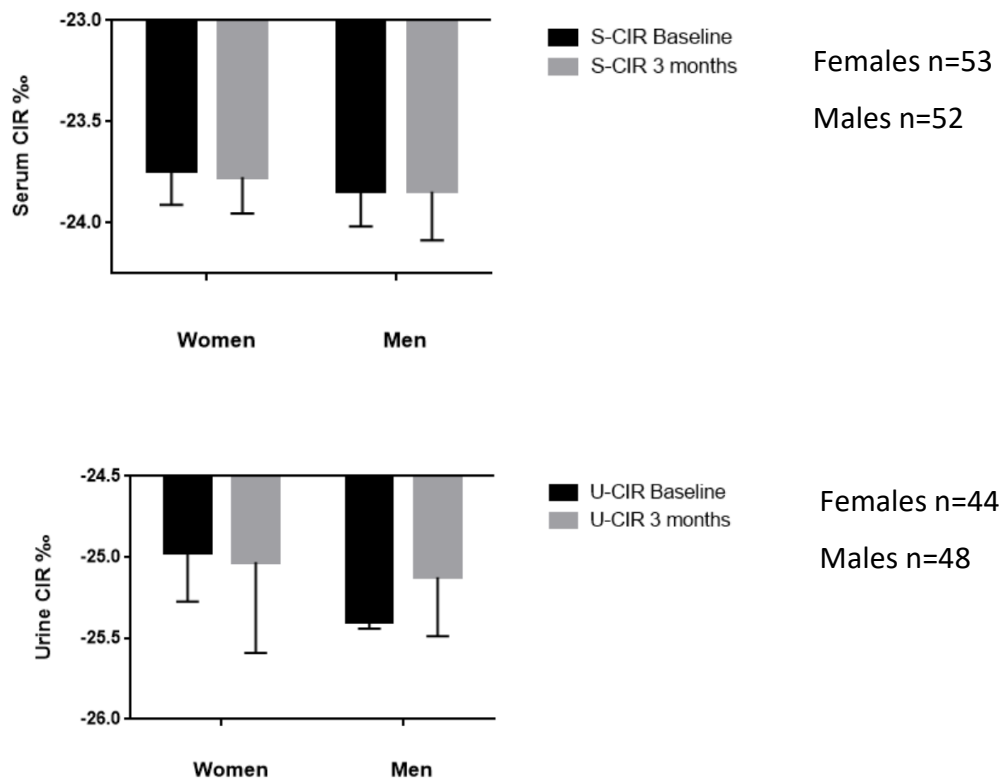
Figure 3: Median and interquartile range of serum and urine CIR at baseline vs. at three months, according to sex. Abbr; CIR= Carbon Isotope Ratio, S-CIR=Serum Carbon Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio.
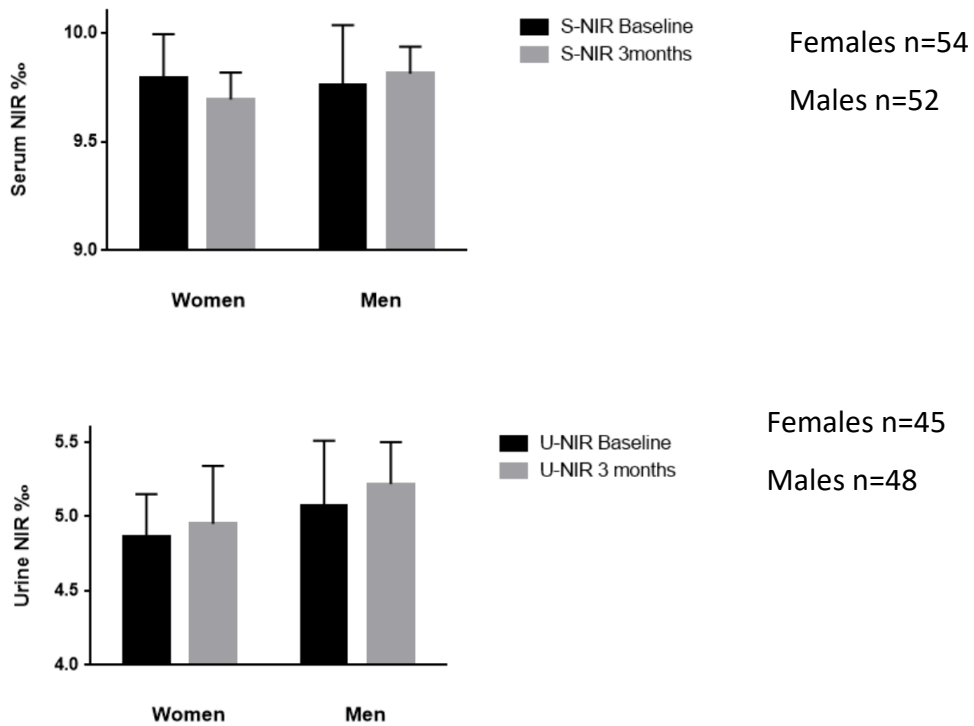
Figure 4 *Median and interquartile range of serum and urine NIR at baseline vs. three months, according to sex. Abbr; NIR= Nitrogen Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio.*
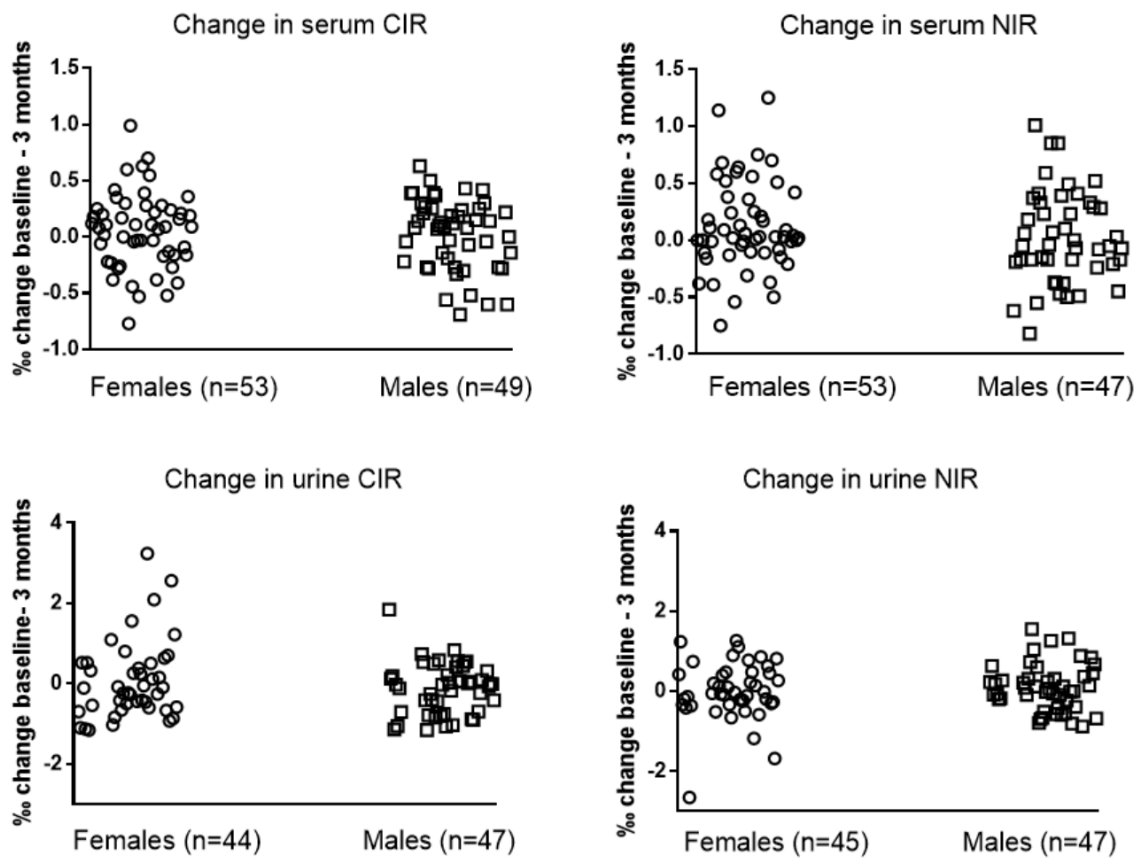
Figure 5 Changes in CIR and NIR from baseline to three months, according to sex. Abbr; CIR=Carbon Isotope Ratio, NIR= Nitrogen Isotope Ratio

4.2 Spearman Correlation

4.2.1 Baseline

Spearman Correlation was performed to calculate the correlation between anthropometric measurements and dietary intake at baseline and stable carbon and nitrogen isotope ratios. S-CIR and U-CIR correlated negatively with weight, WC, and FFM, the strongest correlation was observed with FFM. S-NIR and U-NIR did not correlate with any anthropometric measurements at baseline when all participants were included.

There were few correlations between CIR and NIR with dietary data. In the total population, only APR % correlated with S-NIR (Table 5).

When the participants were split by sex, it appeared that in females, only S-NIR and WC correlated at baseline (Table 6), while in men, weight, FM, and FFM correlated with S-NIR, the strongest correlation seen with weight (Table 7). Serum and urine CIR correlated with all anthropometric measurements at baseline, with the strongest correlation found with FM. U-NIR did not correlate with any anthropometric measurements at baseline.

No correlations between U-CIR and dietary intake were found in females, while APR was correlated with U-CIR in males. U-NIR was correlated with both APR and AMPR in females, while APR and AMPR were correlated with S-NIR in males.

Table 5: Spearman correlation matrix for stable isotope ratios at baseline, using data from all participants (n=83-102). Significant correlations are marked with **bold**

	S-CIR ‰	U-CIR ‰	S-NIR ‰	U-NIR ‰	Weight kg	BMI kg/m²	WC cm	FM kg	FFM kg
Weight kg	**-0.25**	**-0.20**	-0.13	0.08					
BMI kg/m²	-0.10	-0.12	-0.05	0.08	**0.77**				
WC cm	**-0.20**	**-0.25**	0.01	0.09	**0.88**	**0.78**			
FM kg	-0.13	-0.11	-0.11	0.03	**0.76**	**0.90**	**0.71**		
FFM kg	**-0.33**	**-0.24**	-0.06	0.09	**0.73**	**0.25**	**0.61**	0.13	
APR %	0.09	0.17	**-0.20**	0.03	0.03	-0.06	-0.01	-0.04	0.1
AMPR %	0.05	0.13	-0.14	0.16	0.02	-0.02	0.01	-0.04	0.07
Added sugar g/d	-0.07	-0.10	0.05	-0.07	-0.05	-0.02	0.00	0.01	-0.09

Note; Due to missing values, the number of observations varied between 83 and 102. APR is calculated by Animal protein intake / total protein intake. AMPR calculated by AP+MP/total protein intake Abbrevantions; APR=Animal Protein Ratio, AMPR=Animal Marine Protein Ratio, BMI=Body Mass Index, FM=Fat-Mass, FFM=Fat Free-Mass, S-CIR=Serum Carbon Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio, WC=Waist Circumference.

Table 6: Spearman correlation matrix for stable isotope ratios at baseline, using data from female participants (n=40-53). Significant results are marked with **bold**

	S-CIR ‰	U-CIR ‰	S-NIR ‰	U-NIR ‰	Weight kg	BMI kg/m²	WC cm	FM kg	FFM kg
Weight kg	-0.07	0.03	0.02	0.04					
BMI kg/m²	0.18	0.03	0.10	0.14	**0.82**				
WC cm	-0.02	-0.14	**0.21**	0.10	**0.78**	**0.79**			
FM kg	0.01	0.10	-0.03	0.09	**0.96**	**0.87**	**0.78**		
FFM kg	**-0.21**	-0.03	0.02	-0.08	**0.88**	**0.62**	**0.63**	**0.74**	
APR %	0.08	0.09	-0.14	**0.23**	-0.12	**-0.22**	-0.15	-0.18	**-0.028**
AMPR %	0.05	0.05	-0.6	**0.44**	-0.15	**-0.22**	-0.13	**-0.22**	-0.01
Added sugar g/d	-0.17	-0.15	0.05	0.07	0.09	0.01	0.01	0.07	0.07

Note; Due to missing values, the number of observations varied between 40 and 53. APR is calculated by Animal protein intake / total protein intake. AMPR calculated by AP+MP/total protein intake Abbrevantions; APR=Animal Protein Ratio, AMPR=Animal Marine Protein Ratio, BMI=Body Mass Index, FM=Fat-Mass, FFM=Fat Free-Mass, S-CIR=Serum Carbon Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio, WC=Waist Circumference.

Table 7: Spearman correlation matrix for stable isotope ratios at baseline, using data from male participants (n=40-57). Significant results are marked with **bold**

	S-CIR ‰	U-CIR ‰	S-NIR ‰	U-NIR ‰	Weight kg	BMI kg/m²	WC cm	FM kg	FFM kg
Weight,kg	**-0.32**	**-0.28**	**-0.24**	-0.02					
BMI, kg/m²	**-0.37**	**-0.22**	-0.19	0.01	**0.90**				
WC, cm	**-0.31**	**-0.21**	-0.15	-0.02	**0.89**	**0.92**			
FM, kg	**-0.35**	**-0.29**	**-0.22**	0.04	**0.91**	**0.93**	**0.95**		
FFM, kg	**-0.28**	**-0.20**	**-0.20**	-0.16	**0.79**	**0.55**	**0.51**	**0.49**	
APR, %	0.12	**0.21**	**-0.26**	-0.11	0.11	0.07	0.06	0.10	0.18
AMPR,%	0.02	0.19	**-0.23**	-0.05	0.16	0.18	0.15	0.13	0.18
Added sugar,g	-0.00	-0.09	0.08	-0.07	-0.11	-0.03	0.00	-0.05	-0.16

Note; Due to missing values, the number of observations varied between 40 and 57. APR is calculated by Animal protein intake / total protein intake.AMPR calculated by AP+MP/total protein intake Abbr; APR=Animal Protein Ratio, AMPR=Animal Marine Protein Ratio, BMI=Body Mass Index, FM=Fat-Mass, FFM=Fat Free-Mass, S-CIR=Serum Carbon Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio, WC=Waist Circumference

Spearman correlation was performed to calculate the correlation between the stable isotope ratio of carbon and nitrogen at baseline and three months and serum and urine CIR and NIR at three months (Shown in Figures S2-S7 in appendix). Overall, relatively weak correlations were observed (rho from 0.18 to 0.43), with the correlation of U-NIR from baseline and three months in females virtually being absent. (Figure S5).

4.2.2 Three months

We investigated the association of CIR and NIR at three months both with the results from anthropometric measurements taken at three months and dietary intake at three months (Tables 8, 9, 10) and with changes in anthropometric measurements (Tables 11,12,13), each for the total cohort and men and women separately.

Table 8: Spearman correlation matrix for stable isotope ratios at three months, using data from all participants (n=72-118). Significant results are marked with **bold**

	S-CIR ‰	U-CIR ‰	S-NIR ‰	U-NIR ‰	Weight kg	BMI kg/m²	WC cm	FM kg	FFM kg
Weight kg	-0.00	-0.07	-0.13	**-0.21**					
BMI kg/m²	0.11	0.04	-0.17	**-0.31**	**0.76**				
WC cm	-0.02	-0.03	-0.08	**-0.21**	**0.89**	**0.80**			
FM kg	0.09	-0.03	**-0.21**	**-0.40**	**0.74**	**0.88**	**0.72**		
FFM kg	-0.01	**-0.22**	0.01	-0.09	**0.70**	**0.23**	**0.56**	0.07	
Carbohydrate E%	0.11	-0.10	-0.03	**-0.24**	**-0.36**	0.06	-0.16	-0.18	0.07
Animal Protein g/d	**-0.32**	0.17	0.08	**0.25**	0.06	**-0.23**	**0.25**	0.09	-0.09
APR %	**-0.24**	0.11	0.04	**0.24**	0.04	**-0-21**	**0.26**	0.13	-0.08
AP+USPR %	-0.13	0.16	-0.15	**0.23**	0.18	0.04	**0.25**	**0.26**	-0.04
AMPR %	-0.04	0.12	0.12	**0.29**	0.04	**-0.23**	0.17	0.04	0.01

Note; Due to missing values, the number of observations varied between 72 and 118. APR is calculated by Animal protein intake / total protein intake. AP+USPR is calculated by AP+USP/total protein intake.AMPR calculated by AP+MP/total protein intake Abbr; APR=Animal Protein Ratio, AP+USPR=Animal And Unspecific Protein Ratio AMPR=Animal And Marine Protein Ratio, BMI=Body Mass Index, FM=Fat-Mass, FFM=Fat Free-Mass, S-CIR=Serum Carbon Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio, WC=Waist Circumference

Table 9: Spearman correlation matrix for stable isotope ratios at three months, using data from female participants (n=40-57). Significant results are marked with **bold**

	S-CIR ‰	U-CIR ‰	S-NIR ‰	U-NIR ‰	Weight kg	BMI kg/m²	WC cm	FM kg	FFM kg
Weight kg	**-0.20**	0.07	**-0.25**	**-0.42**					
BMI kg/m²	**-0.26**	**0.21**	**-0.23**	**-0.42**	**0.79**				
WC cm	0.15	-0.14	-0.13	**-0.39**	**0.82**	**0.84**			
FM kg	**0.29**	0.12	**-0.28**	**-0.36**	**0.74**	**0.87**	**0.84**		
FFM kg	0.07	-0.18	-0.12	**-0.44**	**0.70**	**0.62**	**0.67**	**0.70**	
Carbohydrate E%	-0.01	**-0.30**	0.00	**-0.23**	-0.05	-0.07	-0.19	-0.09	0.05
Animal Protein g/d	**-0.21**	0.11	0.10	**0.29**	**0.23**	**-0.34**	**0.30**	0.18	0.06
APR %	-0.17	0.09	0.11	**0.25**	0.06	**-0.46**	**0.30**	0.08	0.02
AP+USPR %	-0.08	**0.24**	-0.14	**0.23**	0.19	-0.16	**0.35**	**0.24**	0.10
AMPR %	0.00	0.10	0.19	**0.26**	0.08	**-0.38**	0.21	0.9	0.05

Note; Due to missing values, the number of observations varied between 40 and 57. APR is calculated by Animal protein intake / total protein intake. AP+USPR is calculated by AP+USP/total protein intake.AMPR calculated by AP+MP/total protein intake Abbr; APR=Animal Protein Ratio, AP+USPR=Animal And Unspecific Protein Ratio AMPR=Animal And Marine Protein Ratio, BMI=Body Mass Index, FM=Fat-Mass, FFM=Fat Free-Mass, S-CIR=Serum Carbon Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio, WC=Waist Circumference

Table 10: Spearman correlation matrix for stable isotope ratios at three months, using data from male participants (n=39-61). Significant results are marked with **bold**

	S-CIR ‰	U-CIR ‰	S-NIR ‰	U-NIR ‰	Weight kg	BMI kg/m²	WC cm	FM kg	FFM kg
Weight kg	-0.15	-0.17	-0.17	**-0.28**					
BMI kg/m²	-0.05	-0.15	-0.14	**-0.25**	0.79				
WC cm	-0.14	-0.13	**-0.23**	**-0.30**	0.82	0.91			
FM kg	-0.15	**-0.24**	-0.15	**-0.40**	0.91	0.91	0.9		
FFM kg	0.05	-0.08	-0.07	**-0.48**	**0.47**	**0.48**	**0.48**	0.06	
Carbohydrate E%	0.08	-0.03	-0.08	**-0.21**	-0.10	0.15	-0.05	**-0.25**	0.09
Animal Protein g/d	**-0.41**	**0.30**	-0.02	**0.20**	-0.17	-0.09	**0.21**	0.014	**-0.24**
APR %	**-0.29**	**0.25**	-0.00	**0.32**	-0.03	0.03	**0.25**	0.15	-0.19
AP+USPR %	-0.14	0.14	-0.12	0.09	0.12	**0.22**	0.14	**-0.27**	-0.03
AMPR %	-0.05	0.19	0.04	**0.47**	-0.00	-0.05	0.18	-0.03	-0.11

Note; Due to missing values, the number of observations varied between 39 and 61 APR is calculated by Animal protein intake / total protein intake. AP+USPR is calculated by AP+USP/total protein intake.AMPR calculated by AP+MP/total protein intake Abbr; APR=Animal Protein Ratio, AP+USPR=Animal And Unspecific Protein Ratio AMPR=Animal And Marine Protein Ratio, BMI=Body Mass Index, FM=Fat-Mass, FFM=Fat Free-Mass, S-CIR=Serum Carbon Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio, WC=Waist Circumference

For the purpose of this thesis, only correlations with SIRs at three months were described and commented. S-CIR did not correlate with any anthropometric measurements in the total cohort at three months. U-CIR correlated negatively with FFM, while S-NIR correlated negatively with FM. All anthropometric measurements, except for FFM, correlated with U-NIR at three months. In the total cohort, S-CIR correlated negatively with animal protein and APR. U-NIR correlated with all dietary variables, negatively with carbohydrate E% and the rest positively (Table 8).

When conducting the analysis separated by sex, S-CIR correlated negatively with weight and BMI and positively with FM in females (Table 9). However, in males, S-CIR did not correlate with the anthropometric measurements (Table 10). Among the dietary variables, only animal protein intake (g/d) was negatively correlated with S-CIR in females. In males, animal protein and APR were negatively correlated with S-CIR, with animal protein having the strongest correlation.

 U-CIR only correlated positively with BMI in females while correlating negatively with FM in males. AP+USPR had a positive relationship with U-CIR in females among the dietary variables. Animal protein and APR were positively correlated in males with UCIR.

S-NIR had a negative relationship with weight, BMI, and FM in females, while only correlating negatively with WC in males. The Spearman correlation analysis revealed no correlation between S-NIR and dietary intake, neither in females nor in males.
 All anthropometric measurements correlated negatively with U-NIR in both sexes. Carbohydrate E% showed a negative relationship with U-NIR in both sexes. U-NIR had a positive relationship with animal protein, APR, and AMPR in both sexes. The Spearman correlation revealed a positive correlation in females of U-NIR and AP+USPR.

Table 11: Spearman Correlation Matrix using data from all participants (n=82-105). Stable isotope data were obtained at three months, while changes in anthropometric data were calculated from baseline and three months data. Significant correlations are marked with **bold**

	S-CIR %	U-CIR %	S-NIR %	U-NIR %	Weight Change kg	WC Change cm	FM Change kg
Weight change kg	**-0.22**	-0.19	0.02	0.14			
WC Change cm	**-0.26**	-0.11	0.01	**0.21**	**0.70**		
FM Change kg	-0.14	**-0.25**	0.05	0.17	**0.81**	**0.63**	
FFM Change kg	**-0.23**	-0.08	-0.03	-0.08	**0.60**	**0.43**	-0.02

Note; Due to missing values, the number of observations varied between 82 and 105. Changes were calculated by subtracting three months values with baseline values. Abbreviations; BMI=Body Mass Index, FM=Fat-Mass, FFM=Fat Free-Mass, S-CIR=Serum Carbon Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio, WC=Waist Circumference

Table 12: Spearman Correlation Matrix using data from female participants (n=40-54). Stable isotope data were obtained at three months, while changes in anthropometric data were calculated from baseline and three months data. Significant correlations are marked with **bold**

	S-CIR %	U-CIR %	S-NIR %	U-NIR %	Weight Change kg	WC Change cm	FM Change kg
Weight change kg	**-0.23**	**-0.32**	0.02	**0.21**			
WC Change cm	**-0.32**	**-0.20**	-0.09	**0.23**	**0.63**		
FM Change kg	**-0.23**	**-0.39**	0.07	0.07	**0.81**	**0.63**	
FFM Change kg	**-0.21**	-0.11	-0.03	0.09	**0.60**	**0.43**	**0.21**

Note; Due to missing values, the number of observations varied between 40 and 54. Changes were calculated by subtracting three months values with baseline values. Abbreviations; BMI=Body Mass Index, FM=Fat-Mass, FFM=Fat Free-Mass, S-CIR=Serum Carbon Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio, WC=Waist Circumference

Table 13: Spearman Correlation Matrix using data from male participants (n=42-51). Stable isotope data were obtained at three months, while changes in anthropometric data were calculated from baseline and three months data. Significant correlations are marked with **bold**

	S-CIR %	U-CIR %	S-NIR %	U-NIR %	Weight Change kg	WC Change kg	FM Change kg
Weight Change kg	-0.14	-0.03	0.02	0.10			
WC Change cm	**-0.20**	0.06	0.15	**0.22**	**0.78**		
FM Change kg	0.07	-0.05	0.08	**0.33**	**0.73**	**0.61**	
FFM Change kg	**-0.28**	-0.08	-0.07	**-0.29**	**0.47**	0.19	-0.17

Note; Due to missing values, the number of observations varied between 42 and 51. Changes were calculated by subtracting three months values with baseline values. Abbreviations; BMI=Body Mass Index, FM=Fat-Mass, FFM=Fat Free-Mass, S-CIR=Serum Carbon Isotope Ratio, S-NIR=Serum Nitrogen Isotope Ratio, U-CIR=Urine Carbon Isotope Ratio, U-NIR=Urine Nitrogen Isotope Ratio, WC=Waist Circumference

In the total cohort, the Spearman correlation analysis revealed a negative relationship between S-CIR and changes in weight, WC, and FFM. U-CIR correlated negatively with changes in FM. WC change was the only anthropometric measurement with a negative relationship with U-NIR in all participants (Table 11).

When analyzing the associations separately for sexes, it appeared that in females, all anthropometric measurements correlated negatively with S-CIR (Table 12). In contrast, the Spearman correlation analysis showed no correlation between anthropometric measures and S-CIR for males (Table 13).

Of all anthropometric measurements, FFM change was the only variable not negatively correlated with U-CIR in females. In males, no anthropometric measurements showed a relationship with U-CIR.

The Spearman correlation analysis revealed no correlation between S-NIR and anthropometric measurements, neither in females nor in males.
Weight change and WC change were positively correlated with U-NIR in females, and WC change, FM change, and FFM change positively correlated with U-NIR in males.

4.3 Linear Regression

Linear regression was performed to test if changes in anthropometric measurements (Changes in weight, WC, FM, and FFM) were associated with S-CIR and S-NIR/U-NIR at three months. Linear regression was not performed for U-CIR as the residuals of U-CIR did not seem to be normally distributed, as visualized in the Q-Q plot (Shown in Figure S1 in the appendix). The regression was stratified by sex and conducted multiple adjusted models for each anthropometric measurement. Changes in fat mass and fat-free mass were calculated from BIA measurements.

4.3.1 Serum CIR

Table 14: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with S-CIR at three months as the dependent variable. Only beta-coefficients for weight change, separately for women (n=52) and men (n=49), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	Weight change	-0.021 (0.011)	6%	-0.021 (0.012)	6%
Model 2	Weight change, BMI at baseline	**-0.026 (0.011)**	14%	-0.021 (0.012)	6%
Model 3	Weight change, Carbohydrate E%	**-0.026 (0.012)**	12%	-0.025 (0.014)	10%
Model 4	Weight change, AMPR,	-0.029 (0.015)	9%	-0.12 (0.015)	4%
Model 5	Weight change, BMI, Carbohydrate E%, and AMPR	**-0.047 (0.016)**	23%	-0.020 (0.017)	20%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m², weight change (kg) E%= Energy Percent, R²=Variance in S-CIR explained by the linear regression model, +-SE=+-Standard Error

In models 2, 3, and 5, weight change was negatively associated with S-CIR in women. Adjusting for BMI, energy from carbohydrates, or AMPR increased the beta-coefficient and explained variance. The final model explained 23% of the variance in S-CIR in women. In men, no model revealed an association between S-CIR and weight change.

Table 15: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with S-CIR at three months as the dependent variable. Only beta-coefficients for WC change, separately for women (n=52) and men (n=50), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	WC change	**-0.023 (0.009)**	12%	-0.023 (0.013)	6%
Model 2	WC change, BMI at baseline	**-0.023 (0.008)**	17%	-0.024 (0.013)	7%
Model 3	WC, Carbohydrate E%	**-0.027 (0.009)**	19%	**-0.030 (0.015)**	12%
Model 4	WC change, AMPR	**-0.033 (0.010)**	21%	-0.024 (0.014)	8%
Model 5	WC change, BMI, Carbohydrate E%, and AMPR	**-0.033 (0.011)**	25%	**-0.036 (0.015)**	30%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m^2),) E%= Energy Percent, R^2=Variance in S-CIR explained by the linear regression model, +-SE=+-Standard Error, WC=Waist Circumference (cm).

In all models, change in WC was negatively associated with S-CIR in women, and adjustment for either BMI, energy from carbohydrates, or AMPR increased the beta-coefficient and the explained variance. The final model explained 25% of the variance in S-CIR in women.
In men, changes in WC did not explain variance in S-CIR in the unadjusted model or models adjusted for BMI or AMPR, however, the final model explained 30% of the variance in S-CIR in men.

Table 16: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with S-CIR at three months as the dependent variable. Only beta-coefficients for FM change, separately for women (n=44) and men (n=44), are provided. Significant results are provided in **bold.**

	Variables in the model	Female	R^2	Male	R^2
Model 1	FM change	**-0.034 (0.015)**	11%	0.001 (0.015)	0%
Model 2	FM change, BMI at baseline	**-0.037 (0.015)**	19%	0.002 (0.015)	1%
Model 3	FM change, Carbohydrate E%	**-0.035 (0.016)**	13%	0.004 (0.017)	2%
Model 4	FM change, AMPR	**-0.043 (0.018)**	14%	0.008 (0.016)	2%
Model 5	FM change, BMI, Carbohydrate E%, AMPR	**-0.059 (0.020)**	27%	0.003 (0.016)	14%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m^2),) E%= Energy Percent, FM=Fat-Mass (kg), R^2=Variance in S-CIR explained by the linear regression model, +-SE=+- Standard Error.

In all models, change in fat mass was negatively associated with S-CIR in women, and adjustment for AMPR or the total adjustment (BMI, Carbohydrate E%, and AMPR) increased the beta-coefficient and the explained variance. The final model explained 27% of the variance in S-CIR in women.

In men, changes in FM did not explain variance in S-CIR in the unadjusted model or models adjusted for BMI, Carbohydrate E%, or AMPR, and the final model explained 14% of the variance in S-CIR in men.

Table 17: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with S-CIR at three months as the dependent variable. Only beta-coefficients for FFM change, separately for women (n=42) and men (n=44), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	FFM change	-0.033 (0.031)	2%	-0.040 (0.022)	7%
Model 2	FFM change, BMI at baseline	-0.032 (0.031)	5%	-0.040 (0.024)	7%
Model 3	FFM change, Carbohydrate E%	-0.028 (0.033)	2%	-0.037 (0.024)	8%
Model 4	FFM change, AMPR	-0.036 (0.037)	3%	-0.040 (0.023)	10%
Model 5	FFM change, BMI, Carbohdyrate E%, AMPR	-0.029 (0.042)	4%	-0.041 (0.025)	23%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m^2),) E%= Energy Percent, FFM=Fat-Free Mass (kg), R^2=Variance in S-CIR explained by the linear regression model, +-SE=+-Standard Error.

For FFM change, the linear regression did not find any association with S-CIR in females or males. The explained variance in females was very low, and in males, only model 5 had a slightly elevated explained variance (23%).

4.3.2 Serum NIR

Table 18: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with S-NIR at three months as the dependent variable. Only beta-coefficients for weight change, separately for women (n=53) and men (n=49), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	Weight change	-0.003 (0.011)	0.1%	0.006 (0.011)	0.6%
Model 2	Weight change, BMI at baseline	0.001 (0.011)	6%	0.006 (0.011)	2%
Model 3	Weight change, Carbohdyrate E%	-0.001 (0.013)	1%	0.000 (0.013)	0%
Model 4	Weight change, AMPR	-0.013 (0.014)	3%	-0.008 (0.015)	0.8%
Model 5	Weight change, BMI, Carbohydrate E% and AMPR	-0.008 (0.016)	13%	-0.018 (0.018)	8%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m^2), weight (kg), E%= Energy Percent, R^2=Variance in S-NIR explained by the linear regression model, +-SE=+-Standard Error.

The linear regression showed no association between S-NIR and weight change. The explained variance of S-NIR was low in all models and for both sexes.

Table 19: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with S-NIR at three months as the dependent variable. Only beta-coefficients for WC change, separately for women (n=53) and men (n=47), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	WC change	-0.006 (0.009)	1%	0.015 (0.012)	3%
Model 2	WC change, BMI at baseline	-0.007 (0.008)	7%	0.014 (0.012)	3%
Model 3	WC change, Carbohydrate E%	-0.008 (0.010)	3%	0.016 (0.014)	4%
Model 4	WC change, AMPR	-0.009 (0.010)	3%	0.007 (0.017)	0.6%
Model 5	WC change, BMI, Carbohydrate E% and AMPR	-0.00 (0.010)	13%	0.007 (0.019)	3%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m^2),) E%= Energy Percent, R^2=Variance in S-NIR explained by the linear regression model, +-SE=+-Standard Error, WC= Waist Circumference (cm)

The linear regression showed no association between S-NIR and change in waist circumference. The explained variance of S-NIR was low in all models and for both sexes.

Table 20: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with S-NIR at three months as the dependent variable. Only beta-coefficients for FM change, separately for women (n=44) and men (n=44), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	FM change	0.003 (0.016)	0.1%	0.003 (0.014)	0.1%
Model 2	FM change, BMI at baseline	0.005 (0.016)	5%	0.004 (0.014)	1%
Model 3	FM change, Carbohydrate E%	0.002 (0.017)	0.6%	-0.003 (0.016)	1%
Model 4	FM change, AMPR	-0.004 (0.018)	3%	-0.009 (0.017)	0.9%
Model 5	FM change, BMI, Carbohydrate E% and AMPR	0.001 (0.062)	13%	-0.016 (0.020)	7%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m^2),) E%= Energy Percent, FM=Fat-Mass, R^2=Variance in S-NIR explained by the linear regression model, +-SE=+- Standard Error.

The linear regression showed no association between S-NIR and change in fat mass. The explained variance of S-NIR was low in all models and for both sexes.

Table 21: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with S-NIR at three months as the dependent variable. Only beta-coefficients for FFM change, separately for women (n=42) and men (n=44), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	FFM change	-0.002 (0.031)	0%	0.002 (0.023)	0%
Model 2	FFM change, BMI at baseline	-0.003 (0.030)	5%	0.005 (0.025)	0.2%
Model 3	FFM change, Carbohydrate E%	0.001 (0.033)	0.2%	-0.007 (0.025)	1%
Model 4	FFM change, AMPR	-0.024 (0.033)	3%	0.012 (0.027)	0.6%
Model 5	FFM change, BMI, Carbohydrate E% and AMPR	-0.022 (0.034)	16%	0.008 (0.034)	2%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m^2),) E%= Energy Percent, FFM=Fat-Free Mass, R^2=Variance in S-NIR explained by the linear regression model, +-SE=+- Standard Error.

The linear regression showed no association between S-NIR and fat-free mass change. The explained variance of S-NIR was low in all models and for both sexes.

4.3.3 Urine NIR

Table 22: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with U-NIR at three months as the dependent variable. Only beta-coefficients for weight change, separately for women (n=44) and men (n=45), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	Weight change	0.024 (0.025)	2%	0.013 (0.018)	1%
Model 2	Weight change, BMI	0.030 (0.024)	15%	0.013 (0.018)	2%
Model 3	Weight change, Carbohydrate E%	0.007 (0.028)	5%	-0.007 (0.021)	7%
Model 4	Weight change, AMPR	-0.007 (0.033)	6%	0.024 (0.021)	13%
Model 5	Weight change, BMI, Carbohydrate E%, AMPR	-0.007 (0.036)	24%	0.03 (0.026)	32%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m², weight (kg), E%= Energy Percent, R²=Variance in U-NIR explained by the linear regression model, +-SE=+-Standard Error.

The linear regression showed no association between U-NIR and weight change. The explained variance of U-NIR was low in models 1, 2, 3, and 4 and for both sexes. Even though the explained variance of U-NIR was 24% in females and 32% in males for the final model 5, this cannot be explained by weight change but by the other variables in the model.

Table 23: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with U-NIR at three months as the dependent variable. Only beta-coefficients for WC change, separately for women (n=44) and men (n=47), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	WC change	0.017 (0.018)	2%	0.033 (0.018)	7%
Model 2	WC change, BMI at baseline	0.015 (0.017)	14%	0.032 (0.018)	8%
Model 3	WC change, Carbohydrate E%	-0.002 (0.022)	5%	0.024 (0.020)	9%
Model 4	WC change, AMPR	0.002 (0.022)	6%	**0.048 (0.020)**	23%
Model 5	WC change, BMI, Carbohydrate E%, and AMPR	-0.009 (0.022)	25%	**0.047 (0.022)**	37%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m²,) E%= Energy Percent, R²=Variance in U-NIR explained by the linear regression model, +-SE=+-Standard Error, WC= Waist Circumference (cm)

The linear regression showed no association between U-NIR and change in WC in females and a low explained variance of U-NIR by a change in WC.

In males, changes in WC and AMPR, as well as the full model, explained 23% and 37% of the variance of U-NIR.

Table 24: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with U-NIR at three months as the dependent variable. Only beta-coefficients for FM change, separately for women (n=38) and men (n=39), are provided. Significant results are provided in **bold.**

	Variables in the model	Female	R^2	Male	R^2
Model 1	FM change	0.012 (0.032)	0.4%	**0.053 (0.022)**	13%
Model 2	FM change, BMI at baseline	0.026 (0.031)	15%	**0.061 (0.021)**	24%
Model 3	FM change, Carbohydrate	0.007 (0.034)	5%	0.031 (0.028)	17%
Model 4	FM change, AMPR	-0.017 (0.041)	8%	**0.051 (0.025)**	19%
Model 5	FM change, BMI, Cabohydrate E% and AMPR	0.002 (0.043)	25%	0.057 (0.031)	39%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m²,) E%=Energy Percent, FM=Fat-Mass (kg) R²=Variance in U-NIR explained by the linear regression model, +-SE=+-Standard Error.¨

The linear regression showed no association between U-NIR and change in FM in females and a low explained variance of U-NIR by a change in fat mass.

In males, change in fat mass alone explained 13% of the U-NIR variance. Further adjustment for BMI at baseline increased the explained variance to 24%, and adjustment for AMPR increased the explained variance to 19%.

Table 25: Beta coefficients (+-SE) and total explained variance from the linear regression analysis with U-NIR at three months as the dependent variable. Only beta-coefficients for FFM change, separately for women (n=37) and men (n=39), are provided. Significant results are provided in **bold**.

	Variables in the model	Female	R^2	Male	R^2
Model 1	FFM change	0.027 (0.070)	0.4%	-0.059 (0.035)	7%
Model 2	FFM, BMI at baseline	0.013 (0.067)	12%	-0.045 (0.037)	11%
Model 3	FFM change, Carbohydrate E%	0.012 (0.077)	5%	-0.059 (0.039)	22%
Model 4	FFM change, AMPR	-0.015 (0.078)	10%	0.010 (0.025)	14%
Model 5	FFM change, BMI, Carbohydrate E% and AMPR	-0.061 (0.087)	26%	-0.056 (0.044)	33%

Explanation and Abbreviation: AMPR= Animal marine protein ratio (%), BMI=Body mass index (kg/m²,) E%=Energy Percent, FFM=Fat-Free Mass (kg), R²=Variance in U-NIR explained by the linear regression model, +-SE=+-Standard Error.

The linear regression revealed no association between U-NIR and FFM change. The explained variance of U-NIR was low in models 1, 2, 3, and 4 and for both sexes. Even though the explained variance of U-NIR was 26% in females and 33% in males for the final model 5, this cannot be explained by a change in FFM but by the other variables in the model.

5 Discussion

5.1 Main Findings

The main finding of the current investigation was that both CIR and NIR, either in serum or urine, are only weakly associated with weight loss during the prior three months in people with obesity. In addition to total weight loss, changes in FM and FFM were also measured. Interestingly, although neither SIRs at baseline nor weight loss differed among sexes, we observed strikingly different associations of changes in anthropometric measurements in women or men, respectively. While S-CIR was associated with changes in almost all anthropometric measurements in women, this was not observed in men. On the other hand, U-NIR was associated with anthropometric measurements of weight loss in men but not in women. In linear regression analyses, the explained variance of CIR or NIR by changes in anthropometric measurements was also different in women and men.

5.1.1 Baseline values – association with obesity

Our data suggest some association of stable isotope ratios with obesity. At baseline, S-CIR and U-CIR correlated with all anthropometric measurements in men (Table 7). S-NIR correlated at baseline with weight, FM, and FFM in males and WC in females (Tables 6 and 7). Few studies have investigated the association of stable isotope ratios with measures of obesity, and even fewer studies used serum or urine CIR or NIR (93, 105, 106). A study from Nash in 2013 done in a Yup'ik found no association between BMI and CIR (105), while a small study from Hayasaka in 2016 found a negative correlation between BMI and CIR measured in hair (106). A very small study in anorexia patients found that an increase in BMI resulted in an increase in hair CIR (93). When comparing S-CIR and U-CIR in our population with a population with lower BMIs, our CIR values are generally much lower than those (68, 104, under review). Votrubas' 12-week feeding study population had an average BMI of 27 kg/m2 and reported S-CIR levels of -19.9‰ (68). Other data from the US in a predominantly obese group and high added sugar intake had whole blood CIR of -18.92 (n=296, BMI 33 $\pm$ 9 kg/m2) (107)

Comparing to European data, which are probably less influenced by sugar cane and corn than the US data, reveals that the omnivore participants in (103) with a BMI of 23kg/m^2 on average had much higher S-CIR levels (-22.76‰) and lower S-NIR (9.49‰) compared to our research, where the median BMI was 37 kg/m^2.

Concerning geographic differences, our data can be compared with other studies from Scandinavia or Europe. Swedish data (n=46) reported S-CIR of -22.36‰ and S-NIR 10.42‰ but did not report BMI or body weight. Since samples were collected in 1978 from the general population in Malmø, these participants were probably, on average, not obese (75). A Danish study from 2016 had hair CIR of -20.9 ‰ and an average BMI of 22 kg/m^2 (108). Petzke, with 99 participants and a mean BMI of 25 kg/m2, had hair CIR levels of -19.6 ‰ and NIR 9.9 ‰ (92). Hair CIR levels are normally 2‰ higher than plasma CIR levels (45), but even when considering this, our levels are still lower compared to these studies. Compared to a study with 24-hour urine with a mean BMI of 24 kg/m2 and CIR levels of -26.4 ‰ and NIR 7.0 ‰ (86), our S-CIR levels and U-CIR levels are much higher. We are not aware of any other studies investigating CIR or NIR, specifically in obesity. These results could imply that CIR is affected by overweight/obesity. However, it is not easy to compare as CIR values are different in places where C4 plants are a significant part of the diet, for example, the USA, and overall due to geographical differences in SIR. Further research on this topic would be needed to establish CIR as a candidate biomarker for measurements of obesity.

5.1.2 Three months values-associations with obesity

After three months, there were still associations with measurements of obesity and SIR. However, these were quite different than the associations at baseline. In females, S-CIR did correlate negatively with weight and BMI and positive with FM at three months, while no correlations were found at baseline (Table 9). On the other hand, S-CIR no longer correlated with any anthropometric measurements at three months in males (Table 10). U-CIR was also only related positively to BMI in women and negatively to WC in men. At baseline, U-CIR correlated with no anthropometric measurements in women while correlating with all anthropometric measurements in men. At three months, S-NIR correlated negatively with weight, BMI, and FM in females, instead of only WC, and males only correlated negatively with WC. U-NIR, which did not have any correlations at baseline, did correlate negatively with all anthropometric measurements in both sexes at three months.

These very different correlations at baseline and three months show that these associations should be interpreted with caution. Although it seems likely that there is some relationship between anthropometric measurements for obesity and SIR, it could very well just be random.

5.1.3 Weight change and SIR

Intended weight loss in obesity by dieting is associated with a decrease in triglycerides stored, and their fatty acids, which are depleted of ^{13}C (45, 109) are used as a source of energy instead of food in weight loss. This would suggest that as weight is lost and lipids are used as a source of energy, CIR should decrease. This was not the case in our thesis, as we did not observe any significant changes in CIR. Weight loss did not differ between groups, and we analyzed all participants regardless of the dietary group (Tables 3a and b).

Further, as weight loss is also associated with changes in fat-free mass and thus altered AA homeostasis, the liver oxidizes more AA during weight loss, both derived from protein breakdown and uses the carbon skeleton as a source of energy. The oxidation of AA discriminates against ^{15}N, leaving AA enriched in ^{15}N behind for protein synthesis (45). Therefore, we hypothesized that NIR would increase as the AA containing ^{15}N are oxidized, and their nitrogen either present in serum or excreted, increasing NIR in serum or urine, respectively. We did find associations with U-NIR that agree with our theory (Tables 11-13, 23, and 24). Observations reported by Fueller and Meketo would support this, having found associations with an increase in NIR and weight loss/weight gain when the nitrogen balance in the body changed (93, 94).

However, NIR barely changed from baseline to three months in our population, and neither did we find any associations with S-NIR (Tables 11-13 and 19-21). This could be because the participants consumed enough protein and were not in a negative nitrogen balance. It is known that the nitrogen balance influences NIR values, anabolic states possibly decrease NIR while a catabolic state or high protein diets increase NIR (62, 94, 110, 111). Therefore, maybe only changes from a positive to negative nitrogen balance or the other way would lead to significant changes in NIR.

In our thesis, carbohydrate E% correlated negatively in females with U-CIR and in males with U-NIR (Tables 9 and 10) at three months. As CarbFunc was a dietary intervention study changed carbohydrate intake substantially (Tables 4a and b). Consequently, it could be that the dietary changes were dominating and thus masking any associations with weight loss. We also do not know about acute weight loss in the week before the three-month visit. However, weight loss was objectively measured and continued after the three months, as evident from the visit at six months which is not part of this thesis. Other materials such as red blood cells (RBC) (68) or hair (112) may reflect more extended time periods than serum

or spot urine and could be more suitable to measure long-term changes. However, these materials have not been available in the present study.

Advanced techniques can measure CIR and NIR in single AA in blood and distinguish between essential and non-essential AA. This could be a technique that can possibly differentiate between diet and internal metabolism because essential AA must be derived from dietary intake. In contrast, non-essential AA can be synthesized from different cycles in the body, e.g., the glucose-alanine cycle (69).

The associations of weight change with U-NIR are interesting, as both the amount and the sources of nitrogen in urine may change during weight loss. Nitrogen derived from creatinine (from muscle mass) may differ in NIR compared to urea nitrogen (and thus from AA metabolism). Huelsemann 2017 showed that the NIR of urinary urea was lower than the NIR of urine (see Figure 1), suggesting that urea reflects closer the NIR of dietary intake than creatinine (74). However, urine analyses for urea and creatinine in our urine samples were not available, and no separate measurement of NIR in urea and creatinine. However, this could be interesting in future studies.

S-NIR and U-NIR were significantly different between the three diet groups at three months (p=0.024 and p=0.045, respectively). In the LCHF group, both S-NIR and U-NIR were higher than in the two HCLF groups (Table S2 in Appendix). Energy deficiency will lead to grossly increased nitrogen excretion in the early phase, which will be normalized after a while due to the protein sparing effect of ketone body metabolism. Low carbohydrate intake is associated with higher concentrations of ketone bodies which can replace glucose as a source of energy for the brain (113). In addition to fatty acids, specific AA (lysine, phenylalanine, tyrosine, tryptophan, isoleucine, and leucine) can serve as a source of ketone bodies, which means their carbon skeleton is used for ketone body formation. The amino group containing nitrogen is probably excreted as urea in urine (114). That would explain that the U-NIR in the LCHF group is higher than in the other groups. However, this should be investigated in more detail when data on ketone bodies becomes available.

The current study included obese persons who wanted to loose weight and followed a slightly energy restricted diet with 17 E% protein. Thus, the loss of FFM, as estimated by BIA, was low in the current study. Our study population was obese but otherwise healthy, therefore, the weight loss achieved was primarily related to lost FM (Tables 3a and b). The situation may be quite different in unintended age or disease related weight loss, conditions which are associated with a higher loss of FFM (14, 15). We are unaware of a single study investigating

age-related weight loss and stable isotope ratios. The only studies investigating disease-associated weight change have been done in hyperemesis gravidarum (Fuller) and anorexia nervosa (Mekota) (93, 94). CIR is believed to reflect the dietary energy intake as all dietary components, e.g., carbohydrates, protein, and fat, affect CIR (93). Age-related or disease-associated weight loss is associated with a decreased dietary intake (15). Therefore, one would propose that CIR would decrease in the body, reflecting the change in energy source and using more stored lipids as an energy source. As mentioned before, it is thought that NIR is directly influenced by the anabolism/catabolism state of the body, increasing when the body is in a catabolic state as a direct result of using the body's nitrogen reservoir for protein metabolism (94). Because age-related and disease-associated weight loss is associated with a catabolic state, we propose that NIR would increase.

5.1.4 Differences between men and women

We observed some surprising differences in the associations of stable isotope ratios with changes in anthropometric variables in men and women.

Total weight loss, however, was not different between men and women. Also, dietary macronutrient intake at baseline was barely different between sexes, with men consuming higher absolute amounts of animal protein but no difference in the animal protein ratio.

S-CIR and U-CIR correlations and linear regression findings differed in sex. In females, CIR correlated negatively with all anthropometric measurements, except FFM change with U-CIR, while males had no correlations with anthropometric measures (Tables 12 and 13). FFM change was the only measurement that did not have an association with S-CIR in the linear regression (Table 17). This fits our hypothesis as it was thought that CIR would be related to FM and not FFM. In females, weight and WC change were negatively associated with S-CIR in most models (14 and 15). WC change was associated in models 3 and 5 in males, with the association in model 5 being stronger than in females. FM was only associated with S-CIR in women, also having the overall strongest explained variance in females (27%) (Tables 16).

Unlike S-NIR, U-NIR correlated with anthropometric measurements and dietary intake at three months (Tables 5-10). The different results from the Spearman correlation could be due to varying nutrient turnover, or serum and urine reflecting different periods. Since this is an exploratory analysis with a high number of various statistical analyses, it can not be excluded that this could also be a random occurrence.

U-NIR correlated positively with weight and WC change in women (Table 12). In men, U-NIR correlated with all anthropometric measurements, except weight. The correlations were positive for WC and FM change but negative for FFM change (Table 13). That FM and FFM correlate opposite of each other is favorable for use as a biomarker. In other words, it could be possible to differentiate between FM and FFM changes with this. In the linear regression, no associations were found with U-NIR in females, but both WC and FM change were positively associated in the regression in males (Table 23 and 24). The positive correlations comply with our hypothesis. It was surprising that no other associations were found in females, as the two studies we know about that have investigated SIR and weight loss were done in women, albeit in pregnancy and anorexia, and they measured the NIR in hair. (93, 94).

Animal Protein intake (g/d) correlated stronger with S-CIR in males than in females and only in males with U-CIR, while it correlated only in females with U-NIR (Tables 9 and 10). The stronger correlation in men may directly result from the higher protein intake men had (Table 4b). The correlation between animal protein intake and S-CIR and U-CIR was more potent than the correlation with U-NIR (Tables 8 and 10). This is consistent with O'Brien's findings from a controlled feeding study (83). That animal protein intake correlates with females and males different in CIR and U-NIR could point towards that the sex differences found continuously in this thesis are only a coincidence.

The APR (%) was slightly lower in males (52%) than in females (57%) (Table 4b). Therefore, it is surprising that APR only correlated with S-CIR and U-CIR in males and that the correlation in U-NIR was strongest in males (Tables 9 and 10). At baseline, S-CIR and S-NIR correlated with APR in males and U-NIR in females (Tables 6 and 7). The finding is consistent with another study finding correlations between APR, CIR, and NIR (83). Surprisingly, the correlations for AMPR (%) also differ between sex, as these are similar (females 64%, males 62%). The correlation coefficient was much stronger in men than in women for the correlation between U-NIR and AMPR (Tables 9 and 10), even though the AMPR was slightly higher in women. At baseline, there is also a difference in correlations. AMPR only correlated with U-NIR in females at baseline but with S-NIR in males (Tables 6 and 7).

To our knowledge, no studies have investigated differences in CIR or NIR between sexes. There could be different possible explanations for the observed differences. One explanation

could be that men may have lost weight earlier in the course of the study, and three months were too late to detect the differences, as serum and spot urine reflect a shorter period. Another possible explanation could be that males at three months had higher protein, fat, and fiber intake while having lower carbohydrate E% and added sugar intake change than females, as evident from the dietary data analyses. Indeed, dietary factors were stronger associated with CIR in men than anthropometric measurements.

Other reasons can be the difference in hormones or fat mass between sexes. While we measured fat mass, we can only speculate on hormones. All women were pre-menopausal, thus, the differences in hormones between men and women are evident. We are not aware of any studies that have investigated hormones and SIR.

5. 2 Statistics

As this is an explorative analysis, did we not do a power calculation as little is known about changes in SIR, therefore, no assumed values existed. Consequently, we were reluctant to use P-values, even though we marked significant correlations and results in the linear regression analyses. These significances should not be overemphasized due to the exploratory character of the analyses. Indeed, exploratory analyses should be hypothesis generating but not hypothesis confirming.

The Shapiro (Table S1 in Appendix) and QQ-plot (Figure S1 in Appendix) showed that the residuals for U-CIR at three months were not normally distributed. As the values of CIR are negative, it was impossible to transform them into logarithmic values. As multiple values were outside the distribution, it would have been a considerable manipulation of data to remove these outliers. Therefore the decision was not to perform the linear regression on U-CIR at three months.

It could also be argued whether linear regression analyses was the correct way to analyze the data. Scatterplots of CIR and NIR did not suggest non-linear associations by visual inspections, however, we did not check formally for non-linearity (as this would be beyond the activities of a master thesis), nor did we do GAM analyses (generalized additive models) as these are also beyond the scope of this thesis.

5.3 Strengths and limitations

To the best of our knowledge, we are not aware of other studies that investigated the association of weight loss with stable isotope ratios, except for very small studies in pregnant women with hyperemesis and anorexia (93, 94). Thus, one significant advantage of this study is that no similar studies are existing.

Another strength of the study is the aim of the study, the investigation of a biomarker for recent weight loss. Even though the study did not yield a very clear result, it shows the need for a more objective weight loss assessment, especially in situations when baseline values are not available. This was also the rationale for investigating three-month SIRs with changes in weight, waist circumference, fat mass, and fat-free mass.

Other strengths of the study include the objectively measured weight and height, the availability of body composition data, and the quantitative assessment of dietary intake throughout the study by dietary records and electronic recording, making the dietary assessment in the CarbFunc study reliable and valid (102). SIRs were measured by experienced technicians who were blinded for the weight changes or sex of the samples.

The most significant limitation of this thesis is that it is an exploratory study, investigating variables that had not been considered when the study was planned and thus, were not included in the study's power calculation. Therefore, we were cautious about using or emphasizing p-values, as these may be due to type 1 and type 2 errors. Further, even if the SIRs had been included in the power calculation, it would have been challenging to estimate any change as no preliminary data were available.

Overadjustment in the linear regression could also be a limitation. Models 5, which often yielded the strongest association with CIR, was adjusted for three factors. It can be that this model was adjusted too much, as often, and as 'a rule of thumb' for adjustments, one adjustment per 50 observations is used.

Another limitation was that we did not measure weight change continuously, but only at three months, and thus, we did not know the weight loss curve in the week before the measurement. Future studies should apply more regular weight change measurements, e.g., each week, and compare this with CIR or NIR. In addition, because we divided protein into plant, animal, unspecific and marine protein, is this also a source of error, especially the AP+USPR. Other limitations are that spot urine samples and not 24-hour urine were used for

analysis. However, we used morning urine that reflects metabolic activity during the night and not a random urine sample during the day. The non-availability of material that would allow long-term observations, e.g., hair or RBC, could also be mentioned as a limitation. However, given the limited knowledge on the usefulness of SIRs as a biomarker of weight loss, this study could also be regarded as a starting point.

5.4 Conclusion

In contrast to our hypothesis, CIR and NIR only showed moderate to a weak association with weight loss in obese volunteers. Differences were evident between men and women. As there is no other biomarker of weight loss, the moderate associations of weight change with S-CIR in women and with U-NIR in men should be further explored. Since our baseline results and a comparison with an earlier study of our group in lean people may indicate an effect of obesity with CIR or NIR, the association of CIR and NIR with obesity or body fatness should be further investigated.

More studies are needed to establish CIR as a candidate biomarker for weight loss or changes in body composition. Further investigation should research this in other materials which reflect longer time periods, for example, in RBC or hair. This thesis found a pattern of sex dependency of CIR and NIR. Further research on differences between men and women regarding weight loss, body composition, and dietary intake is necessary.

6 References

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7 Appendix

Table S1 Test of normality with Shapiro Wilk test in all (n=109[*]), p-value <0.05 significant.

	Statistics	Df	P-value
S-CIR_0	0.976	75	0.172
S-CIR_3	0.970	75	0.076
U-CIR_0	0.992	75	0.910
U-CIR_3	0.874	75	<0.001
S-NIR_0	0.960	75	0.019
S-NIR_3	0.986	75	0.583
U-NIR_0	0.944	75	0.002
U-NIR_3	0.985	75	0.509

Note; [*] For S-CIR at three months n=105, For S-NIR at three months n=106, For urine samples n=92. Abbrevations; Df=Degrees of freedom, S-CIR_0=Serum Carbon Isotope Ratio at baseline, S-CIR_3=Serum Carbon Isotope Ratio at three months, U-CIR_0=Urine Carbon Isotope Ratio at baseline, U-CIR_3= Urine Carbon Isotope Ratio at three months, S-NIR_0=Serum Nitrogen Isotope Ratio at baseline, S-NIR_3=Serum Nitrogen Isotope Ratio at three months, U-NIR_0=Urine Nitrogen Isotope Ratio at baseline, U-NIR_3=Urine Nitrogen Isotope Ratio at three months.
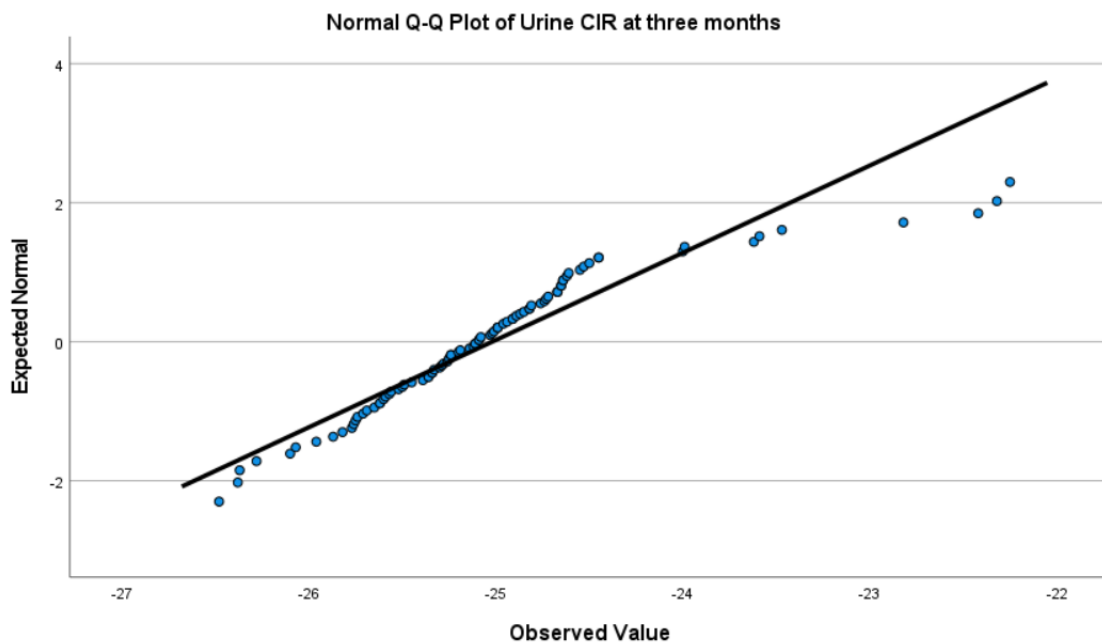
Figure S1 Quantile-Quantile plot (Q-Q Plot) of Urine CIR at three months for all (n=92)
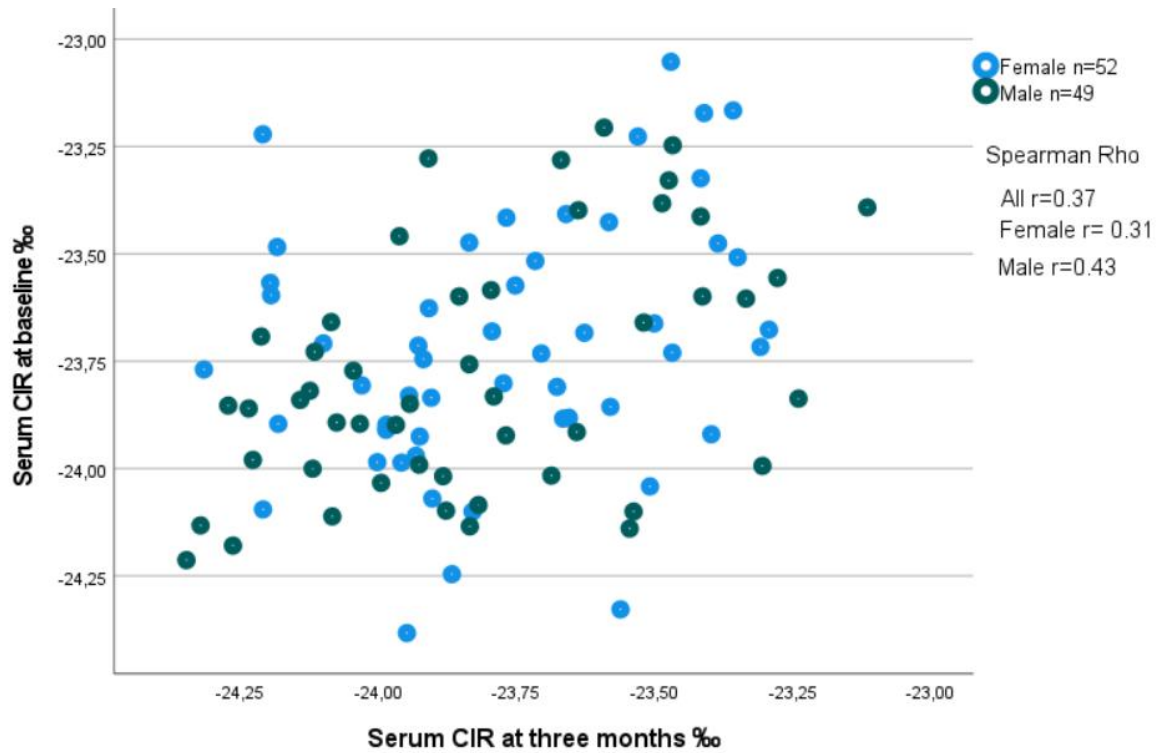
Figure S2 Scatter plot showing the correlation between serum CIR at baseline and three months, separated by sex with Spearman Rho for all (n=101), females (n=52), and males (n=49).
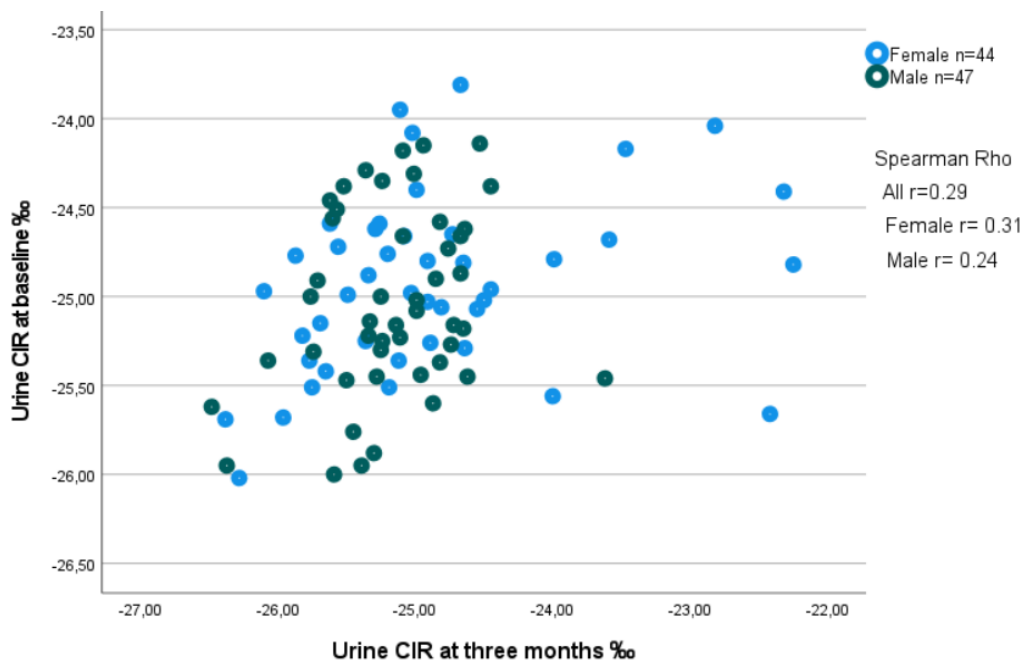

Figure S3 Scatter plot showing the correlation between urine CIR at baseline and three months, separated by sex, with Spearman Rho for all (n=91), females (n=44), and males (n=47).
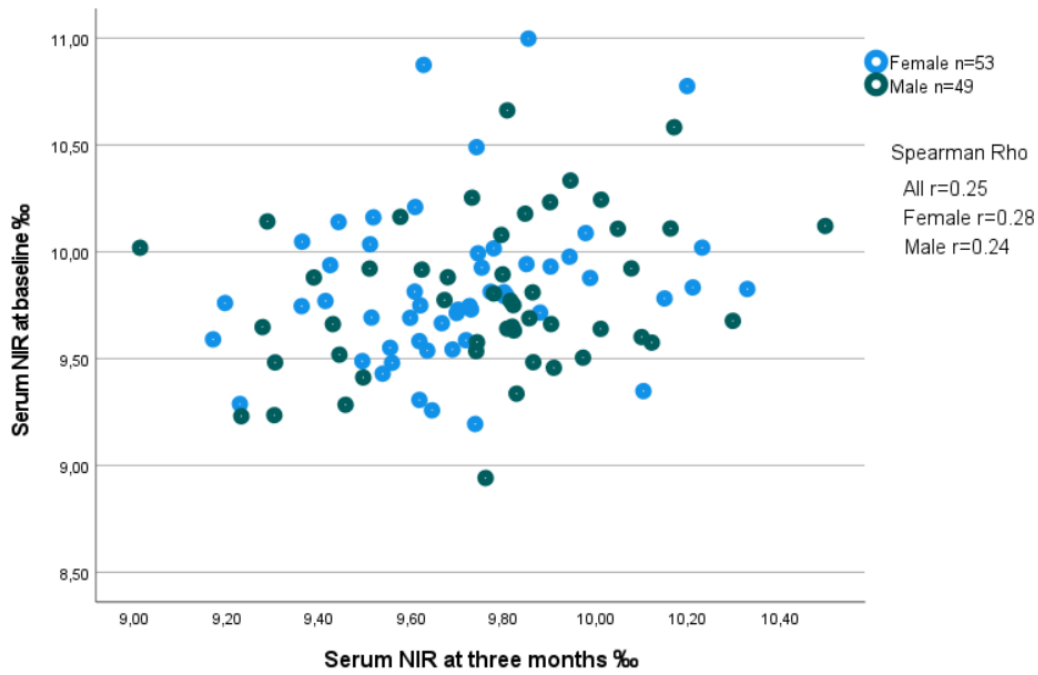
Figure S4 Scatter plot showing the correlation between serum NIR at baseline and three months, separated by sex, with Spearman Rho for all (n=102), females (n=53), and males (n=49)
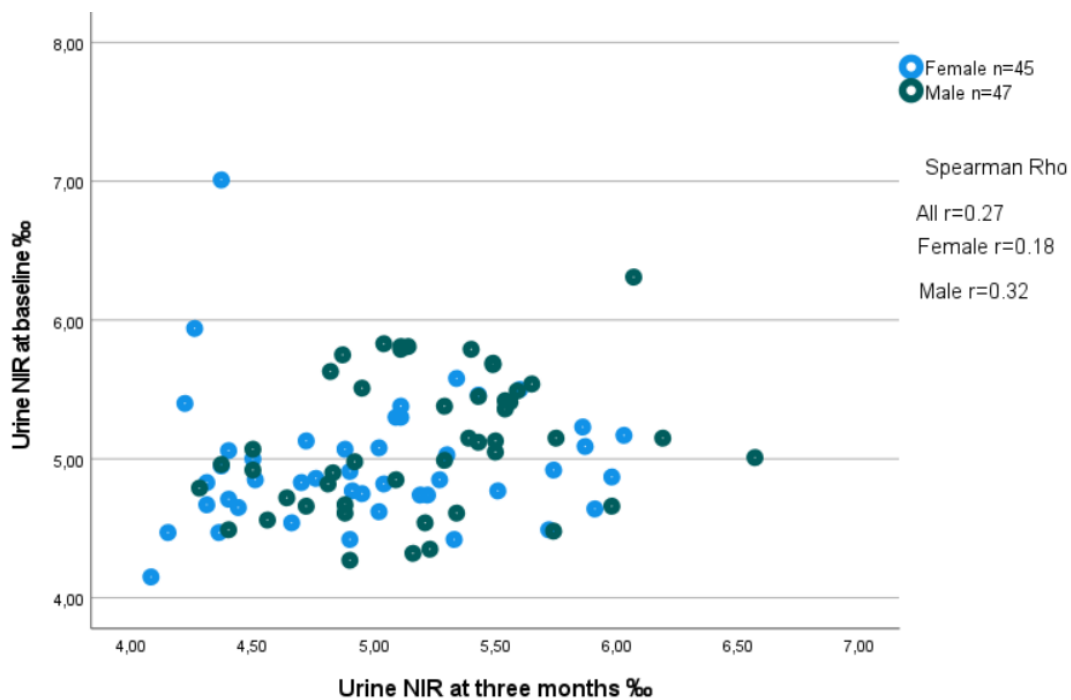

Figure S5 Scatter plot showing the correlation between serum NIR at baseline and three months, separated by sex, with Spearman Rho for all (n=92), females (n=45), and males (n=47)
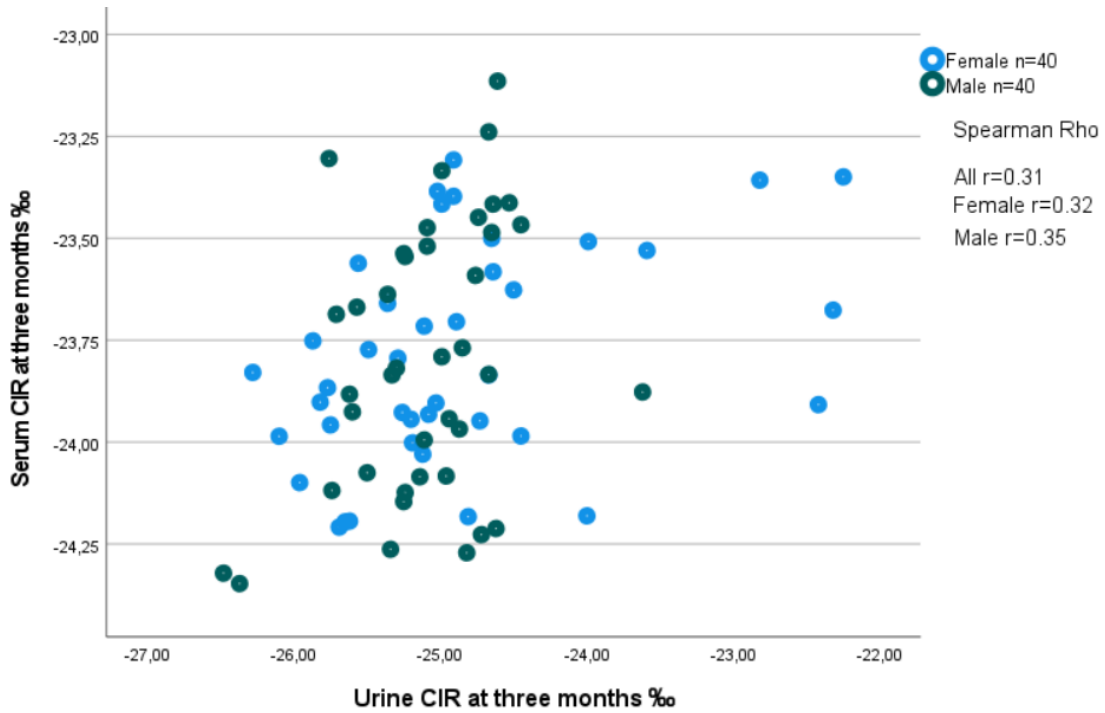
Figure S6 Scatter plot showing the correlation between serum CIR at three months and urine CIR at three months, separated by sex, with Spearman Rho for all (n=80), females (n=40), and males (n=40)
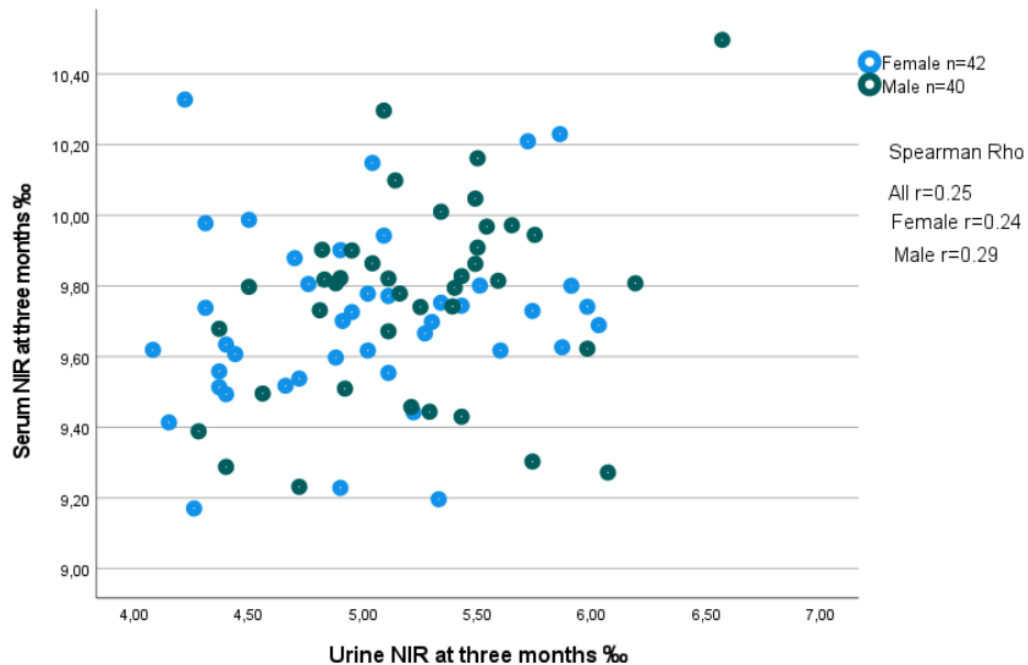

Figure S7 Scatter plot showing the correlation between serum NIR at three months and urine NIR at three months, separated by sex, with Spearman Rho for all (n=82), females (n=42), and males (n=40)

Table S2 Serum and urine CIR and NIR at three months according to diet groups (n=92), median (Interquartile range)

	Acellular HCLF n=34	LCHF n=53	Cellular HCLF n=33
S-CIR %	-23.81 (-24.10- -23.57)	-23.87 (-24.04- --23.52)	-23.71 (-23.92- -23.51)
U-CIR %	-25.24 (-25.59- --24.89)	-25.01 (-25.34- -24.61)	-25.18 (-25.64- --24.80)
S-NIR %	9.68 (9.47-9.86)	9.80 (9.63-9.98)	9.67 (9.52-9.81)
U-NIR %	4.82 (4.40-5.29)	5.32 (4.97-5.74)	4.94 (4.69-5.32)

Note; Missing 7 values from both the acellular and cellular HCLF groups, and 14 values from the LCHF.
Abbrevations; HCLF=High Carbohydrate-Low Fat Diet, LCHF=Low Carbohydrate-High Fat Diet, S-CIR=Serum Carbon Isotope Ratio, S-NIR= Serum Nitrogen Isotope Ratio, U-CIR= Urine Carbon Isotope Ratio, U-NIR= Urine Nitrogen Isotope Ratio