The effect of marine protein hydrolysates on biomarkers related to muscle synthesis and degradation following exhaustive endurance cycling in healthy males

Master's thesis in Clinical Nutrition

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ABSTRACT

Background: To maintain the balance between muscle anabolism and catabolism by influencing the muscle protein synthesis and degradation is crucial to several catabolic diseases to avoid muscle wasting. Previous studies have proposed that marine protein hydrolysates (MPH) have an increased influence on protein synthesis through faster absorption compared to whole proteins like whey, along with positive bioactive functions and other health benefits.

Aim: The overall aim of this thesis is to investigate whether an intake of 20 mg/kg body weight of MPH made from Atlantic cod could have a potential impact on biomarkers related to muscle synthesis and degradation following exhaustive endurance cycling in healthy males.

Methods: This intervention study was performed as a double blind, randomized, placebo-controlled pilot study with crossover design divided into three phases over three separate test days. In total, 14 healthy male volunteers, medium trained and between 40 to 58 years of age were recruited. Phase one consisted of physical assessment and a maximal exercise test. Phase two and three involved the intervention where participants preformed two exhaustive endurance cycling sessions and the ingestion of the intervention drink. The intervention drinks were either MPH or placebo and was only ingested after the first session followed by a standardized meal. Collection of urine was performed to analyze the nitrogen balance, 1-Methylhistidine (1-MH), 3-Methylhistidine (3-MH) and the ratio between 3-MH and creatinine (3-MH/ Creatinine). Blood were collected within the same time intervals throughout both test days whereas 1-MH, 3-MH, creatinine, creatine kinase (CK) and retinol-binding protein 4 (RBP4) were all analyzed.

Results: Urine analysis showed no significant differences between the intake of MPH compared to placebo in nitrogen metabolized ($p = 0.315$), nor in the 3-MH/ Creatinine ratio ($p = 0.066$). The ratio between 1-MH and 3-MH (1- MH/ 3-MH) gave a significant result ($p = 0.028$), however multiple testing showed no significant difference ($p = 0.107$). Linear mixed regression analysis of blood resulted in no significant difference between the two drinks in CK ($p = 0.823$), 1-MH/ 3-MH ($p = 0.595$), 3-MH/ Creatinine ($p = 0.662$) nor RBP4 ($p = 0.052$).

Conclusion: This study has demonstrated the need to further explore and investigate the use of MPH as a supplement to increase muscle synthesis and decrease muscle degradation in humans. Even though this study did not find any significant effect of a single dose containing small amounts of MPH on muscle synthesis and degradation, several results did suggest that there might be improvement through increased protein synthesis with the usage of MPH.

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ABBREVIATIONS

1-MH	=	1-Methylhistidine
3-MH	=	3-Methylhistidine
Anti-HCV	=	Hepatitis C Virus Antibody
ACE	=	Antihypertensive/angiotensin-I Converting Enzyme
BIA	=	Bioeletrical Impedance Analysis
BMI	=	Body Mass Index
BW	=	Body Weight
CI	=	Confidence Interval
CHO	=	Carbohydrates
CK	=	Creatine Kinase
CONJ	=	Conjugate
CPET	=	Cardiopumonary Incremental Excise
CTRL	=	Control
DHA	=	Docosahexanoic Acid
DPI	=	Dietary Protein Intake
DPP-4	=	Dipeptidyl Peptidase-4
E%	=	Percentage of total energy
EDTA-tube	=	Ethylenediaminetetraacetic Acid tube
ELISA	=	Enzyme-Linked Immunosorbent Assay
EPA	=	Eicosatetraenoic Acid
FFM	=	Fat-Free Mass
FM	=	Fat Mass
HBsAg	=	Hepatitis B surface Antigen
HIV	=	Human Immunodeficiency Virus
LC-MS/MS	=	Liquid Chromatography Tandem Mass Spectrometry
LT	=	Lactate Threshold
MCT	=	Medium Chained Triglycerides
MP	=	Marine Peptides
MPH	=	Marine Protein Hydrolysate
NPU	=	Net Protein Utilization
PEW	=	Protein and Energy Wasting
PH	=	Protein Hydrolysates
PVC	=	Peripheral Venous Catheter
RBP4	=	Retinol-Binding Protein 4
REC	=	Regional Committees for Medical and Health Research Ethics
SD	=	Standard Deviation
SMS	=	Short Messenger Service
STD	=	Standard
TBW	=	Total Body Water
TMB	=	Ttramethylbenzidine
VO_{2max}	=	Maximum oxygen uptake
WPC	=	Whey Protein Concentrate

1. INTRODUCTION

Anabolism and catabolism are parts of the body's metabolism where both act at the same time to balance each other. Imbalance between the two could lead to changes in body weight and composition (1). In an anabolic state, the cellular process builds complex molecules from simple molecules to increase or maintain muscle mass. This refers to a state in skeletal muscles where synthesis exceeds degradation (1). A catabolic state on the other hand is the opposite whereas metabolic processes break down complex molecules to simple molecules reducing both total fat- and muscle mass. Referred to as state in skeletal muscle tissue where degradation exceeds synthesis (1).

1.1 MUSCLE SYNTHESIS AND DEGRADATION

The metabolic basis of skeletal muscle growth lies in the relationship between the rates of muscle protein synthesis and muscle protein degradation (2). Expansion of muscles, also termed muscle hypertrophy, is only possible when net protein synthesis occurs (when muscle protein synthesis exceeds degradation) (3). In order to stay healthy, the synthesis of muscle protein is essential to the ongoing growth, maintenance and repair of body tissue (4). On a daily basis, skeletal muscles are exposed to different types of stress that could have a major or minor impact on muscle synthesis. Two important kinds of stress are physical activity and chronic disease.

1.1.1 Physical activity

During physical activity skeletal muscles are often exposed to a great load of stress (3). In all muscular activity, catabolism of the muscle will naturally begin and cannot be stopped (5). Further, as long as a greater amount of protein is available and synthesized at the same time, muscles will be developed and not reduced. Muscular activity and protein synthesis are prerequisite to have a significant development of muscle (5). Through adolescence the synthesis is peaking by the rapid rate of growth of the body, which significantly slows down after reaching roughly 20 years of age. Past this point, muscles will not grow or be strengthened though either sedentary activity, the consumption of particular foods nor supplements alone (5).

Several factors could influence protein requirements in athletes; what kind of exercise, volume, duration and state of training (6, 7), energy density of the diet (8), and carbohydrate content (9) are all relevant factors contributing to this calculation. Research on muscle synthesis and degradation related to physical activity focuses on the type of protein, protein in combination

with carbohydrates and at what time and what rate anabolism occurs compared to catabolism (5).

1.1.2 Chronic diseases

Considering the body is under a lot of stress during chronic diseases, catabolic diseases with rapid weight loss often follows (11, 12). Controlled regulation of muscle mass by maintaining the balance between synthesis and degradation is crucial to several catabolic diseases and unloading to avoid muscle wasting. Muscle wasting, which can lead to severe pathological progression in various diseases and aging, is the result of a decreased rate of protein synthesis and an accelerated rate of protein degradation in skeletal muscles (12). Even though a large portion of protein is stored in the muscles and viscera, the body must strive to protect it from being used as an energy source. During metabolic stress, the body will draw protein out of muscles to meet its needs in contrast to healthy conditions or normal deficits where additional energy is drawn from fat and glycogen stores (10, 11).

-10%	-20%	-30%	-40%
Decreased immunity and increased risk of infection.	Decreased wound healing, increased muscle weakness and increased risk of infection.	Difficulty sitting, pressure ulcers, pneumonia and inability to heal.	Increased risk of death, usually from pneumonia.
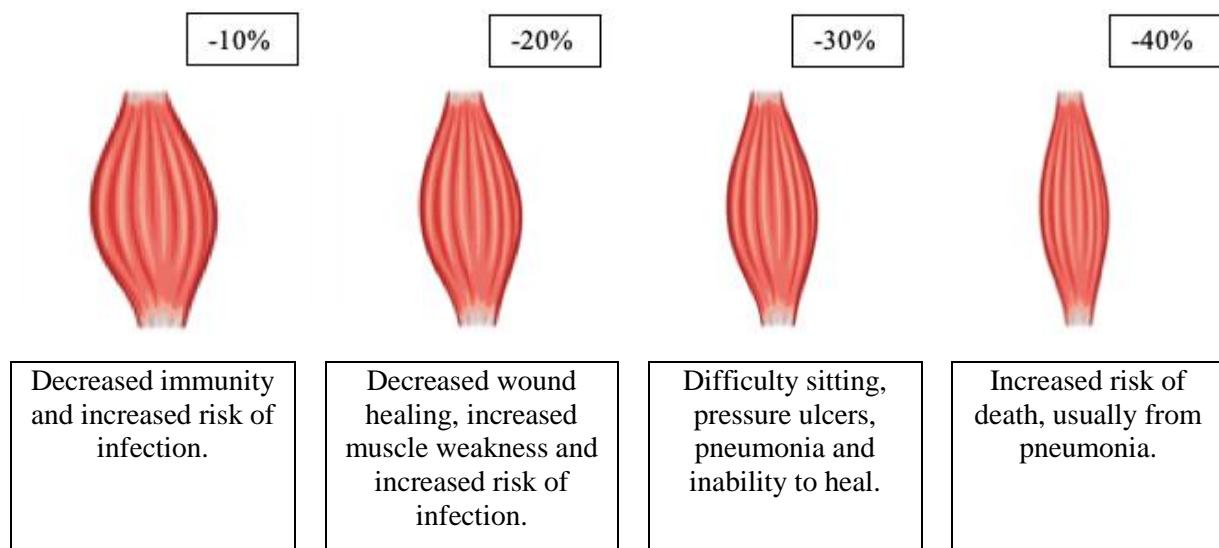
Figure 1: Associated complications to loss of lean muscle mass.

Modified figure from article by Argilies J.M. et.al. (13). The figure shows how pathological progression creates further complications. Already at 10% decreased muscle mass we can see that immunity are decreased and there is an increased risk of infection. This could be crucial to a person who are already struggling with other illnesses or has reduced health because of other age-related health conditions.

Monitoring and assessment of protein and energy nutritional status are also essential to prevent, diagnose and treat protein and energy wasting (PEW). A condition highly prevalent and strongly correlated with increased morbidity and mortality in multiple patient populations (10, 11, 14). PEW is defined as decreased fat mass, reduced somatic protein mass and/or reduced protein pool along with reduced protein and energy intake (11). Monitoring and assessment of PEW is broad and complex, involving indirect measures of visceral protein concentrations, somatic protein stores, energy expenditure and requirements, as well as precise measurements of protein and energy homeostasis (10).

Dietary protein plays a significant role improving muscle protein anabolism and reducing muscle protein catabolism (15, 16). Considering this, optimizing intake and the quality of protein should be a high priority during treatment and prevention of muscle wasting. Previous studies have shown some dietary proteins to be more effective than others. Emerging data have found indications of protein hydrolysates to be significantly more effective at a lower level than that of the whole protein (17, 18). With this in mind, further research is important regarding this area which could improve life quality, health outcomes, and life expectancy in several population groups.

1.2 PROTEIN

The levels of protein in the body is relatively constant throughout the lifespan, despite quantitative and qualitative variations in dietary protein intake (19). Proteins are nitrogen-containing substances that are formed in chains of amino acids and serve as the major structural component of muscles and other tissues in the body. About 10 kilograms of an adult body consist of protein, where approximately 70 % consist of muscle (20).

In total 20 amino acids are identified needed for human growth and metabolism. Twelve of these are termed nonessential, meaning that they can be synthesized by our body. The body are not able to synthesize the remaining eight amino acids, which needs to be consumed through our diets described as essential amino acids. Absence of any of these amino acids will compromise the ability to meet nitrogen and amino acid requirements of growth and maintenance. However, the level and types of protein regarding requirements remain unclear (15, 16).

1.2.1 Protein turnover

The net result of continuous synthesis and degradation of body protein is referred to as protein turnover, which ensures maintenance of optimally functioning proteins as a fundamental biological process (21). To make this process run smoothly, the body is reliant on the availability of protein from the protein pool. Expansion of the protein pool occur either through decreased synthesis, increased degradation or increased dietary protein intake. By contrast, protein pool contraction occur when synthesis increases, degradation decreases or reduced dietary protein intake (22).
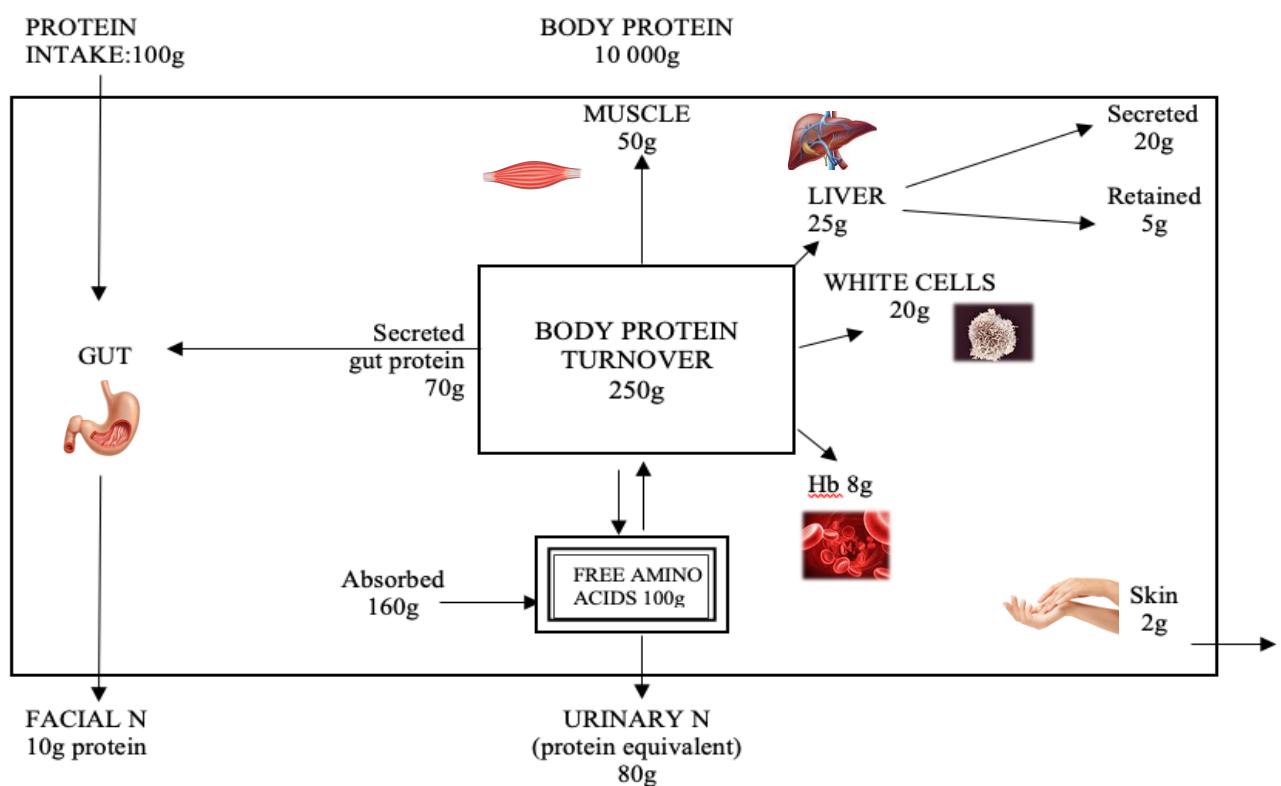
Figure 2: Estimated daily turnover of protein in the body based on a 70 kg male.

Modified figure from published paper by Hellerstein M.K. et.al (23). This figure shows an approximation of how the proteins could be distributed around in the body to be utilized to perform its function.

1.2.2 Functions

All amino acids are based on a common structure consisting of one amino group (NH_2), one organic acid group (COOH), a single hydrogen atom (H), and a side chain (R) all attached to a central carbon atom (24). The side chain is the special component that provides the distinguishing feature. Chains of amino acids differ in length from about 20 amino acid units to many thousands of units. They may be linear or branched and can form complex three-dimensional arrangements. This variety in length and shape of the amino acid chain, including the numerous permutations of the constituent amino acid units, give rise to the wide diversity of proteins in the body with a broad set of functions (25). These functions of protein could be divided into regulatory, structural and energy serving functional groups which are listed in **Table 1**.

1.2.3 Urea cycle

Amino acids from both the diet and the body´s own muscles and tissues are broken down through oxidation (27). During this oxidation process in the liver, ammonia is formed. The urea cycle mainly occurs in the liver and converts highly toxic ammonia to urea (27). Urea is then released into the bloodstream where it travels to the kidneys and is ultimately excreted in the urine. If proper ratios of amino acids are not present at the right time and the body has no possibility of storing amino acids, some amino acids will become oxidized and the nitrogen will be excreted in the urine as urea (27). The body must be supplied with amino acids to replace these even when no net protein synthesis occurs.

Table 1: Protein functions in the human body divided in regulatory, structural and energy serving functional groups.

Group	Function	Description	Example
Regulatory	▪ Enzymes	Enzymes are proteins that aid the thousands of biochemical reactions that take place within and outside of body cells	▪ Digestion ▪ Energy production ▪ Blood clotting ▪ Muscle contraction
	▪ Hormones	Chemical messengers that aid communication between your cells, tissues and organs.	▪ Insulin ▪ Glucagon ▪ Human growth hormone (hGH) ▪ Antidiuretic hormone (ADH) ▪ Adrenocorticotropic hormone (ACTH)
	▪ Maintains proper pH	Proteins play a vital role in regulation the concentrations of acids and bases in the blood and other body fluids.	▪ Haemoglobin
	▪ Balances fluids	Regulation of body processes to maintain fluid balance.	▪ Albumin
	▪ Immune health	Support forming antibodies	▪ Globulin
	▪ Transport nutrients	Transport proteins carry substances throughout the bloodstream – into cells, out of cells or within cells.	▪ Haemoglobin ▪ Glucose transporters (GLUT) ▪ Lipoproteins
	▪ Stores nutrients	Protein also stores different kinds of nutrients like iron.	▪ Ferritin ▪ Casein
Structural	▪ Growth and Maintenance	Under normal circumstances, the body breaks down the same amount of protein that it uses to build and repair tissue.	▪ Muscle ▪ Connective tissue ▪ Numerous specialist cells
	▪ Keratin ▪ Collagen ▪ Elastin	Some proteins are fibrous and provide cells and tissues with stiffness and rigidity.	▪ Hair, skin and nails ▪ Bones, ligaments, tendons and skin ▪ Uterus, lungs and arteries
Energy	▪ Supply the body with energy	Proteins can also be used as energy; however, they are not the primary choice as an energy source. Amino acids from protein supplied in addition to what is needed to build proteins will be used as an energy source, mainly by being oxidized in the liver through the urea cycle.	▪ Fasting ▪ Exhaustive exercise ▪ Inadequate calorie intake

Reference: Healthline (26).

1.3 MARINE PROTEIN HYDROLYSATE

The fact is that protein nutrition is complex. In addition to the amount of protein, multiple other factors could play a significant role in protein synthesis. First, proteins from different sources vary in rates of absorption and quality (51, 52). So far studies have considered whey protein to be the superior one, especially in athletes because of the building and recovery of muscle potential (21, 25). Secondly, proteins could be manipulated and hydrolysed meaning that the protein is broken into peptides. These peptides have been considered to have a more rapid uptake and availability in the body compared to whole proteins (53). Thirdly, as hydrolysed proteins could be significant faster absorbed than intact proteins, they may affect the secretion of hormones like insulin differently from other proteins (53). It is reason to believe that hydrolysed proteins are superior to intact proteins and free amino acids in terms of skeletal muscle protein anabolism (54-56).

1.3.1 Protein hydrolysate

Protein hydrolysates (PH) are produced from purified protein sources by heating with acid or preferably, addition of proteolytic enzymes followed by purification procedures (54). Each PH is a complex mixture of peptides of different chain length providing mainly di- and tripeptides together with free amino acids. Approximately 60% of dietary protein ingested is absorbed along the intestine as di- and tripeptides and have a greater resistance to gastric acid and heat (57). In addition, they are said to be absorbed along a separate route than free amino acids, which could be one explanation of why they might be easier absorbed and taken advantage of in the body (58). Studies have shown that especially hydrolysates of marine proteins have bioactive functions, and positive health effects have been noted in several areas (59).

1.3.2 Marine protein

There is a high potential in marine bioprocessing industries to convert and utilize marine food products and their bi-products as valuable functional ingredients (51). Hydrolysed proteins from fish, marine protein hydrolysates (MPH), have gradually gained more attention due to potential valuable health-related outcomes. Substantial effects of hydrolysed fish proteins on metabolism have been shown in rats (60-62). Marine proteins are leucine rich and well balanced in other essential amino acids (53, 63). Inspired by this, two randomized controlled trial studies with crossover design and great similarities in methods, investigated effects of MPH ingestion during endurance cycling in men (64, 65). Vegge et al. found no influences of MPH on

metabolism. They did however find improved cycling performance in those participants with the lowest exercise capacity, and thus indicated that MPH provided ergogenic effects in less trained athletes (65). On the other hand, results found by Siegler et al. demonstrated metabolic influences of MPH but did not find effects on performance (64).

Furthermore, proteins from seafood may have properties different from those in meat (52). In an intervention study using a crossover design, healthy volunteers consumed meals with similar compositions with exception of the protein source (52). Distribution of fat, carbohydrates and protein were equal in the diet, and in the meals containing meat, cod liver oil was added to balance the long-chained fatty acids in fish. In the study group of which the protein source was fish, researchers found decreased risk factors of cardiovascular disease providing evidence favoring fish to have health benefits. Also, another study showed that 4 weeks of supplementation of 3g fish protein tablets increased the percentage of muscle and decreased the percentage of body fat in overweight adults (66).

1.3.3 Potential health-related outcomes

In everyday life the human body constantly undergoes physiological imbalances and are exposed to extrinsic toxic substances on a regular basis that can disturb mandatory functions. This disturbance could lead to various health problems (67). Meanwhile a lot of processed foods and changes to the raw product cause physical, biological and chemical food spoilage or loss of nutrition characteristics, hydrolysis of proteins to peptides could actually be more beneficial in the body (51). MPH have exhibited potent biological activities like antimicrobial, antiviral, antitumor/cytotoxic, antihypertensive/angiotensin-I converting enzyme (ACE) inhibitory, antioxidant, cardiovascular protective, immunomodulatory neuropeptides, neuroprotective, anti-diabetic, appetite suppressing and other bioactivities (51, 59, 68).

From a nutrition point of view, marine sources compared to other diet sources provide the favorable fatty acid composition EPA (Eicosatetraenoic acid) and DHA (Docosahexaenoic acid) proven to have health benefits (51). Considering the bioactivity's mentioned above, some MPH or their derivates attract the attention of the pharmaceutical and nutraceutical industry, attempting to designing them to be used in treatment or prevention of various diseases (59, 68). A large number are already in different phases of the clinical and preclinical run, but there is still a lot of research and investigation to be performed following this current topic.

1.4 ASSESSMENT OF MUSCLE PROTEIN SYNTHESIS AND DEGRADATION

Protein's unique function in supporting cellular growth and development elevates its significance in nutrition assessment. No specific laboratory value can determine the precise protein status of an individual, and each test has its own limitations. Therefore multiple different measurements are required hence no single method to assess nutritional status and responses to nutritional intervention can be considered as a "gold standard" (10, 28). Some commonly used measures are the following:

1.4.1 Somatic Protein status

Somatic protein status is considered measures of the skeletal muscles. Such measures could be both anthropometric or biochemical in the form of excreted biomarkers linked to muscle degradation in urine or blood (10).

1.4.1.1 Bioelectrical impedance analysis

Bioelectrical impedance analysis (BIA) is a technique proven to be safe, easy to use, and generally acceptable to patients. It is an indirectly measure of the body composition used for determining fluid management, but the use has also increased to evaluate protein-energy status (29). The principle behind is based on a two-compartment model that determines total body water (TBW) and soft tissue (i.e. fat-free mass (FFM) and fat mass (FM)) by using electrical currents. Electrical currents are sent through the body between electrodes attached on the ankles and wrists, or by standing barefoot on a BIA machine holding one handle in each hand. Lean body tissues, which contain body fluids and electrolytes, have highly conductive, low resistance electrical pathways. Skin, bone and fat, on the other hand, are very poor conductors and offer high resistance (10).

Reactance is the opposition to the flow of electrical current due to the electrical capacitance such as is found in the cell membrane wall. Resistance is defined as the extra-cellular and intra-cellular fluid content, while the reactance is based on the cell membrane content (30). In FFM, these fluid compartments are parallel components separated by cell membranes; therefore, parallel models are more accurate for determining their impedance (30).
The relationship between reactance and resistance is called the phase angle (10). Each person's phase angle is a direct measurement of their cell membrane integrity, and the distribution of

water within and outside the cell. It gives an indicator of good health by increasing when there are large quantities of intact cell membranes in the skeletal muscle and body cell mass. Moreover, the phase angle will decrease when a person is ill and cell membranes have been attacked. With increased age, the phase angle also decreases, but if you manage to increase the phase angle, aging is slowed down (30). This device might be superior by its ability to identify patients who have clinically important PEW and a poor prognosis.

1.4.1.2 Nitrogen

Nitrogen is a fundamental main body component of amino acids which the significant amount in the diet is bound in protein (25). It is required for both the production of several nitrogenous compounds involved in a variety of functions (immune mediators, hormones, antioxidant defenses, neurotransmitters etc.) and protein synthesis (19). Thus, the nitrogen content should be both qualitatively and quantitatively normal as well as normally maintained to ensure normal body functions (31).

Nitrogen homeostasis is achieved via complex series of changes to the rates of body protein turnover, amino acid oxidation, urea production and nitrogen excretion that occur during the postprandial and post absorptive periods of the day (31). Respectively, measuring nitrogen intake and loss can be used to study protein metabolism and give a measure of protein balance by analysis of the nitrogen content in urine. Nitrogen balance is commonly divided into three basic states; positive, equilibrium, and negative nitrogen balance further described in **Table 2**.

Table 2: Basic states commonly used about nitrogen balance.

Basic state	Description
Positive	When nitrogen uptake exceeds the excretion and there is an increase in the total body pool of protein, we are talking about a positive nitrogen balance. This state is associated with periods of growth, hyperthyroidism, tissue repair, at rehabilitation centers following abnormal diseases, and pregnancy. It is the optimal state of muscle growth called the anabolic state of the body.
Equilibrium	This state represents the normal state where uptake and excretion of nitrogen are equal, the perfect balance of nitrogen in the body to maintain steady-state. Healthy adults eating a balanced diet.
Negative	Where nitrogen excretion exceeds nitrogen uptake and the body is turned to a catabolic state. The organism loses tissue, nitrogen is drawn away from muscle where it is needed for growth and from vital organs where serious damage can occur. A negative nitrogen balance could be used as a part of clinical evaluation of malnutrition, and are associated with serious tissue injuries, wasting diseases, burns, hyperthyroidism, fever, and during periods of fasting.

Reference: Article published by Tessari P. (31).

A neutral nitrogen balance, defined as the equilibrium between intake and loss of nitrogen, is essential in order to maintain good health (31). It is all relying on two main parts of the metabolism; anabolism and catabolism which anabolism usually is highly dependent on dietary nutrient intake. A dietary protein intake (DPI) of 0.6 g/kg/day is considered sufficient to achieve equilibrium in most adults, when adequate energy intake and protein with a high biological value is ingested (19, 32). On the other hand, compensatory mechanisms allowing more efficient utilization of nutrients through a reduction in protein breakdown and amino acid oxidation are activated if protein and energy requirements are not met through the diet (33, 34).

The adequacy of protein intake can be determined on behalf of a positive or negative protein balance in patients. However, in most cases direct and precise measures of nitrogen balance are not yet available, and indirect measures of protein balance through estimates of net protein utilization (NPU) is necessary (10). A common method used as an indicator involves measuring urine urea nitrogen loss - as 90% of nitrogen is lost through the urine via the kidneys (31). It is also very important to know exactly how much protein the person have consumed to make a correct estimation by using metabolized nitrogen from the urine result. However, there could be differences in excretion following protein quality. Diets containing poor quality protein have been associated with an increased nitrogen losses due to the inefficient utilization of indispensable amino acids (35).

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1.4.1.3 Creatine kinase

Creatine kinase (CK), formerly known as creatine phosphokinase is an intracellular enzyme present in great amounts in the brain, myocardium, and skeletal muscle; smaller amounts occur in other visceral tissues (36, 37). Like other enzymes, CK is leaked into the bloodstream when a cell becomes damaged. If many cells are damaged at the same time, a detectable level of CK and other enzymes could occur in the blood. CK appear different according to the location it is leaked from making it a useful diagnostic tool to detect the damage muscle tissue which can further be used in diagnosis (36). A serum CK test can detect many conditions such as a heart attack, muscle breakdown, and even autoimmune diseases which are attacking certain organs and tissues (36, 37).

On the other side, the small amount of CK that at a regular basis can be found in the blood comes primarily from skeletal muscles (36, 37). Any condition that causes muscle damage and/or interferes with muscle energy production or impact like strenuous exercise and inflammation of muscles (myositis) or muscle diseases (myopathies) such as muscular dystrophy can cause an increase of CK in the blood (37). Because of the many current assay methods in use, there is no standard reference value for serum CK (36). Normal values are best determined locally based on the method employed and the range for healthy controls.

1.4.1.4 3-Methylhistidine

3-Methylhistidine (3-MH) is an integral part of myofibrillar proteins found in both actin and myosin (38). It is formed by methylation of histidine as a posttranslational modification of these myofibrillar proteins. 3-MH is liberated during degradation of myofibrillar protein, meaning it is not metabolized or used in protein synthesis, but excreted unchanged into the urine (39). As long as protein synthesis and degradation stay steady, the amount of 3-MH should reflect an image of the human body muscle mass. 3-MH has been shown to be elevated after physical exercise, arthritis, neuromuscular disorders, terminal stages of serious illness, muscular dystrophy, and muscle wasting (40).

About 45% of the weight of an adult man is compromised by muscle (20). During conditions like injury and starvation, but also neuromuscular, endocrine and malignant disease muscle, mass falls rapidly (41). This rapid change in mass could be a result of both decreased synthesis or increased degradation. The rate of myofibrillar protein breakdown could theoretically be

measured with 3-MH analyzed from urine excretion, but changes in the rate of synthesis of muscle cannot be measured non-isotopically.

As 3-MH in plasma or urine may serve as a marker of muscle degradation, it also reflects dietary meat intake (41, 42). Urinary 3-MH as an indicator of myofibrillar protein catabolism may be compromised in clinical studies, as patients cannot always make correct statements about their food intake. Furthermore, a 72hour meat-free period as the commonly recommended may be insufficient to guarantee that results are not compromised. Renal excretion could be impaired due to functional or organic disorders, or absorption and emptying can be delayed by an earlier meal containing meat (39). In addition, under normal conditions there is a interindividual variation of 3-MH excretion in man. Moreover, it is not necessarily easy to collect 24hour urine in patients precisely (42).

On the other hand, studies have shown that in healthy adults we could get a pretty good estimate. Endogenous versus dietary 3-MH can be distinguished by simultaneous measurement of 1-methylhistidine (1-MH) (39). That is built on several reasons where 1-MH is not formed in humans, but occurs in skeletal muscles of several other species, including dietary meat (39). These two parameters have the same halftime and similar dose-independent kinetics irrespective of dietary source. There is only a small intraindividual variation in their basal excretion, making it possible to separate and estimate the true excretion of 3-MH representing myofibrillar protein breakdown from skeletal muscle away from dietary meat intake with the 1-MH/ 3-MH ratio (39, 40).

Urinary excretion of 3-MH has been used to determine the rate of skeletal muscle degradation as mentioned above (41, 42, 44). However, the presence of 3-MH in the urine does not necessarily reflect the specific breakdown of myofibrillar protein since this amino acid is also released from tissues other than skeletal muscle (45). This method requires several hours of urine collection, and thus may not be useful to detect acute changes in the rate of skeletal protein degradation. Monitoring 3-MH in the plasma has also been evaluated as means of determining the rate of skeletal muscle degradation in addition to urine measures (45). This procedure is thought to be a more sensitive measure of identifying acute changes in the rate of myofibrillar degradation (45). However, the 3-MH present in the plasma may not be entirely of skeletal muscle origin either, as it has also been detected in cardiac and smooth muscle and may be affected by renal function or blood flow (45).

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1.4.2 Visceral protein status

Visceral protein concentrations refer to biochemical markers present in serum or plasma. These markers are circulating proteins that estimate the size of the visceral protein pool in the body (43). Almost all circulating visceral proteins have certain physiologic roles such as serving as carriers or binders, or having an active involvement in vital functions such as the immune system.

1.4.2.1 Retinol-binding protein 4

Retinol- binding protein 4 (RBP4) is an acute-phase respondent synthesized by the liver and serves as the transport protein for vitamin A which forms a complex with prealbumin in blood (46, 47). Once the vitamin has been delivered to the target cell, the molecule also loses its affinity for pre-albumin (48). The free RBP4 molecule is rapidly filtered at the glomerulus and catabolized in the renal tubules after reabsorption by the proximal tubular cells. RBP4 has the smallest body pool and shortest half-life (12hours) of the serum proteins (47, 49).

Assessment of RBP4 is used to determine visceral protein mass in health-related nutritional studies (50). Theoretically, it is most likely better to reflect recent dietary intake of short-term changes and responses to nutrition support interventions, rather than an indicator of overall nutrition status considering the short half-time (49). RBP4 is considered to be one of the more sensitive indicators of protein status in the non-critical ill, where it is a measure of the protein pool available in the body (47).

1.5 AIM OF THE STUDY

The overall aim of this thesis was to investigate whether intake of 20 mg/kg body weight (BW) of MPH made from Atlantic cod could have a potential impact on biomarkers related to muscle synthesis and degradation following exhaustive endurance cycling in healthy males. We wanted to explore if there was possibility that a single dose of MPH in addition to whey protein could be superior to whey protein alone at influencing these biomarkers.

1.5.1 Hypothesis:

H_0: The intake of a single dose of 20 mg/kg BW of MPH will not show significant impact on biomarkers related to muscle synthesis and degradation.

H_1: The intake of a single dose of 20 mg/kg BW of MPH will show significant impact on biomarkers related to muscle synthesis and degradation.

1.5.2 Research questions:

1. Does a single dose of MPH have an impact on nitrogen balance related to muscle synthesis and degradation?

2. Could a single dose of MPH influence the secretion of CK and 3-MH related to muscle degradation?

3. How does a single dose of MPH influence the RBP4 level in blood related to the body protein pool?

2. METHOD

2.1 STUDY DESIGN

This intervention study was performed as a double blind, randomized, placebo-controlled pilot study with crossover design divided into three phases. The intervention was a collaboration conducted by The Department of Clinical Science and The Department of Physiotherapy at Haukeland University Hospital, University of Bergen and The Western Norway University of Applied Science. Data collections and analyzing were carried out from September 2017 to February 2019.

2.2 RECRUITMENT OF PARTICIPANTS

A total of 14 participants were estimated to be the decisive number needed in the study. This calculation was based on a power estimation of mean changes in blood glucose profile (area below the curve) of 20%, alpha of 0.05, 80% effect and a standard deviation (SD) of 10%. Due to a small sample size and the goal of achieving low variation with a homogeneous test group, women were not recruited in this study to avoid possible hormone and anthropometric differences.

The recruitment was conducted by reaching out through advertising in social media, but also with an informing poster sent through email directly to different bicycle clubs in Bergen, July 2017 (**Appendix 1**). All potential participants responding to the advertisement were screened according to the inclusion and exclusion criteria (**Table 3**) of the study through email. This resulted in 14 healthy male volunteers, medium trained with a total amount of 8-12 hours weekly training and cycling as their main exercise activity between 40 to 58 years of age were recruited.

Participants recruited were primarily reached through email, except for a short message service (SMS) reminder on the day before assigned time and date of the intervention days.

Table 3: Study inclusion and exclusion criteria.

Inclusion criteria

- Signed informed consent form (**Appendix 2**).

- Willing to comply with all study procedures and be available for the length of the study.

- The participant is a man between 40 to 58 years of age.

- The participant has a BMI between 19-29.

- The participant is in good general health according to the physician at the screening visit.

Exclusion criteria

- Treated with medication that affects the intestinal function such as H2- blockers, inhibitors, diuretics, antiemetics, antidepressants, or antacids.

- Treated with antibiotics within 3 months prior to screening (oral, parenteral or rectal), but not spray or ointment.

- Treated with steroids within 1 month prior to screening (including oral drink).

- The participant has taken any investigational drugs within one month prior to screening.

- The participant has had surgery or trauma with significant blood loss or has donated blood within the last three months prior to the screening visit.

- Diabetes type 1 or 2, or persistent high blood sugar levels.

- The subject has tested positive for HIV.

- HBsAg or anti-HCV positive.

Abbreviations: BMI = body mass index, HIV = human immunodeficiency virus, HBsAg = Hepatitis B surface antigen, anti-HCV = hepatitis C virus antibody.

2.3 COURSE OF THE INTERVENTION STUDY

The intervention was conducted at The Western Norway University of Applied Sciences sports lab and divided into three phases. Phase number one consisted of physical assessment, health status and baseline measurements of body composition 7-14 days before the first intervention day. Intervention day one and two, referred to as phase two and three, were both build up in the same manner with the participants meeting in the fasting state at their individual assigned time to consume a standardized meal followed by high-intensity cycling test until exhaustion, ingestion of the intervention drink, and blood collection repeated in two different intervals.

2.3.1 Phase one

On their first visit all participants were informed about the aim of the study and how the intervention would proceed. A written informed consent form was handed out and collected before further assessment was made.

Screening of health status was based on self-reported questionnaires and a physical assessment performed by a physician. Following approved physical assessment, a maximal exercise test was preformed to find the lactate threshold (LT) and the estimate of the maximum oxygen uptake (VO_{2max}) through a cardiopulmonary incremental exercise test (CPET). A body composition analysis was performed with an InBody 720 scanner (InBody Co., Ltd, Cerritos, California, USA) accompanied by height and weight measurements. This way all participants were registered with total body weight and height, FFM, FM, muscle mass (MM) and body mass index (BMI) before start-up.

2.3.2 Phase two

To their second visit, participants were allocated a date and time 9-16 days after phase one. The second phase consisted of two sessions of cycling until exhaustion with a four-hour recovery period in between and a diet intervention.

All participants met to their given date and timeslots in the fasting state. Upon arrival information of the logistics and procedures of the day were carefully described. Further, a peripheral venous catheter (PVC) was inserted into an antecubital vein in each participant. Before the standardized breakfast meal was served (section 2.3.4), baseline blood samples in the fasting state were conducted. The participants each received a designated can to collect urine throughout the day. Water intake was also recorded, with a water restriction of 2.5-3.0 L while testing.

One hour after they finished their standardized breakfast, the first exercise session began. Each exercise session was initiated with a 20 minutes warm-up at 60% of VO_{2max}, followed by the first increase of load to reach 90% of VO_{2max} until 5 minutes had passed, and then another increase of load to reach 95 % of VO_{2max} until exhaustion. When the participant reached the exhausted level of both exercise session, venous blood was collected at 0, 15, 30, 60, 90, and 120 minutes accompanied by questionnaires regarding abdominal pain, satiety, hunger, nausea,

desire to eat, and diarrhea (**Appendix 8 and Appendix 9**). Immediately after the exhaustion exercise test was completed and before the blood sample at 0 minutes was collected, the participants each had the choice of choosing one of the intervention test drinks, A or B, without anyone participating in the study knowing which of the drink contained MPH.

Following the blood collection at 120 minutes, a standardized heated dinner meal was immediately served (section 2.3.4) followed by a two-hour long recovery period before the next exhaustion test session, four hours of recovery all together. The next session was performed in the same manner as the first one, with the exception that they did not receive the intervention drink after the exhaustion test. When the last blood sample at 120 minutes was collected, the participants were free to go home.

2.3.3 Phase three

At their third and last visit, the participants met at the same time as their second visit to make the time of the day consistent to avoid circadian variance. They had then been through a seven-day washout period since the last visit, phase two. Each participant was informed that they were going to follow the exact same procedure as their previous visit in this final phase. However, to complete the crossover testing of the study, each participant received the reverse intervention test drink of which they choose in phase two. **Figure 3** shows the flow-chart with an overview of the course throughout phase two and three of the study.
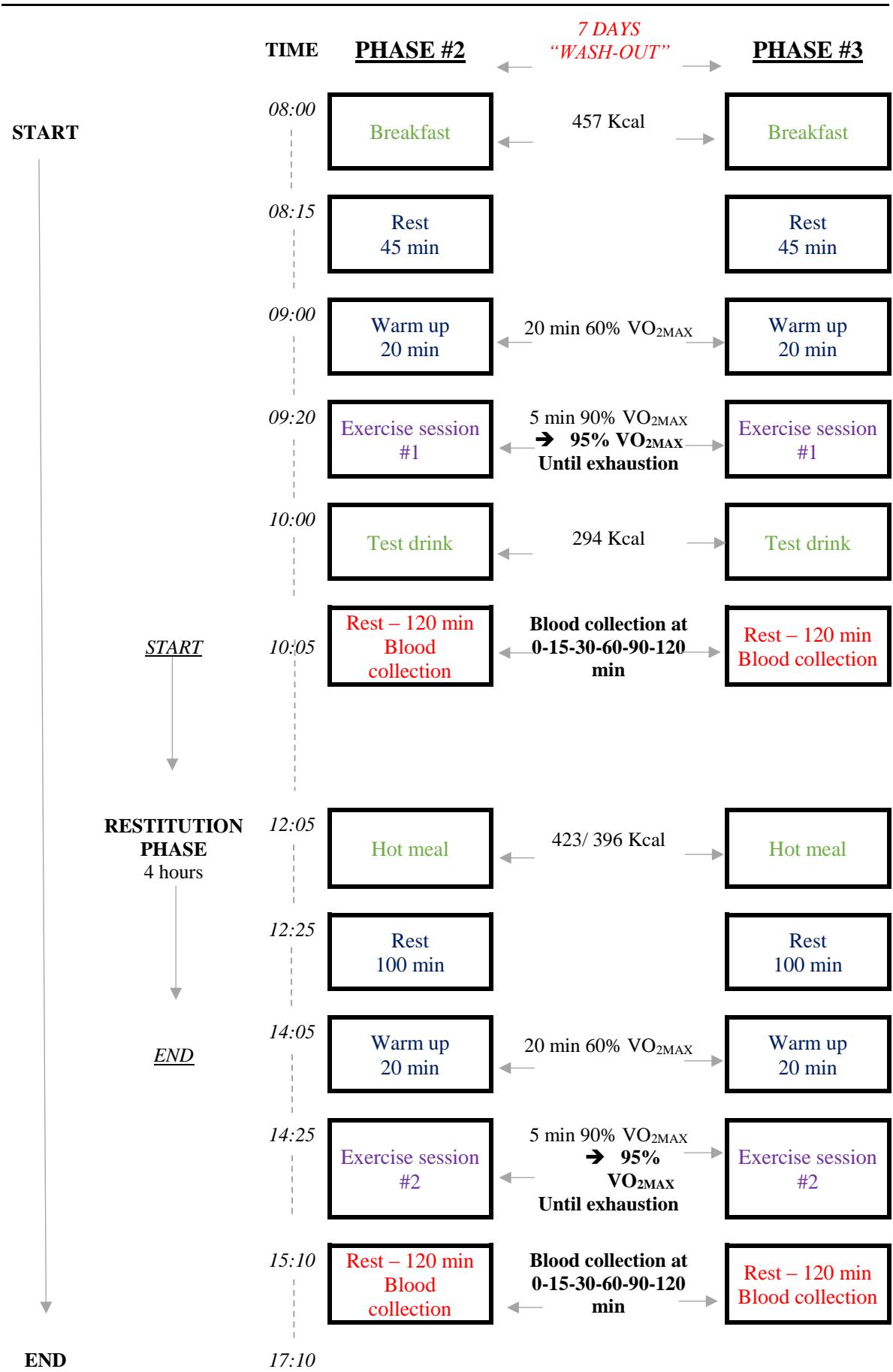
Figure 3: Flow-chart of phase two and phase three of the intervention study.

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2.3.4 Standardized meals

During phase two and three of the intervention, the participants were served two standardized meals each with the same content at both visits. Breakfast was the first meal served right after information about logistics and the procedures of the day and fasting blood samples were collected. This was a standardized meal delivered form a local pastry shop at Kronstad, Bergen. The meal consisted of whole grain baguette with lettuce, pepper, cucumber, ham and cheese with a cup of coffee, tea or a glass of orange juice on the side by choice.

The second meal was served after the last blood sample at 120 minutes past the exhaustion exercise. This was a heated meal with two options because one of the participants turned out to be a pescatarian (excluding white and red meat from their diet, but do include fish and other seafood). The two options turned out to be beef stroganoff (69) and cod with leek sauce (70) both from the same producer, Fjordland AS. An overview of the nutritional value of the composition in both standardized meals are shown in **Table 4** excluding lettuce, pepper, cucumber, tea, coffee and water considering these does not contain any significant amounts of nutrients that would have influenced any of the important values in this study.

Table 4: Nutritional content of the standardized meals given in phase two and three.

Standardized meals	Quantity (g)	Energy (Kcal)	Fat (g)	CHO (g)	Protein (g)
Breakfast;					
Bread	92.6	228.0	2.0	41.9	8.0
Ham	24.9	26.0	0.9	0.0	4.5
Cheese	33.3	117.0	9.0	0.0	9.0
Total:	150.7	457.0	12.3	61.1	22.9
Orange juice	200.0	86.0	0.4	19.2	1.4
Hot meal;					
Beef stroganoff,					
Total:	460.0	409.0	8.3	59.8	26.7
Cod in leek sauce,					
Total:	550.0	391.0	5.5	37.4	30.8

Abbreviation: CHO = carbohydrate

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2.3.5 Test drink; MPH or placebo

In the second and third phase of the study each participant received an intervention drink after the first endurance cycling test was performed. They were given the free choice of choosing between test drink A or B in phase two. This choice then decided which drink they would be given in phase three, as it would be the opposite of what they had chosen in phase two (**Figure 4**). The content ratio between macronutrients were respectively the same in both drinks with 12 E% protein, 22 E% fat, and 66 E% carbohydrate. They were both isoenergetic and isonitrogenous which mean that they contained the same amount of energy and nitrogen avoiding unnecessary variance in nitrogen intake. The energy content was measured to be 3.68 kcal/kg BW and each intervention drink was carefully fitted to each participant to match their BW.
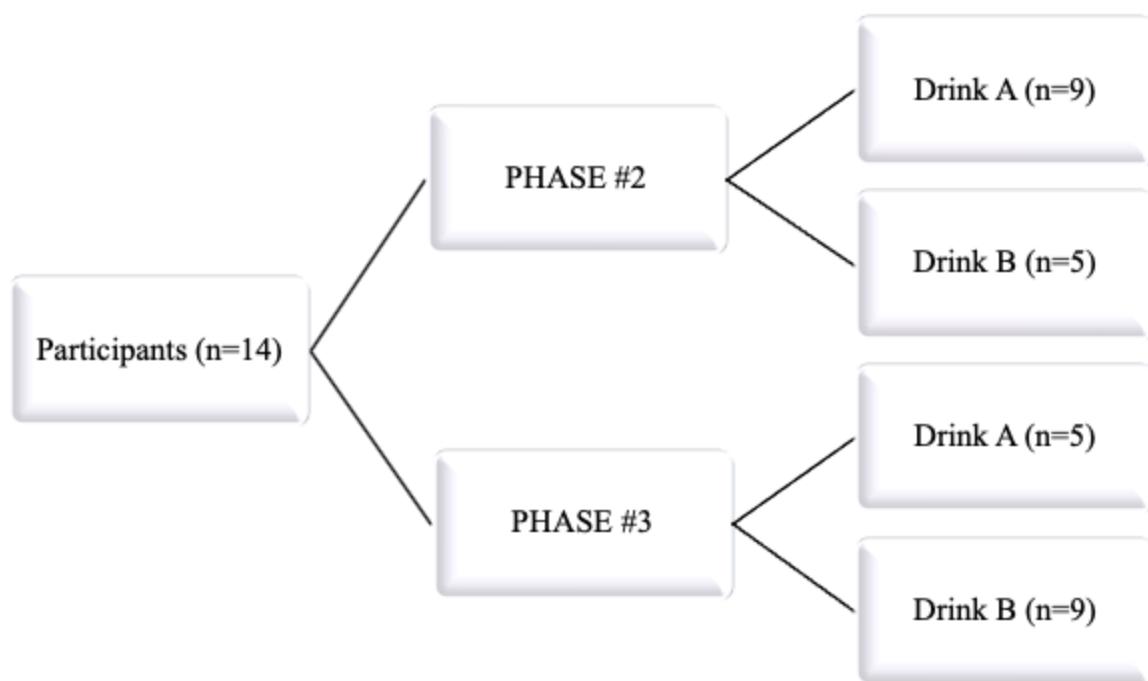
Figure 4: Randomization of the intervention drinks.
In phase two each participant chose if they wanted drink A or B, this resulted in nine participants choosing drink A and five drink B. They then received the opposite in phase three.

One of the intervention drinks were a placebo consisting of whey protein concentrate (WPC80 from Tine ASA, Bergen), vegetable fat (medium chained triglycerides (MCT) fat powder, 70:30 from BERGMAST) and maltodextrins (DE 20-21) derived from corn. The other test drink was the MPH drink were all the main components were equal to the placebo, except for 3.2 E% of

the WPC protein was replaced with MPH made of fish filet from Atlantic cod. 3.2 E% equals about 20 mg of MPH/kg BW.
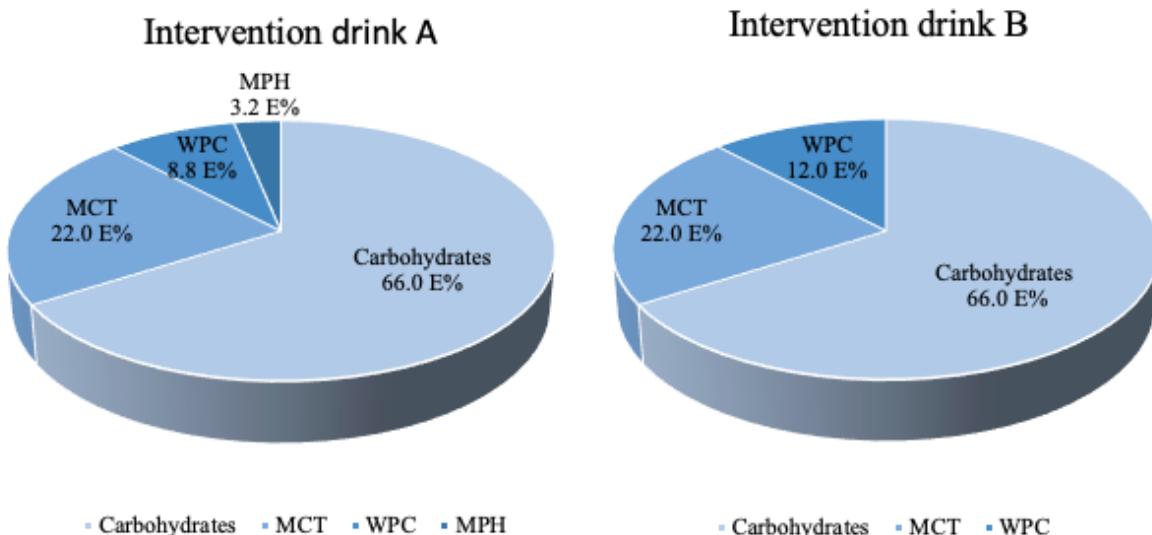
Figure 5: Nutritional content in the intervention drinks.
Intervention drink A: 8.8 E% WPC, 3.2 E% MPH, 22 E% MCT, and 66 E% Carbohydrates.
Intervention drink B: 12 E% WPC, 22 E% MCT, and 66 E% Carbohydrates.
Abbreviation: E%= energy percent, MPH= marine protein hydrolysate, WPC= whey protein concentrate, MCT= medium chained triglycerides.

As the drinks came in powder form, the powder had to be dissolved in cold water into a creamy drink using the ratio of 2 ml cold water to 1 g powder. The final mix between these components was made approximately 30 minutes before the participants had completed their first exhaustion test. Both products were complemented with the technical natural flavor of strawberry and a light pink coloring agent to even out a potential difference in taste and smell, respectively.

2.3.6 Restrictions during the study period

A few restrictions during the intervention period was necessary. Participants were not allowed to drink more than five cups of coffee a day and had to avoid alcohol consumption 48 hours prior to each visit. Each participant was informed to abstain from exercise 24 hours prior to all intervention days. They were told to maintain approximately the same intensity, volume and frequency of their training in between phase two and three as before phase two. This was done to make the starting point as similar as possible. Also, as described in the exclusion criteria

(**Table 3**), medication that could affect the intestinal function was not allowed. To be able to control differences in dietary habits, they were all instructed and informed how to conduct a three-day dietary intake register before phase two and one-day dietary intake registration before phase three.

2.3.7 Blinding

Throughout the intervention all participants, technicians and researches were blinded to which content the different bottles contained until most of the statistical analyses were completed. To make this possible, both the intervention test drink and placebo were in identical opaque glass bottles marked with only the letter A or B and the participants ID when provided from the manufacture Firmenich Bjørge Biomarin AS (Aalesund, Norway).

2.4 DATA COLLECTION

In this study several biomarkers, anthropometric measures, dietary intake records and symptom questionnaires have been performed, analyzed and considered a long with physical capacity. Some of these collected data has been considered to be beyond the scope of this thesis and/ or covered by previous master students. They will therefore not be included nor further discussed.

2.4.1 Urine sampling

At the beginning of both intervention days, each participant was handed a can an instructed to collect urine throughout the day, consuming only controlled amounts of protein. They were told to record intake of water they consumed while at the intervention, with an upper limit intake of 2,5-3,0 L. At the end of both test days we were left with one can from each participant. The urine in each can was measured, stirred and sampled into a smaller can measuring only 100 ml for storage at $-20\ ^oC$ until further analysis.

2.4.1.1 Nitrogen

To measure the amount of excreted nitrogen in the urine, the Kjeldahl method was performed by Nofima AS (Bergen, Norway) (71)

The nitrogen level is determined by the following principle:

1. The sample is dissolved by concentrated sulfuric acid using copper as a catalyst. The nitrogen in the protein gets reduced and converted to ammonium sulfate.
2. Ammonia is released by basic distillation and determined by titration.

First step, weighing, involved transferring 1 gram of each sample to a tube made of glass whereas two catalyst tablets, 15.0 ml concentrated H_2SO_4 were added. The complete solution was dissolved using the following temperature program;

1. Gradually heating to 420 °C during approximately 1 hour.
2. Keep the temperature at 420 °C for 2 hours and 20 minutes.
3. Cool down the sample.

Second step, distillation, separate tubes were filled with distillation fluid, indicator solution, titration acid and distilled water at the distillation unit. The distillation unit automatically adds all solutions by titrating until the dark-colored medium has become clear and colorless.

Each time batch number on indicator solution, titration acid was changed, or a different equipment, chemicals or environment was used, blank test samples had to be performed with 8 blanks and calculations of the average result. The blank value was then deducted from new results.

Estimated intake of nitrogen was calculated by dividing protein intake by 6.25 which is the estimated factor of nitrogen in protein. Nitrogen values were measured by analyzing total urine collected at both intervention days and validated against the controlled protein intake to estimate a measure of nitrogen metabolized. Estimated intake of nitrogen was 9.16g for all participants.

2.4.1.2 1-Methylhistidine, 3-Methylhistidine and creatinine

To measure the levels of 1-MH, 3-MH and creatinine in urine, the liquid chromatography tandem mass spectrometry (LC-MS/MS) method in platform C was performed by Bevital AS (40). The level of the different parameters is determined by the following principle; a detection method that relies on the mass-to-charge ratio of a compound. The method used by Bevital AS is published in this article (72).

2.4.2 Blood sampling

During intervention days phase two and three, blood collection was performed through a PVC (infusion cannula 18G, BD) in the antecubital vein. The blood was conducted by a qualified

nurse and the catheter was washed with 2.5 ml saline solution prior to each blood sampling and a 3-way tap was used. The first sample was taken at baseline in the fasting state upon arrival followed by intervals of 0, 15, 30, 60, 90, and 120 minutes after completion of each exhausting exercise session.

Included in this thesis, data collection in blood was limited to baseline measures upon arrival and 0- and 60-min samples after the second exhaustion excise session. These measures were of the most relevance to the scope and purpose of the selected biomarkers focused on in this thesis. This resulted in six different blood samples from each participant, three from each intervention day with either the consumption of test drink A or test drink B to be able to compare the two. Under the scope of this thesis, CK, 1-MH, 3-MH, creatinine and RBP4 are the blood biomarkers included in analysis and will be further discussed.

Serum

Handling of blood samples to be separated into clean and stable serum samples was performed by filling up serum gel tubes (Vacuette® Ref 454067R, Greiner Bio-One) and mix the content by turning the tube a few times and left in room temperature for about 30 minutes. The separation of serum from clotting factors in blood were performed by centrifugation at 2000 G for 10 minutes. The serum had now been separated as an upper layer in the tube, which could easily be transferred by pipetting into new sterile cryotubes marked with the participants ID and coding of sample.

Serum samples collected and prepared were stored at -20 °C until all samples were collected from all participants each intervention day. At the end of the day samples were transported on ice to Haukeland University Hospital to be stored at -80°C until laboratory tests were ready to be conducted. Relevant for this thesis, CK was the biomarker analyzed in serum.

2.4.2.1 Creatine kinase

Measuring the levels of CK in serum, photometry was used as the method to detection by the Laboratory of Clinical Biochemestry (Labaratorieklinikken) at Haukeland University Hospital (73).

The level of CK is determined by the following principle; quantity measurement of light absorbing analyte in a sample. The exposure of incident light leads to absorption in analytes

which then will reflect light at a lower intensity. The color of the samples is defined by light that is not absorbed and transmitted through which then is measured by photometric instruments.

Plasma

Handling of blood samples to be separated into clean and stable plasma samples was performed by filling up, prepared ethylenediaminetetraacetic acid tubes (EDTA-tubes Vacuette® Ref G454047, Greiner Bio-One) added dipeptidyl peptidase-4 (DPP-4) inhibitor. This inhibitor was not necessary for the analysis preformed in this thesis, but the same plasma was used to analyze Glucagon-like peptide-1 by another master student which required this inhibitor. The content was mixed by turning the tubes a few times followed by centrifugation at 1800 G at 4°C for 10 minutes within 20 minutes of collection. The plasma was then separated from white blood cells, platelets and red blood cells which was pipetted and transferred to sterile cryotubes labeled with the participants ID and coding of sample.

Plasma samples collected and prepared were stored at -20 °C until all samples were collected from all participants each intervention day. At the end of the day, samples were transported on ice to Haukeland University Hospital to be stored at -80°C until laboratory tests were ready to be conducted. Relevant for this thesis 1-MH, 3-MH, creatinine and RBP4 were the biomarkers analyzed in plasma.

2.4.2.2 1-Methylhistidine, 3-Methylhistidine and creatinine

To measure the levels of 1-MH, 3-MH and creatinine in plasma, the LC-MS/MS method in platform C was performed by Bevital AS (40). The level of the different parameters is determined by the following principle; a detection method that relies on the mass-to-charge ratio of a compound. The method used by Bevital is published in this article (72).

2.4.2.3 Retinol-binding protein 4

The collection method used to measure RBP4 was an enzyme-linked immunosorbent assay (ELISA) kit by Immundiagnostik AG (Bensheim, Germany, REF K6110). This two-sided "sandwich" technique is intended for the quantitative determination of free RBP4 as well as RBP4 complexed with transthyretin in plasma. The kit was stored at -4°C, while plasma samples were taken out form the freezer at - 80°C approximately one hour before use. Included

in the kit there were a 96 well microtiter plate (pre-coated), ELISA wash buffer concentrate, sample dilution buffer, conjugate concentrate (CONJ, rabbit anti RBP/RBP4, peroxidase), 2x5 lyophilized standards (STD), two lyophilized controls (CTRL), tetramethylbenzidine substrate (TMB), and ELISA stop solution.

Preparation of reagents

The wash buffer concentrate was diluted with ultra-pure water 1:10 before use. The lyophilized STD and CTRL were reconstituted with 500 ul of ultra-pure water and allowed to dissolve for 10 minutes and mixed thoroughly to ensure complete reconstitution. Before use, also the CONJ was diluted to 1:101 in wash buffer. All other test reagents were ready-to-use.

Preparation of samples

All samples were diluted with sample dilution buffer (SAMPLEBUF) until 1:5 000 before use, this was done in three steps:

- 20 ul sample + 980 ul SAMPLEBUF = 1: 50 (dilution I)
- 5   ul dilution I + 45 ul SAMPLEBUF = 1: 10 (dilution II)
- 10 ul dilution II + 90 ul SAMPLEBUF = 1:10 (dilution III)

This resulted in a final dilution of 1: 5 000. The last dilution was made directly into the well on the 96-microtiter plate.

Assay procedure

All reagents and samples were brought to room temperature (15-30°C) and vortexed. The protocol sheet with positions of standards/controls/samples are showed in **Appendix 7**. In total there was 2x5 STD, two controls and 84 samples. Samples were marked with the given participant identification and coding. The coding consisted of the letter A which represented baseline samples taken in the fasting state upon arrival the first intervention day, while D represented the same sample the second intervention day. The letter C0 represented the blood sample taken 0 min after the second exhaustive endurance cycling test equal to 5 hours after the test drink the first day (7hours after baseline) and F0 the second day, while C3 or F3 represented the same only 60 minutes after the second exhaustive endurance cycling test equal to 6 hours post the test drink (8hours after baseline).

To start of the procedure, the wells were washed thoroughly 5 times with 250 ul wash buffer. Following, 100 ul STD/CTRL/ diluted samples were added to the wells. As mentioned above, the last dilution of the sample was made directly on the plate. The plate was then covered and incubated for 1hour at room temperature on a horizontal shaker. The contents were then discarded, and the plate washed with wash buffer, before 100 ul CONJ was added, covered and again incubated for 1hour at room temperature and on a horizontal shaker. The contents were again discarded, and the plate washed five times. To develop readable absorbance 100 ul TMB substrate was added followed by an 10-20 minutes incubation in room temperature, but this time covered by a foil cap to create a dark environment. After this incubation 100 ul ELISA stop solution was added and vortexed.

The absorption was immediately determined with an ELISA reader (spectra max plus, microplate spectrophotometer) with a wavelength at 405 nm against 650 nm as a reference.
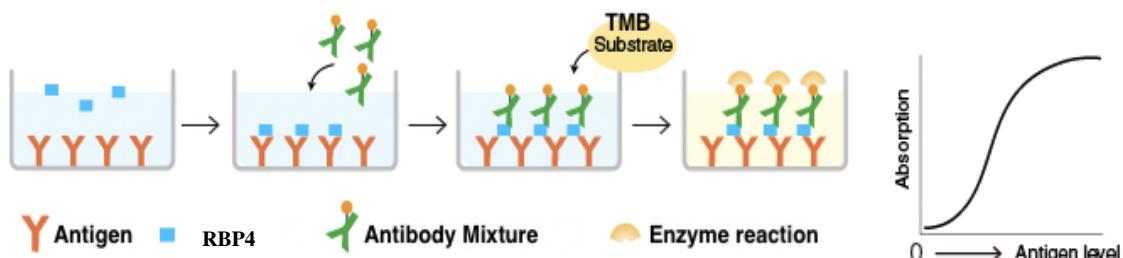
Figure 6: The principle of RBP4 sandwich ELISA method.

In the first incubation step, RBP4 in the samples are bound to polyclonal rabbit anti RBP4 antibodies, immobilized on the microtiter plate. A peroxidase-conjugated anti RBP4 antibody is used for detection and quantification, and tetramethylbenzidine (TMB) as a peroxidase substrate. A dose-response curve of absorbance unit (optical density at 405 nm) vs. concentration is generated using the values obtained from standard. RBP4 present in the participant is determined directly from this curve. Modified figure from MBL life science webpage (74).

2.5 DATA ANALYSIS AND STATISTICS

All data analysis and statistics were performed using Microsoft Excel © 2019 for Mac (Microsoft Corporation, Redmond, WA, USA) and IBM SPSS Statistics for Windows Version 25.0 (IBM Corp. Armonk, NY). All figures are presented based on median values with a percentile of 0.25 as recommended by a statistician.

A descriptive analysis of each participant demographics measured with InBody 720 scanner (InBody Co., Ltd, Cerritos, California, USA) during phase one is presented as mean, SD (±), 95% CI (lower, upper), and range (min, max) values.

Estimated intake of nitrogen was calculated by dividing protein intake by 6.25 which is the estimated factor of nitrogen in protein. Concentration results of nitrogen in the urine analysis performed by Nofirma AS, was converted to gram by calculating the analyte amount in total urine excretion from each participant (1 % N = 10g N / L urine). Nitrogen intake and the analyte level estimated in the urine from each participant was then used to calculated retained nitrogen. Results are presented with a bar graph based on median values with 0.25 percentile.

1-MH, 3-MH and creatinine in urine were analyzed using IBM SPSS and are presented as mean values with 95% confidence interval (CI). 1-MH, 1-MH/ 3-MH, and 3-MH/ Creatinine were not normally distributed, so a non-parametric Wilcoxon singed rank test had to be used. Considering there are more than one dependent variable in the ratio, measures in urine were also controlled for multiple testing with the one-way multivariate analysis of variance (one-way MANOVA).

To analyze CK, 1-MH/ 3-MH, 3-MH/ Creatinine, and RBP4 levels with respect to time and drink, a mixed linear model was used assuming a compound symmetry correlation structure between measurements from the same test person, and testing interactions between the predictors. Results are presented as F-tests with degrees of freedom, p-values accepted as significant with p-value of or below 0.05, and estimated regression coefficients with 95% CI. The Shapiro-Wilk normality test was used to test for normal distribution.

A Spearman's rho non-parametric correlation was performed to examine possible associations between the biomarkers analyzed.

2.6 ETHICS STATEMENT

This intervention was conducted according to the guidelines in the declaration of Helsinki. The policy covers how each potential participant to be included in the intervention shall be informed about the intervention, aim, procedures, favourable and unfavourable effects. It also includes the right to participate and the right to withdraw their consent without any justification, and how the participant information will be handled. All potential participants were carefully informed both oral and written about each point of this policy before the written informed consent had to be fully understood, signed and handed in prior to inclusion of the study.

The study is approved by the Western Norway Regional Committee for Medical and Health Research Ethics (REK 2017/56) of all procedures concerning involvement of human subjects. An ergometer commonly used to determine exercise capacity was used during the endurance exercise test. Considering how this was a strenuous test performance, a physician was present during every phase of the study to lead the safety assessment during every endurance test. The wellbeing of each participant was always prioritized. Any sign of discomfort immediately led to ending of the test preformed.

All personal information collected from each participant was handled with complete confidentiality. Each participant received a personal identification number at their first visit. This was a necessity to sort the data collection and reporting from each participant at every visit and make all samples anonymous throughout the rest of the intervention. All collected data were stored without any identifiable information and with password protection. In addition, researchers were only granted access to necessary data for their specific analysis to be performed.

3. RESULTS

3.1 DEMOGRAPHIC OF THE PARTICIPANTS

The total of 14 healthy male participants recruited conducted the study. Their self-reported lifestyle before and in between intervention days focusing on dietary habits and physical activity did not show any remarkable difference. However, there was one exception where heavy physical work in a job context 1-2 days before the last intervention day had been performed. Analysis results influenced by this were excluded in further analysis covered in this section. Regarding all figures included in this section, the two drinks will be separated by color. Blue will be representing the MPH drink, while grey will be representing the placebo drink.

Detailed baseline demographics of the study population are listed in **Table 5**. The average age of the participants was 46 years with a range from 40 to 58. Measured mean height was 180.8 cm (SD ± 4.1), weight 80.1 kg (SD ± 6.4), and a BMI of 24.5 kg/m^2 (SD ± 2.2). Further inbody anthropometric measures preformed with BIA showed an FFM of 66.6 kg (SD ± 3.7), FM of 13.5 kg (SD ± 4.5, 16.6%, SD ± 4.4), and MM of 37.7 kg (SD ± 2.3).

Table 5: Demographics of the participants in phase one, n=14.

Characteristics	Mean	SD (±)	95% CI (lower, upper)	Range (min, max)
Age (years)	46	5	43, 49	40, 58
Height (cm)	180.8	4.1	178.4, 183.2	176.0, 192.0
Weight (kg)	80.1	6.4	76.4, 83.8	71.2, 96,2
BMI (kg/m^2)	24.5	2.2	23.3, 25.8	21.4, 29.4
FFM (kg)	66.6	3.7	64.5, 68.7	60.4, 71.5
FM (kg)	13.5	4.5	10.9, 16.1	5.8, 24.7
FM (%)	16.6	4.4	14.4, 19.1	7.8, 25.7
MM (kg)	37.7	2.3	36.4, 39.0	33.9, 40.9

Abbreviations: BMI=body mass index, FFM=fat free mass, FM=fat mass, MM=muscle mass, SD=standard deviation, CI= confidence interval.

3.2 URINE ANALYSIS

Total urine was collected and registered during a period of about 9hours while the participants were present in phase two and three. Registered amount of urine was later used to estimate the analyte level in total urine with the analysis results from both Nofima AS and Bevital AS. All urine results are presented as analyte level in total collected urine. Urine samples from participant 13 were not collected at neither of the intervention days. Amount of collected urine was not registered from participant 14 after consuming MPH, making it difficult to calculate the analyte level resulting in the exclusion of nitrogen metabolized in this measure.

3.2.1 Nitrogen

To calculate the nitrogen balance, the controlled and estimated nitrogen amount of 9.16 g in consumed intake during the intervention day in each participant was used. The analyte level of nitrogen in the total urine was calculated by converting % N to g/L (1% N = 10 g/L) and multiplied with total amount of urine (L) collected in each participant. Results are presented in **Figure 7** which compare the two drinks according to intake, nitrogen metabolized, and calculated nitrogen retained in the body.

The analysis result following the MPH drink showed that nitrogen metabolized was significantly lower ($p < 0.001$) from the controlled nitrogen intake, as well in the placebo drink ($p = 0.001$). Nitrogen analysis regarding the intake of MPH showed a slightly lower level of metabolized nitrogen of 5.91 g compared to the nitrogen level metabolized of 7.42 g following the placebo drink. However, results show no significant difference in nitrogen levels metabolized between the two intervention drinks with a p-value of 0.315.

Retained nitrogen was calculated to be 3.25 g following the MPH drink and 1.74 g following the placebo drink, respectively.
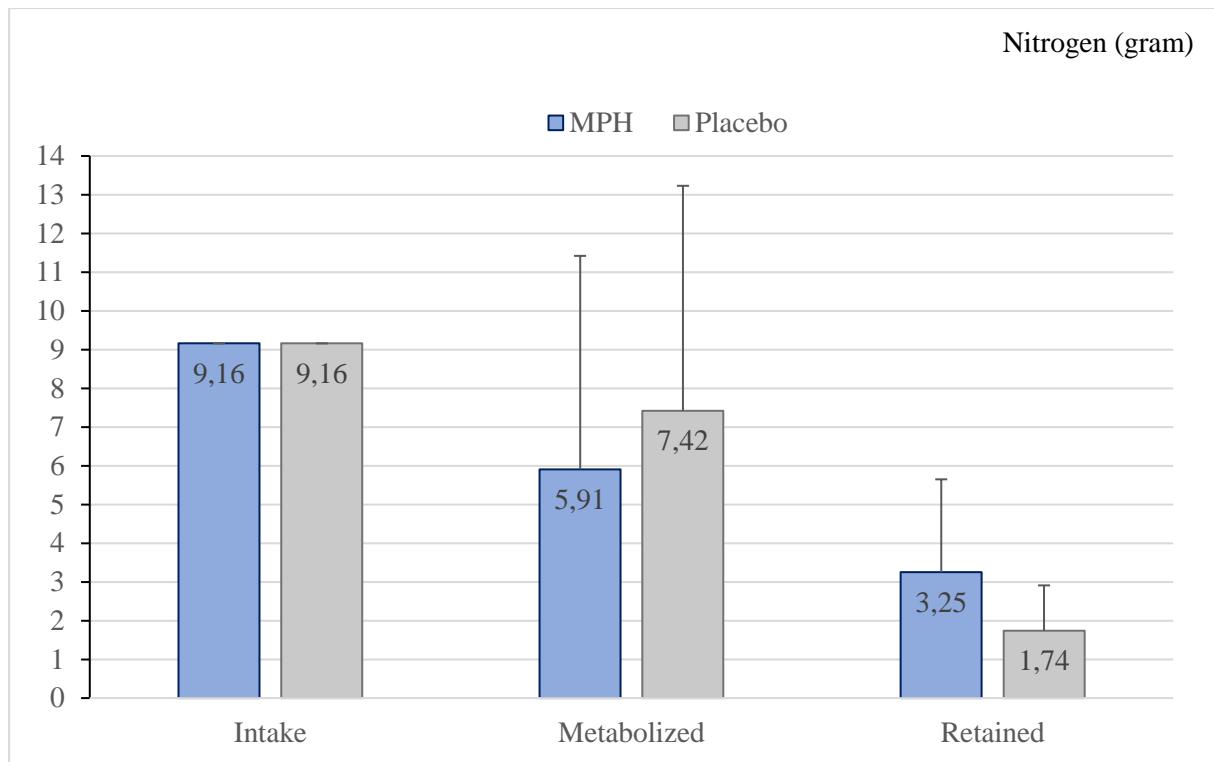
Figure 7: Nitrogen intake, metabolized and retained after consuming MPH or placebo drink. *Nitrogen intake estimated from controlled intake throughout the day. Nitrogen metabolized were measured in urine samples after intake of MPH over a 9hour period in each participant. Retained nitrogen was calculated by using the intake measure and metabolized levels of nitrogen analyzed in the samples. Values are listened as median values with a percentile of 0.25.*

3.2.2 1-Methylhistidine, 3-Methylhistidine and creatinine

In the analysis of 1-MH, 3-MH and creatinine, several values were missing leaving only n= 13 for mean measures, and n=9 participants in calculations of the ratios see **Table 6**. 1-MH did not fulfill the requirement of a normal distribution, so a non-parametric Wilcoxon signed rank test had to be used. Results showed that neither of the biomarkers are significantly different. 1-MH was mainly analyzed to get the true measure of 3-MH by the ratio 1-MH/ 3-MH since the participants did not eat a meat-free diet before testing. Thus, 1-MH/ 3-MH ratio will be used as the relevant measure further. This ratio showed a significant difference with p-value at 0.028 between the two drinks. However, with the correction of the one-way MANOVA, the 1-MH/ 3-MH ratio did not show a significant difference (p = 0.107).

Table 6: 1-MH, 3-MH and creatinine measures from urine, n= 13[1].

Parameter	Mean	95% CI (lower, upper)	P-value*	MANOVA
1-MH [1,3]	350.90	208.01, 493.79	0.110	
3-MH [1]	231.67	204.16, 259.18	0.815	
Creatinine [1]	7.41	6.65, 8.16	0.686	
1-MH/ 3-MH[2,3]	1.53	0.84, 2.23	0.028*	0.107
3MH[4]/ Creatinine[2,3]	0.24	0.10, 0.38	0.066	0.121

Statistical accepting P < 0.05 as significant.
[1]*Missing values from participant 13.*
[2]*Missing value from participant 1,2,3, 13, and 14 in ratio 1-MH/ 3-MH, and 3-MH/ Creatinine.*
[3]*Was not normally distributed, therefore a non-parametric related-samples Wilcoxon Signed Rank test was used.*
[4]*1-MH/ 3-MH*

Figure 8 displays calculations of the 1-MH/ 3-MH ratio from both drinks in urine based on median values with a percentile of 0.25. The bar graph shows how the MPH test drink resulted in a higher ratio than the calculated ratio following the placebo test drink. Despite this, the one-way MANOVA gave no significant difference between the two drinks (p = 0.107).
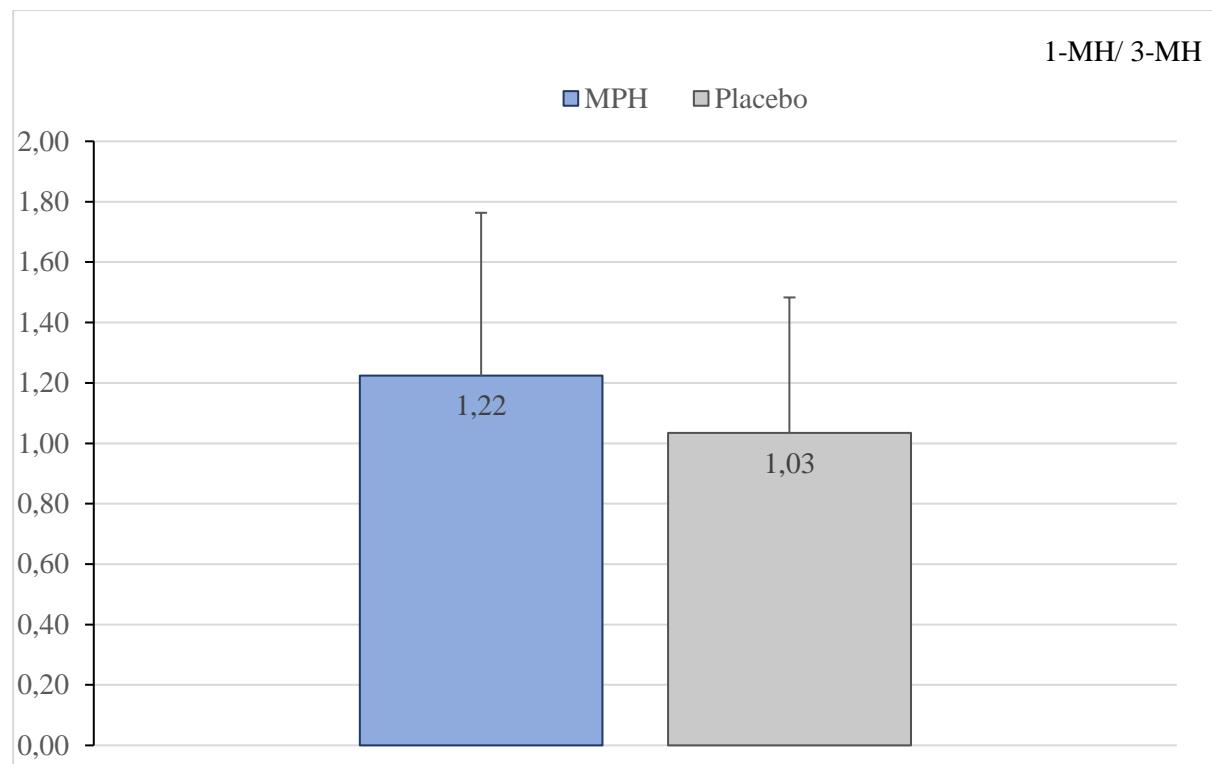
Figure 8: 1-MH/ 3-MH ratio from both MPH and placebo drink.

Calculated ratio from 1-MH and 3-MH measured in urine following either MPH or placebo test drink collected over a 9hour period in each participant. Values are listened as median values with a percentile of 0.25.

Figure 9 displays calculations of the 3-MH and creatinine ratio, where 3-MH is the true value representing the 1-MH/ 3-MH ratio, from both drinks in collected urine. Opposite to the 1-MH/ 3-MH ratio, this bar graph shows a lower ratio following the MPH test drink, and a higher ratio following the placebo test drink, but there was no significant difference (p = 0.121).
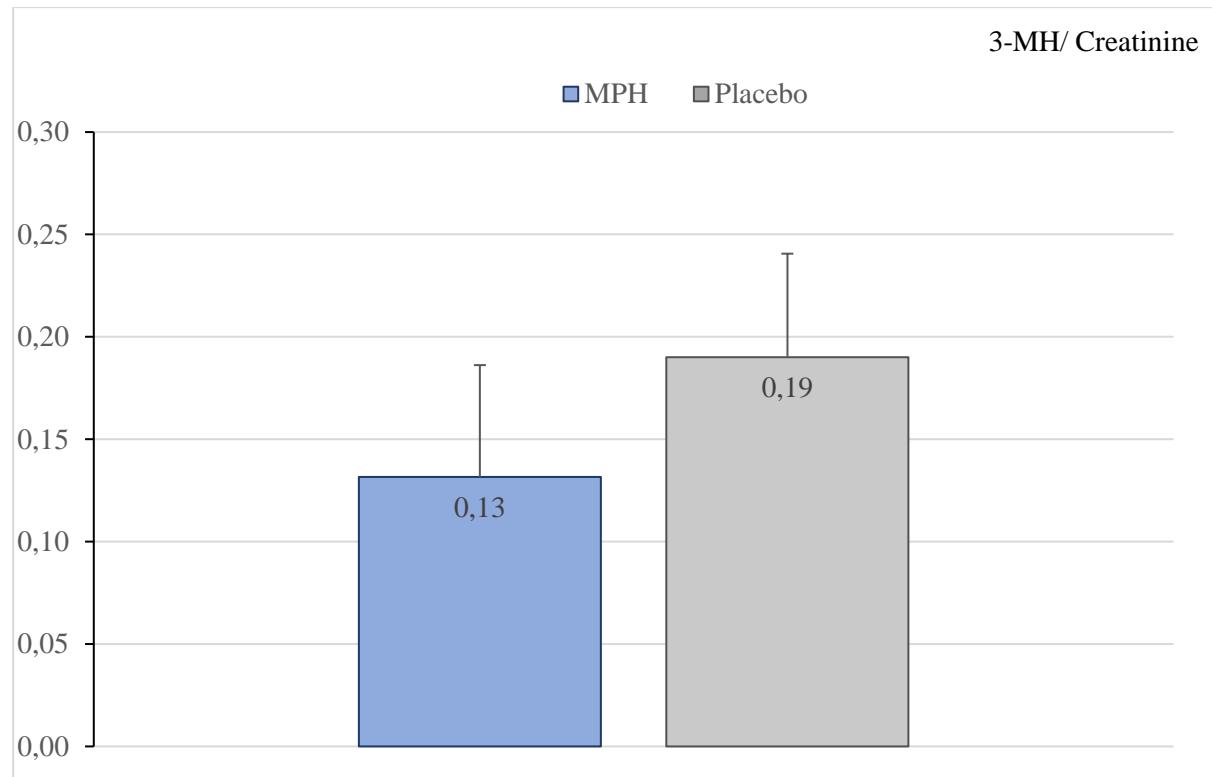
Figure 9: 3-MH/ Creatinine ratio from both MPH and placebo drink.

Calculated ratio from 3-MH (1-MH/ 3-MH) and creatinine measured in urine following either MPH or placebo test drink collected over a 9hour period in each participant. Values are listened as median values with a percentile of 0.25.

3.3 BLOOD ANALYSIS

Blood samples included in all analysis are collected from baseline, 7hours and 8 hours post baseline. Baseline is in the fasting state upon arrival while 7hours and 8hours are equal to 0- and 60 min post second exhaustion endurance cycling session. The same measures are used at both intervention days to be able to use each participant as their own control. A mixed linear regression analysis was conducted for all biomarkers in this section. This type of analysis will consider differences between the two drinks combined in relation to the independent variables time (baseline, 7h, 8h) and drink (MPH or placebo). Regarding line charts displayed in **Figure 10, 11, 12** and **13,** between the two first time measures, time elapse over a 7hour period displayed with a non-continuous line. Between the second- and third-time measure, time elapse over a 1hour period displayed with a continuous line.

3.3.1 Creatine Kinase

Participant 12 was excluded from the analysis because of measurements following high physical demand related to work only a couple days before testing resulted in false measures. The analysis resulted in no significant difference between drinks ($p = 0.823$), but there was a significant difference between time measures ($p < 0.001$). Results are listed in **Table 7.**

Table 7: Estimates of fixed effects[1] CK analysis in serum n=13[2].

Parameter[1]	Estimate	95% CI (lower, upper)	F	P-value*
Intercept	4.74	4.55, 4.92	5256.15	< 0.001*
Time			42.57	< 0.001*
Baseline	0.00[3]	0.00[3]		
7h	0.20	0.11, 0.29		
8h	0.10	0.02, 0.17		
Drink			0.05	0.823
Placebo	0.00[3]	0.00[3]		
MPH	0.03	-0.16, 0.22		
Time x Drink			0.87	0.441
Baseline x MPH	0.00[3]	0.00[3]		
7h x MPH	0.00	-0.12, 0.12		
8h x MPH	-0.03	-0.12, 0.06		

Figure 10 display changes in CK levels in the blood with median measures and percentile 0.25. Both drinks follow the same curve with a slight increase after 7hours followed by a small decrease 1hour later. Although the MPH drink seemed to have increased a little more than placebo, mixed model analysis did not show any significant difference between the two drinks (p = 0.823).

	Baseline	7h	8h
MPH	113	142	125
Placebo	121	135	124
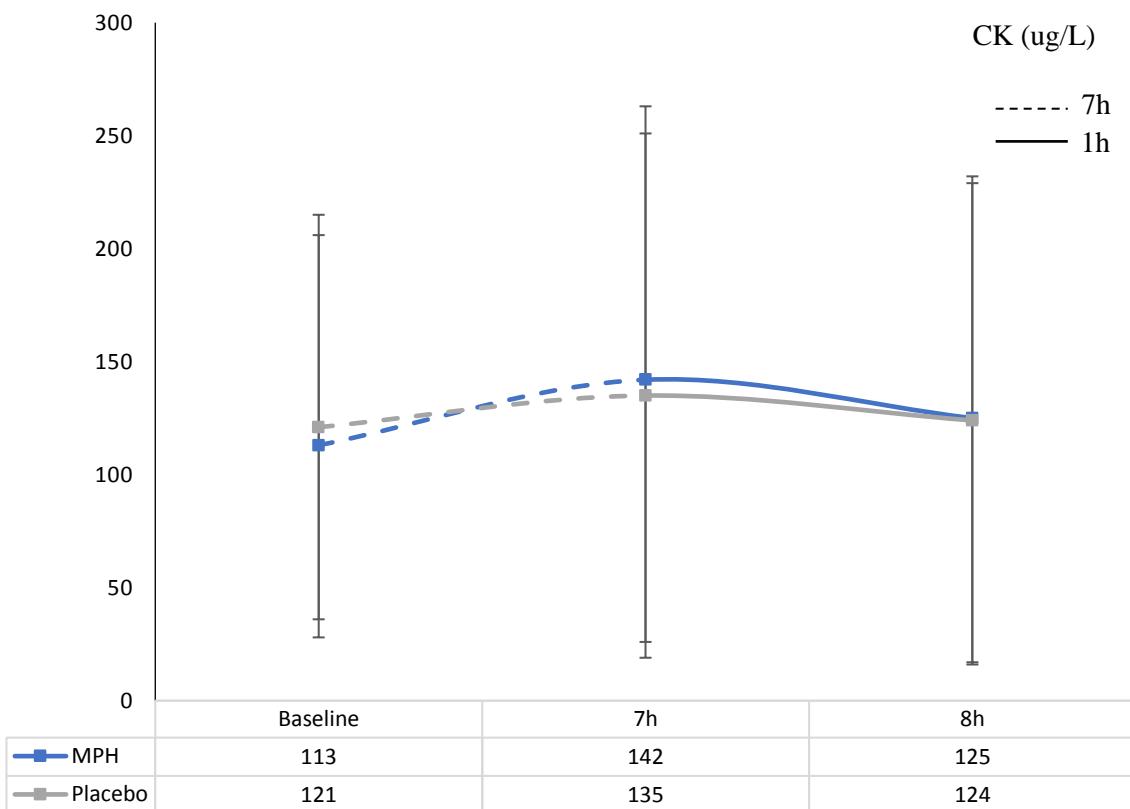
Figure 10: Serum levels of CK measured following both the MPH and placebo drink.

7h represent 0 min and 8h represent 60 min post second exhaustion endurance cycling session, without participant 12 from baseline. The non-continuous line represents a 7hour period, while the continuous line represents a 1hour period. Values are listened as median values with a percentile of 0.25.

3.3.2 1-Methylhistidine, 3-Mmethylhystedine and creatinine

Presented in **Table 8** are median measures and percentile 0.25 of 1-MH, 3-MH and creatinine from all three-time measures after both drinks. Neither of the values between drinks are significant. On the other side, all measures seemed to have increased between baseline and measure point 7h, while they decreased 1hour later. As the ratio 1-MH/ 3-MH ratio are a more true value of 3-MH secreted in human muscle, this ratio will be preferred to use in further analysis.

Table 8: Median 1-MH, 3-MH and creatinine measures from plasma at baseline, 7- and 8 hours post baseline equal to 0 – and 60 min post second exhaustion endurance cycling session.

Variable	MPH		Placebo		
	Median	Percentile[1]	Median	Percentile[1]	P-value[2]
1-MH					
Baseline	3.70	2.26	5.26	2.11	0.794
7h	4.31	3.63	5.50	2.78	0.467
8h	4.21	3.23	5.18	2.89	0.536
3-MH					
Baseline	4.29	3.85	5.22	4.39	0.128
7h	6.22	5.15	5.90	5.13	0.723
8h	5.81	5.48	5.78	5.64	0.719
Creatinine					
Baseline	80.35	72.43	79.40	76.25	0.625
7h	100.00	91.13	93.65	89.23	0.175
8h	92.40	90.10	90.85	88.93	0.131

[1]*Percentile 0.25*

[2]*Mixed linear regression analysis*

Abbreviation: MPH = marine protein hydrolysate, 1-MH = 1-Methylhistidine, 3-MH = 3-Methylhistidine, h = hours.

Results from the 1-MH/ 3-MH analysis are listed in **Table 9**. The analysis resulted in no significant difference between drinks (p = 0.595), but there was a significant difference between time measures (p = 0.006).

Table 9: Estimates of fixed effects[1] 1-MH/ 3-MH analysis in plasma n=14.

Parameter[1]	Estimate	95% CI (lower, upper)	F	P-value*
Intercept	0.03	-0.55, 0.61	0.03	0.861
Time			7.80	0.006*
Baseline	0.00^2	0.00^2		
7h	-0.08	-0.43, 0.26		
8h	-0.14	-0.47, 0.19		
Drink			0.30	0.595
Placebo	0.00^2	0.00^2		
MPH	0.07	-0.57, 0.70		
Time x Drink			1.13	0.352
Baseline x MPH	0.00^2	0.00^2		
7h x MPH	0.13	-0.08, 0.33		
8h x MPH	0.11	-0.09, 0.30		

Statistical significance was evaluated using two-sided pair wise t-test accepting P < 0.05 as significant.
[1]*Dependent variable: ln1-MH3-MHratio*
[2]*Referance*
Abbreviation: CI = confidence interval, F = fishers test, h = hours.

Figure 11 displays changes in 1-MH/ 3-MH levels in the blood with median measures and percentile 0.25. Both drinks follow the same decreasing curve between measures. There was no significant difference between the two drinks (p = 0.595).

|	Baseline	7h	8h
MPH	1,02	0,82	0,75
Placebo	1,03	0,77	0,71
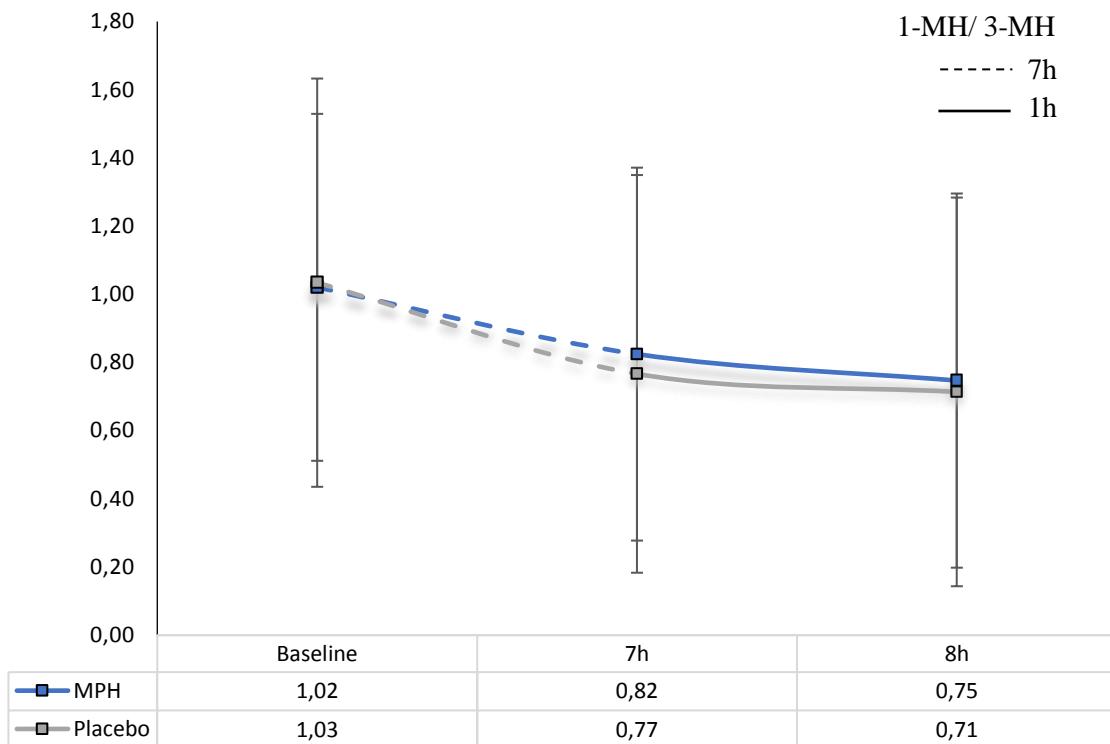
Figure 11: Plasma levels of 1-MH/ 3-MH ratio measured following both the MPH and the placebo drink.

7h represent 0 min and 8h represent 60 min post second exhaustion endurance cycling session. The non-continuous line represents a 7hour period, while the continuous line represents a 1hour period. Values are listened as median values with a percentile of 0.25.

Results from the 3-MH/ Creatinine analysis are listed in **Table 10**. 3-MH represent the true value, the ratio between 1-MH/ 3-MH. The analysis resulted in no significant difference between drinks (p = 0.662).

Table 10: Estimates of fixed effects[1] 3-MH[2]/ Creatinine analysis in plasma n=14.

Parameter[1]	Estimate	95% CI (lower, upper)	F	P-value*
Intercept	-4.35	-4.91, -3.78	732.53	< 0.001*
Time			1.74	
Baseline	0.00^3	0.00^3		0.215
7h	-0.25	-0.61, 0.12		
8h	-0.27	-0.61, 0.06		
Drink			0.20	0.662
Placebo	0.00^3	0.00^3		
MPH	0.08	-0.55, 0.71		
Time x Drink			0.32	0.731
Baseline x MPH	0.00^3	0.00^3		
7h x MPH	0.06	-0.15, 0.26		
8h x MPH	0.08	-0.13, 0.28		

Statistical significance was evaluated using two-sided pair wise t-test accepting P < 0.05 as significant.
[1]*Dependent variable: ln3-MHCreatininratio*
[2]*1-MH/ 3-MH*
[3]*Referance*
Abbreviation: CI = confidence interval, F = fishers test.

Figure 12 displays changes in 3-MH/ Creatinine levels in the blood with median measures and percentile 0.25. Both drinks follow the same decreasing curve between the first and second measure, while it evens out between the second and third measure. There was no significant difference between the two drinks (p=0.662).

|	Baseline	7h	8h
MPH	0,013	0,009	0,007
Placebo	0,012	0,007	0,007
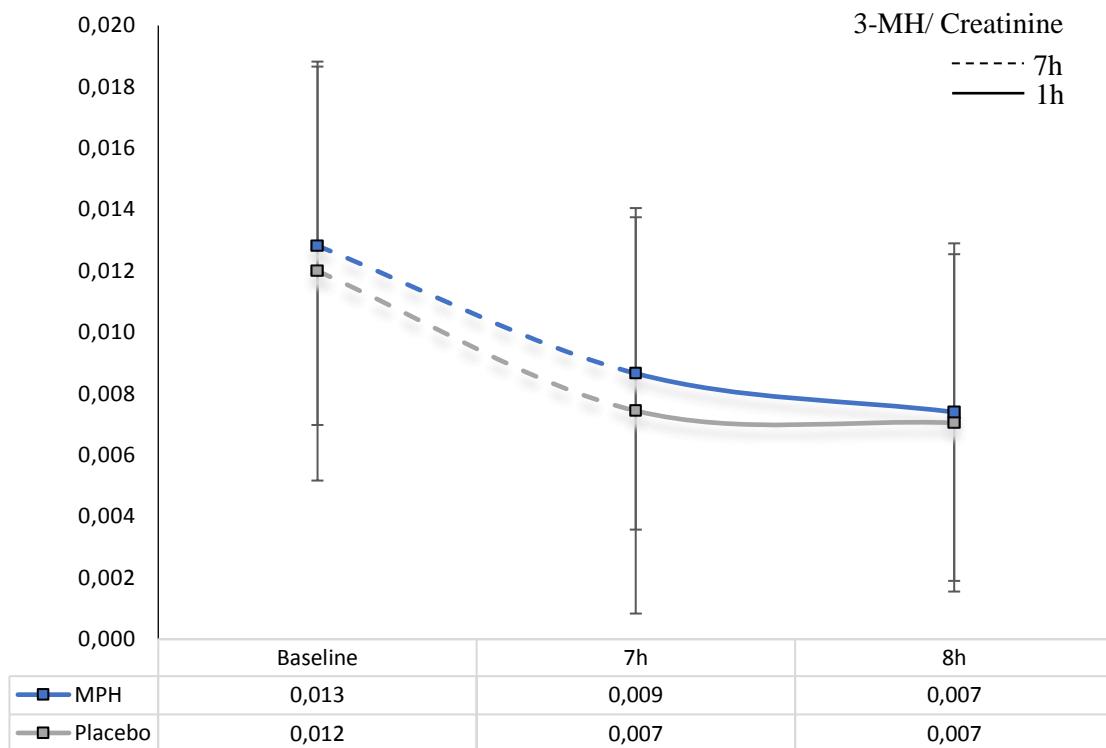
Figure 12: Plasma levels of 3-MH/ Creatinine ratio measured following both the MPH and the placebo drink.

7h represent 0 min and 8h represent 60 min post second exhaustion endurance cycling session.

The non-continuous line represents a 7hour period, while the continuous line represents a 1hour period. Values are listened as median values with a percentile of 0.25.

3.3.3 Retinol-binding protein 4

The ELISA technique resulted in several measures outside the reference range. Values outside the reference range were all set to 0.06 during analysis of the results. RBP4 results were analyzed following a linear mixed regression analysis with respect to the independent variables time and drink all presented in **Table 11**. The analysis resulted in an almost significant difference between the two drinks with a p-value of 0.052.

Table 11: Mixed model RBP4[1] analysis in plasma n=14.

Parameter[1]	Estimate	95% CI (lower, upper)	F	P-value*
Intercept	16.37	8.36, 24.39	51.12	< 0.001
Time			1.13	0.353
Baseline	0.00[2]	0.00[2]		
7h	4.17	-2.36, 10.70		
8h	1.99	-6.28, 10.26		
Drink			4.58	0.052
Placebo	0.00[2]	0.00[2]		
MPH	-1.28	-8.70, 6.13		
Time x Drink			0.74	0.497
Baseline x MPH	0.00[2]	0.00[2]		
7h x MPH	-3.77	-11.30, 3.77		
8h x MPH	-5.84	-17.94, 6.26		

Statistical significance was evaluated using two-sided pair wise t-test accepting $P < 0.05$ as significant.
[1]*Dependent variable: RBP4*
[2]*Referance*
Abbreviation: CI = confidence interval, F = fisher test.

Figure 13 displays changes in RBP4 levels in the blood with median measures and percentile 0.25. Both drinks have close to the same baseline measure with a median of 16.0 mg/L post MPH and 15.2 mg/L in placebo. The second measure showed decreasing levels of RBP4 following the MPH drink (median:14.8 mg/L) while it increases following placebo (median: 21.7 mg/L). Between the second and third measure, MPH seemed to have decreased to a level far below baseline. Placebo also seemed to have decreased between the second and third measure, but still at a level above baseline measure. The analysis did not show any significant difference between the two drinks (p=0.052).

	Baseline	7h	8h
MPH	16,0	14,8	7,4
Placebo	15,2	21,7	18,2
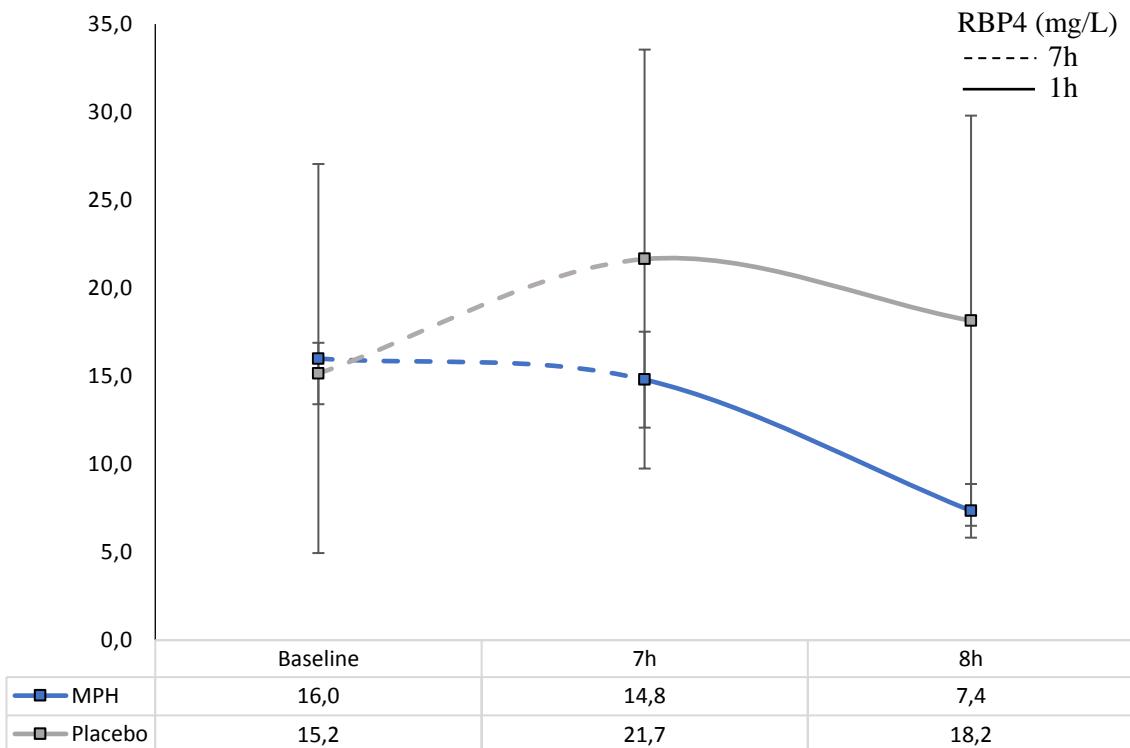
Figure 13: Plasma levels of RBP4 measured following both the MPH and placebo drink.
7h represent 0 min and 8h represent 60 min post second exhaustion endurance cycling session. The non-continuous line represents a 7hour period, while the continuous line represents a 1hour period. Values are listened as median values with a percentile of 0.25.

3.4 CORRELATIONS

To evaluate the relationship between nitrogen balance, CK, 3-MH and RBP4 a Spearman's rho nonparametric correlation was performed. Neither of the analysis gave any significant results and the inclusion of results were therefore considered to be superfluous in this thesis but are included in the **Appendix 6.**

4. DISCUSSION

This is likely the first study to examine the influence of MPH from Atlantic cod on muscle synthesis and degradation post exhaustive endurance cycling. This thesis suggests that a single dose (20 mg/kg BW) of MPH will have an impact on biomarkers related to muscle synthesis and degradation in addition to the superior whey protein. The main findings in this study is that supplementation with a single dose of MPH in addition to whey protein did not show any significant difference between supplementation with only whey protein on increasing muscle synthesis and decreasing muscle degradation. However, several biomarkers did indicate some differences between MPH and whey protein in favour of MPH.

The discussion is divided into three sections. In the first section the main findings of this study are discussed, and results are compared to related studies. In the second section, strengths and limitations of the recruitment and participants, study design, method, urine and blood sampling and biomarkers are discussed. The third section do concern an evaluation of the requirement for further research on MPH.

4.1 DISCUSSION OF FINDINGS

4.1.1 Nitrogen balance

Controlled intake of protein and measurements of nitrogen excreted in urine, gives us an estimate of how much protein was retained in the body. The urine analysis of nitrogen metabolized following the MPH drink showed a significant ($p < 0.001$) lower level of metabolized nitrogen from the nitrogen intake. This indicates a positive nitrogen balance where muscle synthesis is stimulated, and degradation suppressed. Increased muscle synthesis contributes to a faster restitution period post exercise and increase muscle strength related to better prognosis during chronic diseases (3, 21, 75). However, analysis of urine following the placebo drink also showed a significant difference between intake and metabolized nitrogen ($p = 0.001$). This indicates that both intervention drinks have had a positive influence on nitrogen balance in this study population, which could be explained by the fact that both drinks consisted of high-quality protein consumed at the right time.

No significant difference was found when comparing the two drinks ($p = 0.315$). On the other hand, analysis does indicate a slight difference in favor of MPH as in most participants (n= 8/12) nitrogen metabolized was higher following the placebo drink. In other words, more

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protein was retained and available for muscle syntehsis in the body following the MPH drink. A study performed by Paullain M.G. et.al showed a significant difference in both retention and excretion of nitrogen between whole protein and protein hydrolysates in rats (55). Another study by Hays N.P. et.al also found a significant difference in lean body mass and nitrogen excretion in elderly women following intake of whey protein versus protein hydrolysate (56). With this to consider, there is reason to believe that if the study had included a larger number of participants and a better representation of the general population, results might have shown the two drinks to be significantly different.

4.1.2 1-Methylhistidine/ 3-Methylhistidine and 3-Methylhistidine/ Creatinine

The metabolized nitrogen in urine reflects the impact on nitrogen balance but indicate only net protein breakdown. Measurement of 3-MH in the urine enables the estimated contribution of increased muscle catabolism to the negative nitrogen balance associated with muscle wasting and nitrogen loss (41). Analysis on urine showed no significant differences between drinks concerning these biomarkers. This might be explained by the fact that the collection period of urine was too short to see any differences as these biomarkers usually peak 12 to 24 hours after exercise. Even though there was no significant difference, the 1-MH/3-MH ratio seemed higher in the MPH group, indicating that excretion of 3-MH from degenerating muscle was reduced. Supporting this, the 3-MH/ Creatinine ratio was measured to be lower following the MPH drink compared to the placebo drink.

Considering that the collection period of urine is such a limiting factor, measurements of 1-MH, 3-MH, and creatinine was also analyzed in blood to support our results. Like Paul G.L. e.t a.l found out (76), all three biomarkers seemed to have slightly increased 0 min after the second exercise session, while they decreased again only 1hour later following both drinks. The fact that these biomarkers seemed to have increased then decreased could indicate that the protein degradation began during exercise, but then stopped as the physical demand slowed down and sufficient protein was available for synthesis. On the other side, this increase in the single biomarkers might be explained by the meat containing meal they were served during both intervention days, between baseline and the second measure. Supporting this, results of the ratios showed only decreasing values displayed in **Figure 11** and **Figure 12**.

In addition to that the excretion of 3-MH in healthy individuals are proportional to the fat free body mass, creatinine serves as a useful measure as it is produced in the skeletal muscle (77). The more skeletal muscle a person has, the more creatinine will be excreted. Expressing 3-MH relative to creatinine helps to account for possible changes in kidney functions that may occur during and post exercise (39). The ratio 3-MH/ Creatinine has been proposed as an indicator of fractional muscle protein breakdown (77).

Both results showed decreased levels of the ratios which might be explained by that fact that during exhaustive endurance cycling, there was a temporary failure of protein synthesis, rather than an increase in muscle degradation. This theory is supported by Carter E.M. et.al. (41), which states that identifying the catabolic contribution to overall protein loss may show that in other muscle wasting conditions failure of protein synthesis should be a more important focus than muscle degradation (41).

Results from the linear mixed regression analysis showed no significant difference between the two drinks considering the 1-MH/ 3-MH ratio. It did however show a significant difference between time which means that the levels did significantly change between measures ($p = 0.006$). It was no significant difference between the 3-MH/ Creatinine ratio in the two drinks. Similarity between the urine and plasma results are that the 1-MH/ 3-MH ratio was still higher following the MPH drink. Further, the 3-MH/ Creatinine ratio did not support the higher 1-MH/ 3-MH result in plasma like it did in urine, as the 3-MH/ Creatinine turned out to be higher following the MPH drink than the placebo drink in plasma.

4.1.3 Creatine kinase

The linear mixed regression analysis of CK serum revealed no significant difference between the two drinks ($p = 0.823$). Considering CK serum levels are measured to peak 24 hours after exercise by previous studies (36, 76), a longer measurement period could be necessary to discover differences between the two drinks.

The analysis revealed a highly significant difference between time ($p < 0.001$), whereas CK levels have changed significantly between time measures indicating an early increase in CK levels. In addition, this study chose to only focus on a few measurements where there was a 7hour period between the two first measures and a 1hour period between the second- and third

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measure. In between the two first measures, two alike exhaustive excise sessions had been performed. Considering other studies have shown how CK easily adopt to similar high physical demand, higher CK levels may have occurred following the first exercise session (78, 79).

Results show that between baseline and the second measure, CK levels rise. This indicates that right after exercise, CK measures increase. The MPH drink seemed to have caused a slightly more increased CK level than the placebo drink. However, the restitution period between the two exercise sessions could have been too short which gave increased CK levels at the second measure, before it decreased at the third measure for both drinks to a level right above baseline level. Another possible explanation of the decreased CK level between the second- and third measure could be due to an acute phase response where muscle degenerate quickly right after exercise but slows down as the muscles cool down.

Analysis regarding time x drink did not find any significant difference ($p = 0.441$), which again could be explained by the short measurement period mentioned above. Considering that others have looked at measurements up to 48 hours post exercise, a longer time measure would be relevant (76, 80). Another theory is that both drinks consisted of the same amount of high-quality protein which was already in the body at the second- and third measure and did not differ enough in its capability to increase muscle synthesis and/or decrease muscle degradation.

4.1.4 Retinol-binding protein 4

Blood analysis preformed with a linear mixed regression analysis showed no significant difference between time measurements ($p = 0.353$). The analysis showed an almost significant difference between drinks with a p-value of 0.052, although time x drink did not show any significance (p= 0.497). The almost significant measure in drink indicates that the protein pool in the body could have been significantly different between the two drinks if more participants and a higher dose of MPH was used. RBP4 has a half-life of 12hours which means that a longer measurement period would not have been very beneficial in this biomarker (46, 49).

Interestingly, between baseline and the second measure, RBP4 levels raised following the placebo drink while RBP4 decreased following the MPH drink. One theory is that this happened because more of the protein was already absorbed into the muscle following the MPH drink. This theory is supported by the fact that the nitrogen metabolized also indicated a lower level

of nitrogen metabolized in the MPH drink than the placebo. Between the second and third measure, both drinks showed a decreased level of RBP4 in the blood. The placebo measure stayed at a level above baseline, while the MPH seem to have dropped at a higher rate and decreased below baseline. As the RBP4 levels is said to be a measure of the total protein pool in the body (49), this suggest that more protein was absorbed into the muscle and available for muscle synthesis following the MPH drink compared to the placebo drink.

4.2 STUDY STRENGTHS AND LIMITATIONS

This study had several strengths and limitations, some which are discussed beneath divided into recruitment and participants, study design, method, urine and blood sampling and biomarkers.

4.2.1 Recruitment and participants

This study to focused on a narrow group of the population; healthy male, medium trained with a total amount of 8 to 12 hours weekly training and cycling as their main exercise activity. This was done to make the study group as homogenous as possible and give the study strength. Recruitment was completed through flyers and social media posting on local cycling-club websites in Bergen, Norway. Since both the inclusion criteria and recruitment was narrow, several limitations did occur.

Considering the small sample size with only men included in the study, findings cannot be generalized and might not be applicable to women. Furthermore, participants represented a part of the population with a high level of physical activity compared to the general population. High physical activity is related to increased muscle mass which could have impacted the effect of MPH. The general population and most patient groups usually have a normal or below normal amount of muscle mass which could impact both synthesis and degradation of muscles differently.

The original inclusion criteria with men between 40 to 50 years of age had to be extended to 40 to 58 years. This was a consequence of few volunteers and a some withdraws during the recruitment period. Records of how many that were assessed during the recruitment or where participants were recruited from were not performed. As a result, there is no record of the amount of people reached, how they were reached or any information exceeding the fact that they either did or did not fit the inclusion and exclusion criteria of the study.

4.2.2 Study design

The intervention was based on a double blind, randomized, placebo-controlled pilot study with crossover, divided into three phases. The reasoning the study design is its numerus strengths. First, double-blinding excludes any personal interest regarding the intervention or other bias that could occur with knowledge about the intervention drink or the placebo. Both drinks were delivered from the manufacture in opaque glass with white powder and marked with the letter A or B and the participants intervention number on the cover.

Secondly, the randomization was performed by each participant individually. The first intervention day, each participant chose if they wanted to have the drink marked with letter A or B. They then received the opposite at the second intervention day. This way personal opinions about the drinks are limited. Also, it limits the influence of participants having a better knowledge the second time they go through the same testing.

Thirdly, using cross-over were the participants act as their own control decreases differences in metabolism rates, muscle mass, performance etc. in between participants as it ensures a greater biological homogeneous sample.

Another important factor of using this study design was the wash-out period. Considering the same participants received the two different drinks, a long enough wash-out period was crucial. As there was a risk of the carry-over effect between phase two and three that could have affected the second result, an estimated wash-out period based on animal-studies related to the nitrogen balance was used (55). This estimated period was based on the assumption that there would not be a difference in the carry-over effect between animals and humans, which could be a limitation.

In addition, it is worth mentioning that this was a pilot study involving only a small study population which could both be a strength and limitation. Systematic errors and missing values may have had a significant impact on our findings, considering the collected data were so small and sensitive to each measure. The intervention would most likely have benefited from a wider inclusion of the population with a much larger sample size, even though this was not possible in this particular study.

4.2.3 Method

Intervention drinks were individualized by estimation of the protein requirements based on body mass in each participant as a strength to the study. However, the same measure was not possible to be performed in the standardized meals each participant received during test days. The same meals were served to all participants except for one participant that received another main meal (see section 2.3.4), making the individualized test drink differences too small to account for.

There was only a small difference between the intervention drinks considering whey protein was the main protein source in both could be the main limitation of this study. A bigger difference between the drinks might have showed different results. In addition, only 3.2 E% in a single dose right after the first exhaustion cycling session was used. Several doses over a longer time period might also have influenced the results differently. However, this study focused on finding differences between a small dose of hydrolyzed protein compared to a more regular dose of whole protein.

Restitution past challenging exercise sessions is dependent on several factors for the next 48hours, where dietary intake is one main component in muscle synthesis and the rate of degradation. Collected data from 0- and 60 min past second session in blood combined with urine samples collected over a 9hour period will only represent the acute response and not the actual response of total synthesis and degradation. These limitations do probably have an impact on our findings, making our findings better fitted as indications rather than results of MPH.

Nitrogen metabolized was analyzed by Nofima AS, while 1-MH, 3-MH and creatinine were analyzed by Bevital AS and CK measured at the Laboratory of Clinical Biochemestry. These operators all used standardized methods which insured high accuracy and contributed to a higher strength regarding the results. RBP4 was measured preforming the ELISA. A method with good strength and stability; however human errors could have occurred. There was only one kit available to perform the procedure, which also caused a limitation whereas a mean measure between two tests could have increased the reliability. In addition, several measures were outside the reference range. This would have been accounted for if the procedure had been performed a second time by using a less diluted sample. As there was not enough serum to

perform the ELISA after other analysis had been performed, we had to use plasma although previous studies usually used serum (10). This could also have had an impact on the results.

It was decided that each participant would complete a diet registration from the last three days before phase two and one day before phase three. Because of many limitations to the recording, the data received was used as an indicator and not used in direct calculations. The inclusion of a complete diet registration could have been a good strength to the study, especially since several of the biomarkers used in this thesis are influenced by diet.

4.2.4 Urine and blood sampling

Each participant was allocated their individual time for both test days. All blood samples were collected by a qualified nurse following a standardized approach. Samples were collected through a peripheral venous catheter to ensure the collection of enough blood and accuracy which gave the sampling good strength. Because participants were given different timeslots, natural circadian rhythms in the human body could vary between participants which were not accounted for.

Moreover, the estimated nitrogen balance through controlled intake of protein and urine collection over a 9hour period caused a weak indication of the true balance. Total collection of urine a few days before the intervention in addition to a longer collection period during the intervention would have given the results better strength. A more throughout dietary registration or controlled diet over a longer time period could also have been performed. Other studies have collected data during a longer period past performance, since restitution of muscle usually last and are still affected about 48hours after. The same with an accurate measure of protein intake (10). However, this collection would still only give a crude estimate of the true nitrogen balance, hence measuring nitrogen balance requires meticulous detail which was not possible to accomplish in this study.

4.2.5 Biomarkers

All parameters looked at in this thesis was not accounted for when the study took place but was decided to be used when the sample collection was complete. Consequently, important factors have been impacted and then corrugated as much as possible. One example was that 3-MH would not be an accurate measure if the participants had been eating meat a few days in advanced. In addition, the standardized meals during intervention day one and two both

contained meat. As mentioned earlier, other studies have showed that the calculated 1-MH/ 3-MH ratio do give a good estimation of muscle degradation in humans with less interference from degradation of consumed meat (39, 40). This correction gave the results more strength and was a crucial part for the inclusion of these results.

A second example is how nitrogen balance techniques are well known to overestimate nitrogen intake and underestimate nitrogen losses. Estimates are highly dependent on the assumed amount of nitrogen miscellaneous losses that are measured to be fairly constant (19). This study did not account for these losses in neither of the testing. The assessment of the nitrogen losses through the skin (urea), the loss of nitrogen gas after denitrification by the colonic microflora and in the expired air (ammonia) cannot be measured using the Kjeldahl method (31). This could have had an impact that should be accounted for, although such testing would have been too demanding for this study.

4.3 FUTURE ASPECTS

This study has demonstrated the need to further explore and investigate the use of MPH as a supplement to increase muscle synthesis and decrease muscle degradation in humans. Even though this study did not find any significant effect of a single small dose containing MPH on muscle synthesis and degradation, several results did suggest that there might be improvement through increased protein synthesis with the usage of MPH.

To further explore this topic, several improvements to the limitations in this study should be corrected for. Recruitment of a more representing part of the general population with a bigger sample size would be recommended along with a more careful selection of participants. Multiple doses of MPH over a longer period could give better results. A longer measurement period with a more thoroughly collection of urine and blood samples could give more reliable measures especially concerning nitrogen metabolized, CK and 3-MH, in addition to a more controlled diet.

5. Conclusion

Intake of MPH following exhaustive endurance cycling did not show any significant difference on biomarkers related to muscle synthesis and degradation. However, nitrogen retained in the body tended to be higher in the MPH group. Also, RBP4 measures of the total protein pool in the body seemed to have decreased levels following the MPH drink. These results could indicate a faster uptake of protein in the muscles supporting muscle synthesis. This study also found a higher 1-MH/ 3-MH ratio in both urine and plasma samples which indicated less excretion of 3-MH. This could imply a reduced muscle degradation following the MPH drink.

In this study, the decreased results of CK, 1-MH/ 3-MH and 3-MH/ Creatinine levels suggest a temporary failure of muscle synthesis rather than decreased muscle degradation. Identification of the catabolic contribution to overall protein degradation could therefore be less important during muscle wasting than increasing protein synthesis. As such, a possible way to prevent catabolic diseases could be to further study potential solutions to increase protein synthesis.

Considering that this thesis did not achieve significant results, there is no evidence that MPH have an impact on biomarkers related to muscle synthesis and degradation. On this basis, H_1 is rejected while H_0 is proven, stating that the intake of a single dose of 20 mg/kg BW of MPH did not show significant impact on biomarkers related to muscle synthesis and degradation in this study.

6. References

1.   Sheffield-Moore M, Urban RJ. An overview of the endocrinology of skeletal muscle. Trends in endocrinology and metabolism: TEM. 2004;15(3):110-5.
2.   Rothman S. How is the balance between protein synthesis and degradation achieved? Theoretical biology & medical modelling. 2010;7:25.
3.   Tipton KD, Wolfe RR. Exercise, protein metabolism, and muscle growth. International journal of sport nutrition and exercise metabolism. 2001;11(1):109-32.
4.   Dardevet D, Remond D, Peyron MA, Papet I, Savary-Auzeloux I, Mosoni L. Muscle wasting and resistance of muscle anabolism: the "anabolic threshold concept" for adapted nutritional strategies during sarcopenia. TheScientificWorldJournal. 2012;2012:269531.
5.   Science WS. Muscle protein synthesis World of Sport Science: Encyclopedia.com; [Available from: https://www.encyclopedia.com/sports/sports-fitness-recreation-and-leisure-magazines/muscle-protein-synthesis.
6.   Dohm GL, Williams RT, Kasperek GJ, van Rij AM. Increased excretion of urea and N tau -methylhistidine by rats and humans after a bout of exercise. Journal of applied physiology: respiratory, environmental and exercise physiology. 1982;52(1):27-33.
7.   Gontzea I, Sutzesco P, Dumitrache S. [Influence of adaptation to effort on nitrogen balance in man]. Archives des sciences physiologiques. 1962;16:127-38.
8.   Goranzon H, Forsum E. Effect of reduced energy intake versus increased physical activity on the outcome of nitrogen balance experiments in man. The American journal of clinical nutrition. 1985;41(5):919-28.
9.   Richardson DP, Wayler AH, Scrimshaw NS, Young VR. Quantitative effect of an isoenergetic exchange of fat for carbohydrate on dietary protein utilization in healthy young men. The American journal of clinical nutrition. 1979;32(11):2217-26.
10.   Pupim Lara B. MCJ, Ikizler T. Alp Assessment of Protein and Energy Nutritional Status. In: Inc. E, editor. Nutritional Management of Renal Disease: Department of Medicine, Division of Nephrology, Vanderbilt University, Medical Center, Nashville, TN, USA; 2013. p. 137-58.
11.   Allison SP. Malnutrition, disease, and outcome. Nutrition (Burbank, Los Angeles County, Calif). 2000;16(7-8):590-3.
12.   Sato T, Ito Y, Nagasawa T. Regulation of skeletal muscle protein degradation and synthesis by oral administration of lysine in rats. Journal of nutritional science and vitaminology. 2013;59(5):412-9.
13.   Argiles JM, Campos N, Lopez-Pedrosa JM, Rueda R, Rodriguez-Manas L. Skeletal Muscle Regulates Metabolism via Interorgan Crosstalk: Roles in Health and Disease. Journal of the American Medical Directors Association. 2016;17(9):789-96.
14.   Bistrian BR, Blackburn GL, Vitale J, Cochran D, Naylor J. Prevalence of malnutrition in general medical patients. Jama. 1976;235(15):1567-70.
15.   Millward DJ, Pacy PJ. Postprandial protein utilization and protein quality assessment in man. Clinical science (London, England : 1979). 1995;88(6):597-606.
16.   Munro HN. Adaptation of mammalian protein metabolism to amino acid supply. The Biochemical journal. 1969;113(2):1p-2p.
17.   Morifuji M, Kanda A, Koga J, Kawanaka K, Higuchi M. Post-exercise carbohydrate plus whey protein hydrolysates supplementation increases skeletal muscle glycogen level in rats. Amino acids. 2010;38(4):1109-15.

18.   van Loon LJ. Application of protein or protein hydrolysates to improve postexercise recovery. International journal of sport nutrition and exercise metabolism. 2007;17 Suppl:S104-17.

19.   Tomé D, Bos Cc. Dietary Protein and Nitrogen Utilization. The Journal of nutrition. 2000;130(7):1868S-73S.

20.   Munro HN. Nutrition and muscle protein metabolism: Introduction. Federation proceedings. 1978;37(9):2281-2.

21.   Witard OC, Wardle SL, Macnaughton LS, Hodgson AB, Tipton KD. Protein Considerations for Optimising Skeletal Muscle Mass in Healthy Young and Older Adults. Nutrients. 2016;8(4):181.

22.   Chou CJ, Affolter M, Kussmann M. A nutrigenomics view of protein intake: macronutrient, bioactive peptides, and protein turnover. Progress in molecular biology and translational science. 2012;108:51-74.

23.   Hellerstein MK, Munro, H.N. Interaction of liver and muscle in the regulation of metabolism in response to nutritional and other factors. In the liver. Biology and Pathobiology. 1988;2nd edition:965-83.

24.   Berg JM TJ, Stryer L. Proetin structure and function. In: W.H. F, editor. Biochemistry. New York2002.

25.   Hoffman JR, Falvo MJ. Protein - Which is Best? Journal of sports science & medicine. 2004;3(3):118-30.

26.   Van De Walle G. 9 Important functions of protein in your body Healthline: Healthline; 2018 [Available from: https://www.healthline.com/nutrition/functions-of-protein.

27.   Barmore W, Stone WL. Physiology, Urea Cycle. StatPearls. Treasure Island (FL): StatPearls Publishing

StatPearls Publishing LLC.; 2019.

28.   Identifying patients at risk: ADA's definitions for nutrition screening and nutrition assessment. Council on Practice (COP) Quality Management Committee. Journal of the American Dietetic Association. 1994;94(8):838-9.

29.   Buchholz AC, Bartok C, Schoeller DA. The validity of bioelectrical impedance models in clinical populations. Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition. 2004;19(5):433-46.

30.   Kyle UG, Bosaeus I, De Lorenzo AD, Deurenberg P, Elia M, Gomez JM, et al. Bioelectrical impedance analysis--part I: review of principles and methods. Clinical nutrition (Edinburgh, Scotland). 2004;23(5):1226-43.

31.   Tessari P. Nitrogen Balance and Protein Requirements: Definition
and Measurements. In: Cachexia and Wasting: A Modern Approach [Internet]. [73-9]. Available from: https://link-springer-com.pva.uib.no/book/10.1007%2F978-88-470-0552-5.

32.   Anonymous. Energy and protein requirements. Wordl health organ: FAO/WHO/UNU Expert consultation; 1985.

33.   Price SR, Mitch WE. Metabolic acidosis and uremic toxicity: protein and amino acid metabolism. Seminars in nephrology. 1994;14(3):232-7.

34.   Motil KJ, Matthews DE, Bier DM, Burke JF, Munro HN, Young VR. Whole-body leucine and lysine metabolism: response to dietary protein intake in young men. The American journal of physiology. 1981;240(6):E712-21.

35.   Calloway DH, Margen S. Variation in Endogenous Nitrogen Excretion and Dietary Nitrogen Utilization as Determinants of Human Protein Requirement. The Journal of nutrition. 1971;101(2):205-16.

36.   Cabaniss DC. Creatine Kinase. Clinical Methods: The history, Physical, and Laboratory Examinations 3 rd edition. Boston: Butterworths; 1990.

37.   AACC. Creatine Kinase (CK) Lab tests online - Your trusted guide2016 [Available from: https://labtestsonline.org/tests/creatine-kinase-ck.

38.   Johnson P, Harris CI, Perry SV. 3-methylhistidine in actin and other muscle proteins. The Biochemical journal. 1967;105(1):361-70.

39.   Sjolin J, Hjort G, Friman G, Hambraeus L. Urinary excretion of 1-methylhistidine: a qualitative indicator of exogenous 3-methylhistidine and intake of meats from various sources. Metabolism: clinical and experimental. 1987;36(12):1175-84.

40.   Bevital. 3-Methylhistidine and 1-methylhistidine Bergen: Bevital; [Available from: https://folk.uib.no/mfapu/Pages/BV/BVSite/analyteinfo/mHist.html.

41.   Elia M, Carter A, Bacon S, Winearls CG, Smith R. Clinical usefulness of urinary 3-methylhistidine excretion in indicating muscle protein breakdown. British medical journal (Clinical research ed). 1981;282(6261):351-4.

42.   Lukaski HC, Mendez J, Buskirk ER, Cohn SH. Relationship between endogenous 3-methylhistidine excretion and body composition. The American journal of physiology. 1981;240(3):E302-7.

43.   VM S. Fundementals of nutrition. Introduction to clinical nutrition. New York1998. p. 1-13.

44.   Sjolin J, Stjernstrom H, Henneberg S, Andersson E, Martensson J, Friman G, et al. Splanchnic and peripheral release of 3-methylhistidine in relation to its urinary excretion in human infection. Metabolism: clinical and experimental. 1989;38(1):23-9.

45.   Tipton KD, Hamilton DL, Gallagher IJ. Assessing the Role of Muscle Protein Breakdown in Response to Nutrition and Exercise in Humans. Sports medicine (Auckland, NZ). 2018;48(Suppl 1):53-64.

46.   Goodman DS. Retinoid-binding proteins. Journal of the American Academy of Dermatology. 1982;6(4 Pt 2 Suppl):583-90.

47.   Hsiung TP. Protein Assessment 2016 [Available from: http://www.tomhsiung.com/wordpress/2016/08/biochemical-assessment-of-nutrition-status/.

48.   Ong DE. Cellular retinoid-binding proteins. Archives of dermatology. 1987;123(12):1693-5a.

49.   Miura N, Kitamura H, Kameko M. [Evaluation of the reference range of retinol-binding protein (RBP) levels by the latex turbidimetric immunoassay]. Rinsho byori The Japanese journal of clinical pathology. 2009;57(3):195-9.

50.   Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature. 2005;436(7049):356-62.

51.   Advances R. Marine protein hydrolysates: their present and future perspectives in food chemistry – A review. RSC Advances. 2013;1(1):1-100.

52.   Aadland EK, Lavigne C, Graff IE, Eng O, Paquette M, Holthe A, et al. Lean-seafood intake reduces cardiovascular lipid risk factors in healthy subjects: results from a randomized controlled trial with a crossover design. The American journal of clinical nutrition. 2015;102(3):582-92.

53.   Nobile V, Duclos E, Michelotti A, Bizzaro G, Negro M, Soisson F. Supplementation with a fish protein hydrolysate (Micromesistius poutassou): effects on body weight, body composition, and CCK/GLP-1 secretion. Food & nutrition research. 2016;60:29857.

54.   Manninen AH. Protein hydrolysates in sports nutrition. Nutrition & metabolism. 2009;6:38.

55.   Poullain MG, Cezard JP, Roger L, Mendy F. Effect of whey proteins, their oligopeptide hydrolysates and free amino acid mixtures on growth and nitrogen retention in fed and starved rats. JPEN Journal of parenteral and enteral nutrition. 1989;13(4):382-6.

56.   Hays NP, Kim H, Wells AM, Kajkenova O, Evans WJ. Effects of whey and fortified collagen hydrolysate protein supplements on nitrogen balance and body composition in older women. Journal of the American Dietetic Association. 2009;109(6):1082-7.

57.   Koopman R, Crombach N, Gijsen AP, Walrand S, Fauquant J, Kies AK, et al. Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo digestion and absorption rate when compared with its intact protein. The American journal of clinical nutrition. 2009;90(1):106-15.

58.   Manninen AH. Protein hydrolysates in sports and exercise: a brief review. Journal of sports science & medicine. 2004;3(2):60-3.

59.   Cheung RC, Ng TB, Wong JH. Marine Peptides: Bioactivities and Applications. Marine drugs. 2015;13(7):4006-43.

60.   Miguel M. CM, Recio I., Aleixandre A. . Food Chemistry. 2009. p. 211-4.

61.   Lavigne C, Marette A, Jacques H. Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats. American journal of physiology Endocrinology and metabolism. 2000;278(3):E491-500.

62.   Lavigne C, Tremblay F, Asselin G, Jacques H, Marette A. Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats. American journal of physiology Endocrinology and metabolism. 2001;281(1):E62-71.

63.   Ullrich SS, Fitzgerald PC, Schober G, Steinert RE, Horowitz M, Feinle-Bisset C. Intragastric administration of leucine or isoleucine lowers the blood glucose response to a mixed-nutrient drink by different mechanisms in healthy, lean volunteers. The American journal of clinical nutrition. 2016;104(5):1274-84.

64.   Siegler JC, Page R, Turner M, Mitchell N, Midgely AW. The effect of carbohydrate and marine peptide hydrolysate co-ingestion on endurance exercise metabolism and performance. Journal of the International Society of Sports Nutrition. 2013;10:29.

65.   Vegge G, Ronnestad BR, Ellefsen S. Improved cycling performance with ingestion of hydrolyzed marine protein depends on performance level. Journal of the International Society of Sports Nutrition. 2012;9(1):14.

66.   Vikoren LA, Nygard OK, Lied E, Rostrup E, Gudbrandsen OA. A randomised study on the effects of fish protein supplement on glucose tolerance, lipids and body composition in overweight adults. The British journal of nutrition. 2013;109(4):648-57.

67.   K. SE. Encyclopaedia of Occupational Health and Safety. Encyclopaedia of Occupational Health and Safety.

68.   Venkatesan J, Anil S, Kim SK, Shim MS. Marine Fish Proteins and Peptides for Cosmeceuticals: A Review. Marine drugs. 2017;15(5).

69.   AS F. Biff Stroganoff: Fjordland AS; [Available from: https://www.fjordland.no/produkter/kjott/biff-stroganoff.

70.   AS F. Torsk med purreløksaus, gulrøtter og poteter [Available from: https://www.fjordland.no/produkter/fisk/torsk.

71.   AS N. Nofima AS [Available from: https://nofima.no/.

72.   Midttun O, Kvalheim G, Ueland PM. High-throughput, low-volume, multianalyte quantification of plasma metabolites related to one-carbon metabolism using HPLC-MS/MS. Analytical and bioanalytical chemistry. 2013;405(6):2009-17.

73.   Analyseoversikten. CK: Helse Bergen HF Haukeland universitetssjukehus; [cited 2019 26.03]. Available from: https://analyseoversikten.no/analyse/166.

74.   science ML. The principle and method of ELISA ruo.mbl.co.jp [cited 2019 03.26]. Available from: http://ruo.mbl.co.jp/bio/e/support/method/elisa.html.

75.   Sergi G, Coin A, Enzi G, Volpato S, Inelmen EM, Buttarello M, et al. Role of visceral proteins in detecting malnutrition in the elderly. European journal of clinical nutrition. 2006;60(2):203-9.

76.   Paul GL, DeLany JP, Snook JT, Seifert JG, Kirby TE. Serum and urinary markers of skeletal muscle tissue damage after weight lifting exercise. European journal of applied physiology and occupational physiology. 1989;58(7):786-90.

77.   Seashore JH, Huszar G, Davis EM. Urinary 3-methylhistidine/creatinine ratio as a clinical tool: correlation between 3-methylhistidine excretion and metabolic and clinical states in healthy and stressed premature infants. Metabolism: clinical and experimental. 1981;30(10):959-69.

78.   Buckley JD, Thomson RL, Coates AM, Howe PR, DeNichilo MO, Rowney MK. Supplementation with a whey protein hydrolysate enhances recovery of muscle force-generating capacity following eccentric exercise. Journal of science and medicine in sport. 2010;13(1):178-81.

79.   Baird MF, Graham SM, Baker JS, Bickerstaff GF. Creatine-kinase- and exercise-related muscle damage implications for muscle performance and recovery. Journal of nutrition and metabolism. 2012;2012:960363.

80.   Dohm GL, Kasperek GJ, Tapscott EB, Beecher GR. Effect of exercise on synthesis and degradation of muscle protein. The Biochemical journal. 1980;188(1):255-62.

APPENDIX 1. RECRUITMENT POSTER.

Firmenich

HELSE **BERGEN**
Haukeland University Hospital

ER DU SYKLIST,
OG HAR LYST TIL Å DELTA I
FORSKNINGSPROSJEKT?

På Haukeland universitetssjukehus og Høgskulen på Vestlandet planlegges det et forskningsprosjekt for å undersøke effekten av et proteintilskudd på restitusjon hos syklister. Vi ønsker derfor å komme i kontakt med syklister som kan tenke seg å delta i studien. Aktuelle deltakere er: **Menn, i alderen 40-50 år, som sykler 8-12 timer i uken.**

Hva innebærer deltakelse?

Målsettingen med dette prosjektet er å undersøke effekten av et marint proteintilskudd etter en hard treningsøkt på sykkel. Vi vil studere om dette kan bedre blodsukkerprofilen, og om det kan øke restitusjonsevnen.

Deltakelse innebærer oppmøte på Høgskulen på Vestlandet 3 dager i løpet av 3 uker:

- *Testdag 1:* En ettermiddag gjøres innledende undersøkelser med testing av maksimalt oksygenopptak.
- *Testdag 2 og 3:* To påfølgende lørdager, eller to påfølgende søndager, skal det så gjennomføres:
  - Sykling på 90-95% av VO_{2max} inntil utmattelse
  - 4 timer restitusjon med inntak av ernæringsdrikke og mat
  - Ny sykkeløkt på 90-95% av VO_{2max} inntil utmattelse

Det vil være lege, fysioterapeut og sykepleier til stede under testingen. Under syklingen vil det bli målt blodsukker og laktat via et stikk i fingeren. Mellom de to sykkeløktene på testdag 2 og 3 vil det i tillegg bli tatt urin- og blodprøver.

Deltakelse vil være aktuelt fra september og utover høsten 2017.

Kunnskap om egen fysisk form – og bidrag til forskning

Dersom du deltar får du mye kunnskap om egen fysisk form, samtidig som du kan være med å bidra til viktig forskning om sykling, restitusjon og ernæring. Det er frivillig å delta i prosjektet og du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke til deltakelse.

Du vil få dekket transportutgifter til og fra Høgskulen på Vestlandet, samt et gavekort på kr. 500. Prosjektet er godkjent av Regional komite for medisinsk og helsefaglig forskningsetikk.

Ønsker du mer informasjon, eller ønsker å melde din interesse?
Kontakt Ingunn Mjøs: ingunnmjoes@hotmail.com

APPENDIX 2. WRITTEN CONSENT FORM.

PEPTID-TILSKUDD TIL SYKLISTER

Dette er et spørsmål til deg om å delta i et forskningsprosjekt for å undersøke om proteintilskudd kan bedre evnen til å restituere seg etter en hard sykkelbelastning.

HVA INNEBÆRER PROSJEKTET?

Flere studier viser at inntak av proteintilskudd i tillegg til et standardisert karbohydratmåltid etter å ha gjennomgått hard trening bedrer restitusjon, utholdenhet og yteevne. I en nylig utført studie utført i Uppsala ble det vist at tilskudd av proteiner fra torsk bedret blodsukkerprofilen med 20 % etter et standardisert måltid hos 12 friske studenter sammenlignet med en kontrollgruppe hvor det ikke ble gitt tilskudd.

Målsettingen med dette prosjektet er å undersøke effekten av proteintilskudd sammen med et standardisert måltid, servert etter en hard treningsøkt på sykkel. Vi vil studere om dette kan bedre blodsukkerprofilen, og om det kan øke restitusjonsevnen. Studien er designet som en dobbel-blindet studie, hvor du vil motta drikke henholdsvis med og uten proteintilskudd etter belastningstest på sykkel.

Fjorten friske menn i alderen 40-50 år skal inkluderes. Forsøkspersonene skal være middels trente personer fra en lokal sykkelklubb i Bergensområdet som trener 8-12 timer i uken.

I studien er det oppmøte tre dager i løpet av tre uker hvor det til sammen skal gjennomføres fem tester på ergometersykkel. Første gang (testdag 1) gjennomføres en maksimal belastningstest på ergometersykkel hvor blant annet melksyreterskel/laktatprofil og maksimalt oksygenopptak måles. Denne testen vil ta ca. 1 time.

Uken etter første test (testdag 2), vil det gjennomføres ny sykkeltest. Før testingen starter vil du få et standardisert måltid og deretter 45 min. pause før selve syklingen starter. Etter avsluttet test vil du motta restitusjonsdrikke med eller uten proteintilskudd. Dette trekkes tilfeldig. I tillegg vil du få mat, og frem til neste test 4 timer senere vil det bli tilrettelagt for hvile/restitusjon. Du skal gjøre to sykkeltester denne dagen, begge med høy intensitet som er beregnet etter resultat fra maksimaltesten som ble gjennomført på testdag 1.

En uke etter testdag 2 (testdag 3) skal du inn til ny testing og gjennomføre samme prosedyre som uken før. Denne gangen gis det proteintilskudd eller placebo i omvendt rekkefølge sammenlignet med testdag 2. Det må beregnes 9 timer både til testdag 2 og 3.
Under sykkeltestene skal du sykle med et munnstykke for å måle oksygenopptak og karbondioksidutskillelse. Det skal tas blodprøve med et stikk i fingeren for måling av

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blodsukker og laktat. Testdagene skal det i tillegg måles effekt av proteintilskuddet både i blod og urin.

Blodprøver blir tatt et fra en vene i albuen tid; før, og ved tid 0, 15, 30, 60, 90 og 120 minutter etter avslutning av tester for å undersøke hormon- og blodsukkerprofil, og urin samles i en beholder.

Vi vil også registrere hva du spiser og drikker i 24 timer. Effekten av kosttilskuddet gitt sammen med det standardiserte måltidet på viktige tarmhormoner vil gi oss verdifulle opplysninger om hvordan dette kan påvirke restitusjonsevnen.

MULIGE FORDELER OG ULEMPER

Det vil være lege, fysioterapeut og sykepleier til stede under testingen, Under syklingen vil det bli målt blodsukker og melkesyre via et stikk i fingeren. Mellom de to sykkeløktene på testdag 2 og 3 vil det bli tatt blodprøver via en veneflon som blir satt inn i en vene i albuen.

Det forventes ingen ubehag av proteintilskuddet.

FRIVILLIG DELTAKELSE OG MULIGHET FOR Å TREKKE SITT SAMTYKKE

- Det er frivillig å delta i prosjektet. Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke. Dersom du trekker deg fra prosjektet, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner. Dersom du senere ønsker å trekke deg eller har spørsmål til prosjektet, kan du kontakte: Prosjektleder, Professor, MD, Trygve Hausken, Universitetet i Bergen: trygve.hausken@helse-bergen.no Førsteamanuensis, PhD, fysioterapeut, Bente Frisk, Haukeland universitetssjukehus, Fysioterapiavdelingen: bente.frisk@hvl.no eller MSc student/, fysioterapeut Ingunn Mjøs, Haukeland universitetssjukehus, Fysioterapiavdelingen: ingunnmjoes@hotmail.com

Du vil få dekket parkering og transportutgifter til og fra Høgskolen på Vestlandet på testdagene, samt et gavekort på kr. 500.

HVA SKJER MED INFORMASJONEN OM DEG?

Informasjonen som registreres om deg skal kun brukes slik som beskrevet i hensikten med studien. Du har rett til innsyn i hvilke opplysninger som er registrert om deg og rett til å få korrigert eventuelle feil i de opplysningene som er registrert.

Alle opplysningene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger gjennom en navneliste. Prosjektleder har ansvar for den daglige driften av forskningsprosjektet og at

opplysninger om deg blir behandlet på en sikker måte. Informasjon om deg vil bli anonymisert eller slettet senest fem år etter prosjektslutt.

HVA SKJER MED PRØVER SOM BLIR TATT AV DEG?

Prøvene som tas av deg skal oppbevares i en forskningsbiobank. Generell forskningsbiobank for fordøyelsessykdommer, 2012/553, Labbygget UIB, Trygve Hausken

FORSIKRING

Forsøkspersonen dekkes gjennom Haukeland universitetssjukehus sin forsikring.

GODKJENNING

Prosjektet er godkjent av Regional komite for medisinsk og helsefaglig forskningsetikk, REK (2017/56].

Prosjektleders navn og kontaktinfo:
Trygve Hausken, Medisinsk avdeling, Haukeland universitetssjukehus. 5021 Bergen og Klinisk institutt 1, Universitetet i Bergen
Trygve.hausken@helse-bergen.no

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JEG ER VILLIG TIL Å DELTA I PROSJEKTET

Sted og dato	Deltakers signatur
	Deltakers navn med trykte bokstaver

Jeg bekrefter å ha gitt informasjon om prosjektet.

Sted og dato	Signatur
	Rolle i prosjektet

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APPENDIX 3. STUDY PROTOCOL.

Firmenich

HELSE BERGEN
Haukeland University Hospital

Effekt of marine peptide supplementation on recovery following endurance cycling

1. Background

Dietary proteins may play a significant role in reducing muscle damage and improving muscle recovery (i.e. muscle repair and muscle function) in both heavy resistance and in endurance training and competitions (1, 3, 7).

Some dietary proteins have been reported to be more effective than others are, and whey protein considered to be the superior one (3, 6). Emerging data suggest that also protein hydrolysates may be effective, and at significantly lower dietary concentrations than that of the whole protein (). In a recent unpublished study with rats using casein as the basic dietary protein 20% replacement of the dietary protein by marine protein hydrolysate from cod muscle increased the daily nitrogen retention in the animals by 17% (16).

A large number of studies have pointed at the branched chain amino acid leucine (9, 10, 13) – or its metabolite hydroxymethylbutyrate (HMG) (11, 12) - as the bioactive component triggering muscle protein synthesis via the mTOR system; proteins containing high levels of leucine is therefore presumed to be more effective in stimulating muscle protein synthesis than leucine-low proteins.

However, not only amino acid composition and digestibility may affect proteins′ nutritional quality, the rate of digestion and amino acid absorption may also affect their bioactivity. In the case of leucine it is shown that muscle protein synthesis only may be triggered if the ingested protein is able to produce a minimum peak level in the blood (9).

Hydrolysed proteins are pre-digested proteins and mostly composed of peptides of low molecular weight. About 60% of the dietary protein is absorbed in the intestine as di- and tri-peptides, and along a different route than that of the free amino acids (14, 15). Consequently, pre-digested proteins are significantly faster absorbed than native proteins, and may therefore more easily trigger protein synthesis than the corresponding amount of native proteins.

1

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In addition, hydrolysed proteins may affect the secretion of hormones like insulin, and gastrointestinal hormones such as GLP-1 (Glucagon-Like Peptide-1) and ghrelin differently from other proteins like whey and casein, and even at very low dietary concentrations (2, 4, 17); such effects have very recently been linked to peptides containing branched chain amino acids, particularly leucine and isoleucine (4).

Both insulin and GLP-1 are vital for muscle protein synthesis by linking GLP-1 to insulin as a trigger of the latter initiating protein synthesis and stimulating the transport of glucose into the muscle both to energize protein anabolism, and to rebuild its glycogen stores.

Studies have shown that dietary proteins, peptides and amino acids may affect the secretion of insulin as well as uptake of glucose into muscle tissue (5, 7, 17), other studies have shown that marine proteins and peptides may affect muscle protein synthesis, also in humans.

It may therefore be hypothesized that pre-digested proteins like marine protein hydrolysates rich in leucine produce a leucine peak faster and at lower dietary concentrations than that of a native protein. Secondarily that marine protein hydrolysates added to proteins may act synergistically with such proteins at low dietary protein concentrations. Third, that marine protein hydrolysates may reduce inflammation and stimulate the secretion of gastrointestinal hormones – in the end positively affecting the ratio muscle protein anabolism to catabolism by increasing the rate of muscle repair and reducing damage of muscle tissue following strong physiological stress.

The current study uses cyclists as test subjects since they represent a physical demanding sport requiring both high endurance capacity and muscle strength. In the recovery phase following exhaustive physical activity there is a need both for rebuilding the muscle energy stores, for muscle repair and for compensatory muscle cell development. Therefore, marine peptide combined with dietary protein will be given in order to examine the capacity to recover after exhaustive endurance cycling.

In order to eliminate any effect of gender difference only male test subjects will be invited to participate in the study.

2

2. Objectives

Main objective:

To investigate the effect of marine peptide combined with dietary protein on the capacity to recover after exhaustive endurance cycling as measured by exercise capacity and blood glucose homeostasis.

Hypothesis: Supplementing foods with low concentration of marine protein hydrolysate improves recovery after exhaustive endurance cycling and blood glucose homeostasis in male endurance cyclists.

Secondary objectives:

To investigate the effect of marine peptide combined with dietary protein on gastrointestinal hormones (GLP 1, Ghrelin, PYY, Insulin-like peptide 5, Glucagon)

3. Methods

Study Design

This is a randomized, double-blinded, placebo controlled study with crossover design. The participants will be recruited from the local cycle clubs in Bergen, Norway.

Partcipants

Healthy male volunteers (n=12), 40–50 years of age, medium trained subjects with cycling as their main exercise activity, with a total amount of 8-12 hours weekly training will be recruited in this trial. Due to a small sample size and avoidance of hormone changes between individuals, no women will be recruited. The hormone changes can influence the rating of hunger and satiety. This recruitment approach is assumed to attain optimal stability and low variation between subjects.

Inclusion criteria
- Signed informed Consent
- The subject is a man between 40-50 years
- The subject has a body mass index (BMI) between 19–29

Exclusion criteria

- The subject has had surgery or trauma with significant blood loss or has donated blood within the last 3 months prior to the screening visit
- Diabetes type 1 or 2, or persistent high blood sugar levels
- The subject has tested positive for human immunodeficiency virus (HIV)
- Hepatitis B surface antigen (HBsAg), or hepatitis C virus antibody (anti-HCV)
- The subject has taken any investigational drugs within 1 month prior to screening
- Treated with antibiotics within 3 months prior to screening (oral, parenteral or rectal), but not spray or ointment
- Treated with steroids within 1 month prior to screening (including oral treatment)
- Treated with medication that effects the intestinal function such as, H2- protonpump, inhibitors, diuretics, antiemetics, antidepressants, antacids,

Intervention

The experimental set up is divided in three phases (Figure 1):

Phase 1: Pretesting, physical assessment testing, 7-14 days before exercise intervention with peptide supplement

Before start of the cardiopulmonary incremental exercise test (CPET) following measurements will be done:

- Body composition measurement: BMI, fat free mass index (FFMI), fat mass index (FMI)
- Blood samples tests
- Dietary registration

Restrictions:

- Coffee /tea: No more than 5 cups of coffee are allowed per day during the study period.
- Alcohol: It is not allowed to drink any alcohol 48 hours prior to each visit.
- Medications: As described in the exclusion criteria the following types of medications are prohibited and not allowed during and before the study:
  - Medication that effects the intestinal function such as, antibiotics during the last 3 months, H_2-receptor blockers, proton pump inhibitors, antidiuretics, antiemetics, antidepressants, benzodiazepines, analgesic morfine derivatives or anticholinergic drugs within 1 month prior to screening

4

Phase 2: Starts 7 -14 days after pretesting

In phase 2 the subjects will perform two exhaustive exercise sessions. First, one session until exhaustion followed by dietary intervention. Thereafter the subjects will rest for 6 – 8 hours before performing a second exercise session until exhaustion.

There will be no exercise for a minimum of 24 h before the first exhaustive bout of exercise, and the test subjects will record their dietary intake over the last 24 h prior to phase 2.

Fasted venous and capillary blood samples will be taken and subjects will receive a light breakfast with a standardized energy and composition followed by preparation for the performance test including insertion of a veneflon into an antecubital vein. The first exercise will be performed 1 hour after breakfast.

The exercise session will be initiated with a 4 min warm up at 50, 55 and 60% of maximal oxygen uptake (VO_{2max}) respectively. Subjects will then cycle at 80 % of VO_{2max} until exhaustion. The exercise load (Watt) at the given intensities is determined on the basis of the measurements of VO_{2max} in phase 1. Exhaustion is defined when subjects are unable to maintain the desired pedal frequency (self-selected pedal frequency - 10 rpm) at the desired workload despite the third verbal encouragement to maintain the pedal frequency.

Oxygen uptake ($\dot{V}O_2$), carbon dioxide output ($\dot{V}CO_2$), respiratory exchange ratio (RER) and lactate will be measured every 5 minutes during exercise, and at exhaustion. The rating of perceived exertion (Borg RPE 6-20) will be registered simultaneously.

After cycling until exhaustion, blood samples will be taken, and the subjects will drink the dietary supplementation immediately. After 6-8 hours of resting, exercise until exhaustion and measurements are repeated, following the same procedures as described above.

The first blood samples will be taken before drink consumption, and capillary and venous blood samples will be taken after 15, 30, 60, 90 and 120 min of the recovery period.

After the first 2 hours of the recovery period subjects will be given a standardized meal (composed of pasta, grained meat and tomato sauce).

Diets

The drink will be supplied in equal portions (0.8 g carbohydrate and 0.2 g protein per kg body weight per hour and adjusted to provide 20 mg MP per kg bodyweight) every 30 min for 2 hours. Drinks will be served in opaque flasks and will be flavoured to be indistinguishable by taste and smell.

The diets will be taken as beverages, basically in the form of a powder composed of whey protein concentrate (WPC80), maltodextrins (DE 20-21) from corn, vegetable fat (MCT fat powder) with and without supplementation with marine protein hydrolysate made of fish fillet from Atlantic cod; the powder will be dissolved in cold water to form a creamy drink. Mixing ratio: 1 g powder plus 2 ml cold water.

The dietary distribution of protein, fat and carbohydrate is 20, 11 and 65 %, respectively; the energy content is 4.4 Kcal/gram dry matter. Flavouring agent, acidifier and natural colouring will be added as technical ingredients. The diets will be given a natural strawberry flavour to level out any taste differences between diets. The diets will be provided, randomly numbered from by Firmenich Bjørge Biomarin AS, Aalesund/Norway.

The diets are composed as follows:
Diet A: (CHO+WPC) Whey protein concentrate + plus carbohydrate (maltodextrins) + fat (MCT); 20 g of protein from WPC80, in addition 200 ml water.
Diet B: (CHO+WPC+MP) diet in which 3.2 % of the whey protein is replaced by marine peptides (marine protein hydrolysate) in terms of crude protein (N x 6.25), in addition 200 ml water.

All diets will be given in amounts equivalent to 20 mg of peptides/kg bodyweight/dosage during the 120 min intervention period following exhaustive exercise, consequently the amount given will be adjusted to the participants' body weight.
1) CHO +PROT composed of maltodextrin and whey protein at a ratio of 80/20 or
2) CHO+PROT+MP in which part of the whey protein is replaced by marine peptides in terms of Nx6.25. The intervention diets (CHO+PROT) and (CHO+PROT+MP) were isocaloric and isonitrogenous.

Phase 3: Cross over testing

Test subjects will return after a 7 days (max 14) wash out period to repeat the procedure in Phase 2. The only difference in the aforementioned protocol is the administration of the alternative beverage. For example, if the subject consumed the (CHO+PROT)-beverage in the first intervention in Phase 2 they will consume the (CHO+PROT+MP)-beverage when Phase 2 is repeated after the wash out period.

Outcome measurements and assessment tools

Questionnaire
Before and after the diet intervention the participants will fill out a questionnaire related to hunger and satiety, with a linear scale of 0-100 mm for:

- Hunger
- Satiety
- Abdominal pain
- Nausea
- Diarrhoea
- Desire to eat

Blood and urine analysis
Details regarding analytical parameters will be discussed; tentatively the following analyses/calculations will be carried out:

- Blood glucose (Hb1Ac)
- Blood Urea Nitrogen (BUN)
- Amino acids
- Urine urea
- Urine creatinine
- Prealbumin
- Inflammatory factors TNFalfa, cytokines
- Calculation of carbohydrate and fat oxidation (RER)
- Calculation of nitrogen balance urea in blood and urea
- Cycle time at the exercises in Phase 2 and Phase 3
- GLP-1 (Glucagon-like peptid -1), Ghrelin, PYY (Peptide Y-Y), Glucagon, Insulin, ILP-5 (Insulin-like factor 5)
- CPK (Creatine Phosphokinase).

Dietary registration
3 days Food registration

Assessment of exercise capacity

The participants will conduct cardiopulmonary incremental exercise tests (CPET) on a cycle ergometer to establish the relationship between work (Watt) and $\dot{V}O_{2max}$. The test procedure will start with a warm-up phase for 5 min., thereafter start the test at 100 W increasing by 25 Watt every 1 min until exhaustion. The tidal volume (V_T), breathing frequency (B_f), $\dot{V}O_2$, $\dot{V}CO_2$, and heart rate (HR) will be measured on a breath by breath basis and averaged over 20 second intervals. Ventilation ($\dot{V}_E$) and V_T were corrected to the body temperature pressure saturated (BTPS) condition, and $\dot{V}O_2$ and $\dot{V}CO_2$ to the standard temperature pressure dry (STPD) condition. The subjects can choose a pedaling frequency between 70 to 95 RPM (pedaling rates), and the self-selected pedaling frequency will be used throughout the study.

The participants will grade their level of exhaustion during the CPET by using the Borg RPE Scale.

There will be no diet intervention between phase 2 and 3.

Statistical analyses and sample size calculation

Descriptive analyses will be used to characterize the study population. Analyse of variance and linear regression will be used to analyse the data.

With an estimated change in mean blood sugar profile (area under the curve) of 20 %, power of 80 %, alpha of 0.05 and a standard deviation of 10 % the calculations estimated that a total of 14 participants had to be included in the study.

4. Ethical considerations

The patients will be given written and oral information when invited to take part in the study, and informed that participation is entirely voluntary. At any time of the follow-up period they can withdraw from the study without giving any reasons. The study will be approved by the Western Norway Regional Committee for Medical and Health Research Ethics. The recommendations from the Helsinki declaration will be followed. Exercise test on cycle ergometer is a common part of evaluation of exercise capacity. These tests are strenuous, but the participants' well-being will always be the main focus, and the tests will be stopped if the participants show any form for discomfort.

5. Organisation and collaboration

- Project leader, Professor, MD, Trygve Hausken, University of Bergen, Dept. of Clinical Science
- Professor Einar Lied, Firmenich.
- PhD/physiotherapist, Bente Frisk, Haukeland University Hospital, Dept. of Physiotherapy,
- MSc student/ Physiotherapist Ingunn Mjøs, Haukeland University Hospital, Dept. of Physiotherapy
- Physiotherapist Elfrid Herre Staveland, Haukeland University Hospital, Dept. of Physiotherapy
- Professor, MD, Einar Thorsen, University of Bergen, Dept. of Clinical Science
-

Project organisation

Haukeland University Hospital, Dept. of Physiotherapy and University of Bergen, Dept. of Clinical Science.

6. References

1. Buckley JD, Thomson RL, Coates AM, Howe, PRC, DeNichilo MO, Rowney, MK; *Supplementation with a whey protein hydrolysate enhances recovery of muscle force-generating capacity following eccentric exercise.* J Sci Med Sport (2010); **13**, 178-181

2. Nobile V, Duclos, E, Michelotti A, Bizzaro G, Negro M, Soisson F; *Supplementation with a fish protein hydrolysate (Micromesistius poutassou): effects on body weight, body composition, and CCK/GLP-1 secretion.* Food Nutr Research (2016), **60**, 29857 – http://dx.doi.org/103402/fnr.v60.29857.

3. Witard OC, Wardle SL, Macnaughton LS, Hodgson AB, Tripton K ND; *Protein considerations for optimising skeletal muscle mass in healthy young and older adults.* Nutrients (2016), **8**, doi:10.3390/

4. Ullrich SS, Fitzgerald PCE, Schober G, Steinert RE, Horowitz M, Feinle-Bisset C. *Intragastric administration of leucine or isoleucine lowers the blood glucose response to a mixed-nutrient drink by different mechanisms in healthy, lean volunteers.* Am J Clin Nutr (2016) doi: 10.3945/ajcl.116.140640.

5. Petersen BL, Ward LS, Bastian ED, Jenkins AL, Campbell J, Vuksan V. *A whey protein supplement decreases post-prandial glycemia.* Nutr J (2009), **8**, doi: 10.1186/1475-2891-8-47.

6. Hoffmann JR, Falvo MJ. *Protein – which is best?* J Sports Sci Med (2008), **3**, 118-130.

7. Saunders MJ, Kane MD, Todd MK. *Effects of a carbohydrate-protein beverage on cycling endurance and muscle damage.* Med Sci Sports Exerc (2004), **36**, 1233-1238.

8. Norton LE, Layman DK. *Leucine Regulates Translation Initiation of Protein Synthesis in Skeletal Muscle after Exercise.* J Nutr (2006), **136**, 533S-537S

9. Churchward TA, Breen L, DiDonato DM, Hector AJ, Mitchell CJ, Moore DR, Stellingwerff T, Breuille D, Offord EA, Baker SK, Phillips SM. *Leucine supplementation of a low-protein mixed macronutrient beverage enhances myofibrillar protein synthesis in young men: a double-blind, randomized trial.* Am J Clin Nutr (2014), **99**, 276-286.

10. Pasiakos SM, McClung HL, McClung JP, Margolis LM, Andersen LM, Cloutier GJ, Pikosky MA Rood RA, Young AJ. *Leucine-enriched essential amino acid supplementation during moderate steady state exercise enhances postexercise muscle protein synthesis.* Am J Clin Nutr (2011), **94**, 809-818.

11. Manzano M, Giron MD, Salto R, Sevillano N, Rueda R, Lopez-Pedrosa JM. *Is β-hydroxy-β-methylbutyrate (HMB) the bioactive metabolite of L-leucine (LEU) in muscle? Molecular evidence and potential implications.* Abstract : European Society for Clinical Nutrition and Metabolism 31st Congress; Vienna, Austria; August, 2009. Abstract P267

12. Alon T, Bagchi D, Preuss HG. *Supplementing with beta-hydroxy-beta-methylbutyrate (HMB) to build and maintain muscle mass: a review. Res Commun Mol Pathol Pharmacol.* 2002;111:139-152.

13. Suryawan A, Jeyapalan AS, Orellana RA, Wilson FA, Nguyen HY, Davis TA. *Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation.* Am J Physiol Endocrinol Metab (2008), **295**, E868-E875.

14. Grimble GK. *Physiology of nutrient absorption and patterns of intestinal metabolism.* In: Payne-James JJ, Grimble GK, Silk DBA (eds); Artificial nutrition support inh clinical practice (1985), 73-97. Arnold, London.

15. Grimble GK. *Mechanisms of peptide and amino acid transport and their regulation.* Nestle Nutr Workshop Ser Clin Perform Programme (2000), **3**, 63-

APPENDIX 4. NITROGEN INTAKE METABOLIZED AND RETAINED FOLLOWING MPH AND PLACEBO DRINK IN EACH PARTICIPANT.

Nitrogen (gram) per viral ID

MPH
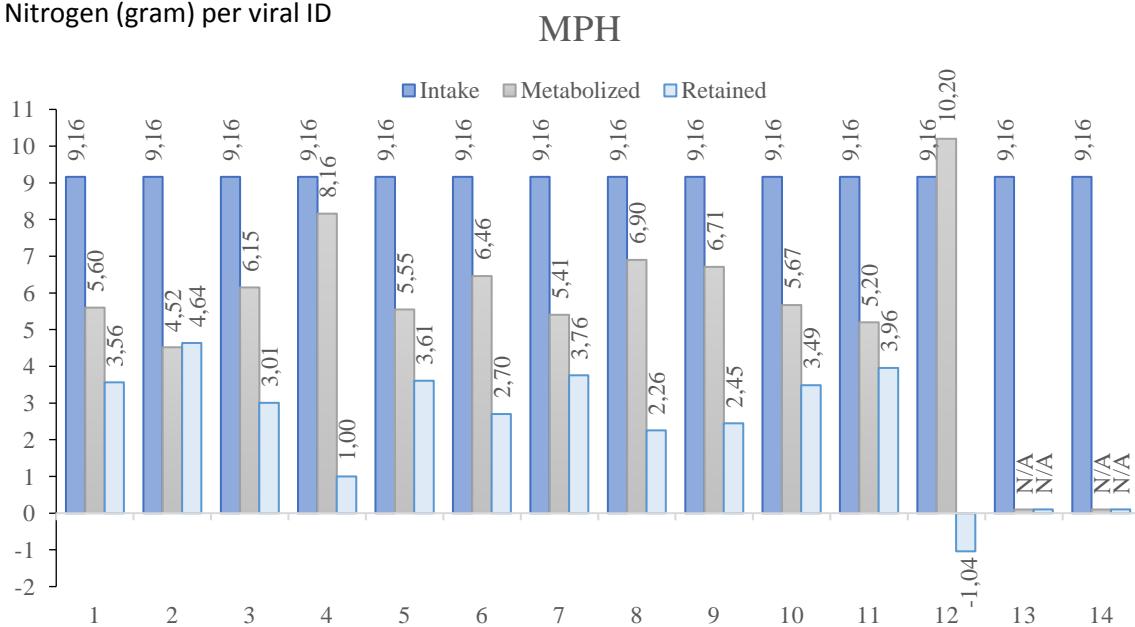
Nitrogen intake, metabolized and retained after consuming MPH drink.
Nitrogen intake estimated from controlled intake throughout the day. Nitrogen metabolized and retained nitrogen measured in urine samples after intake of MPH over a 9 hour period in each participant.

Nitrogen (gram) per viral ID

Placebo
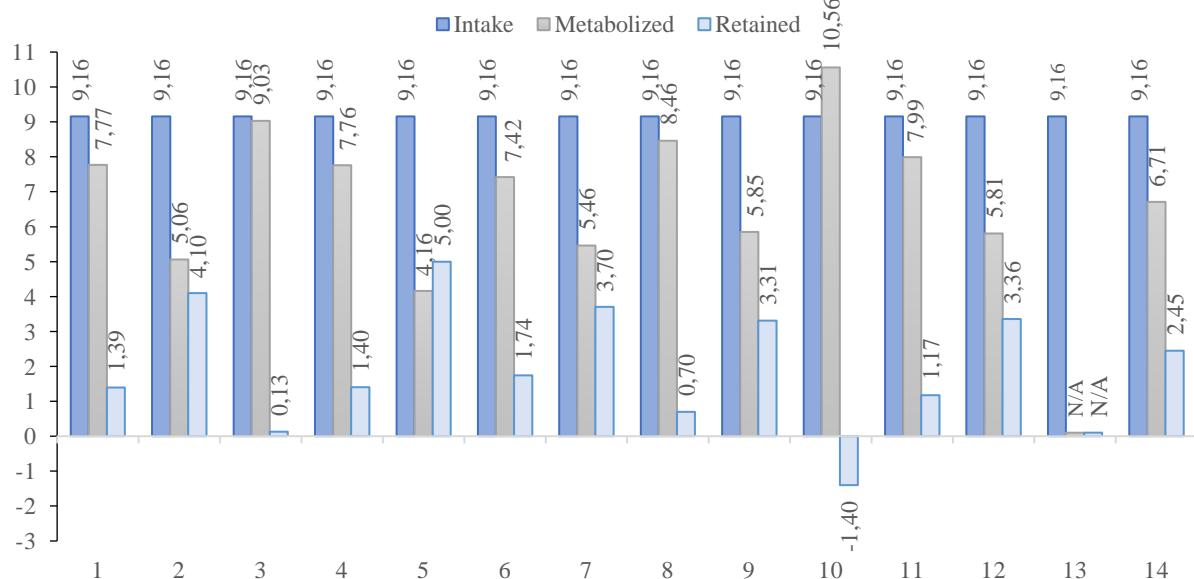
Nitrogen intake, metabolized and retained after consuming placebo drink.
Nitrogen intake estimated from controlled intake throughout the day. Nitrogen metabolized and retained nitrogen measured in urine samples after intake of placebo over a 9hour period in each participant.

Nitrogen metabolized (gram) per viral ID
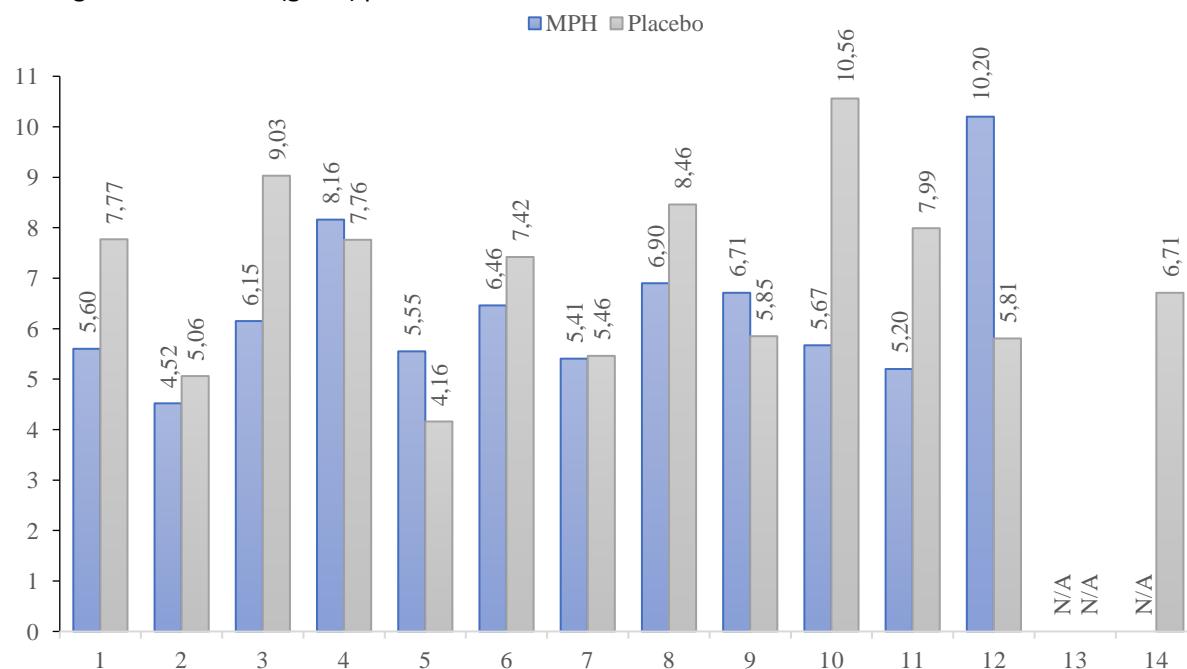
Comparing nitrogen metabolized presented as analyte level in total amount of urine from each participant between drinks.
Measured in urine samples after intake of MPH and placebo post exhaustion endurance cycling collected over a period of 9hours in each participant at two different intervention days.
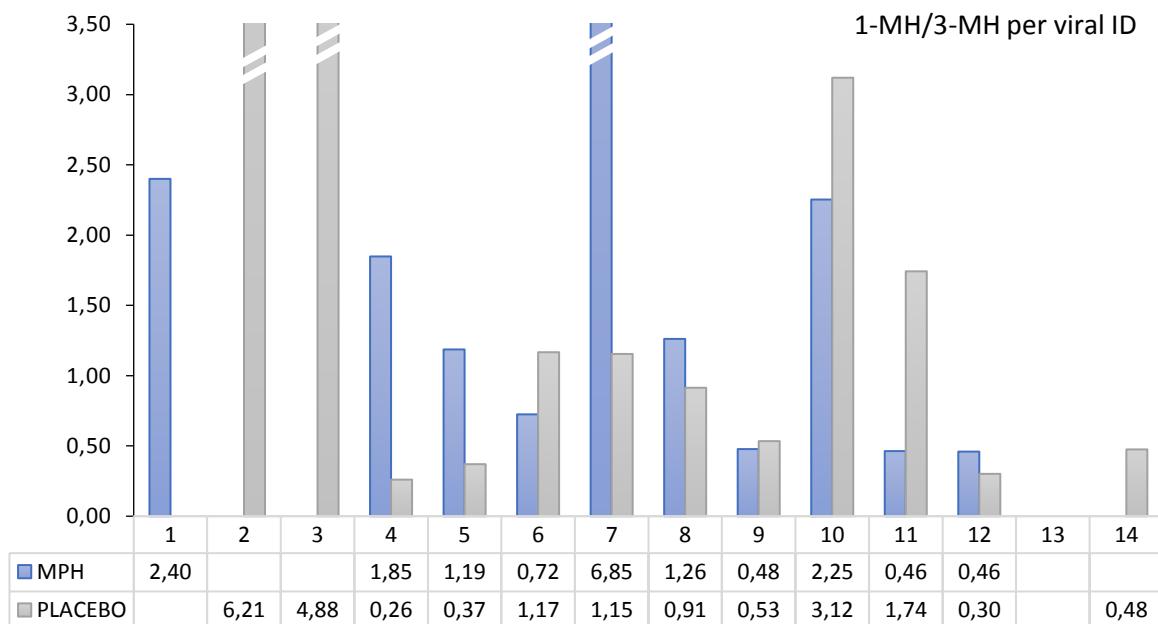
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MPH	2,40			1,85	1,19	0,72	6,85	1,26	0,48	2,25	0,46	0,46		
PLACEBO		6,21	4,88	0,26	0,37	1,17	1,15	0,91	0,53	3,12	1,74	0,30		0,48

1-MH/ 3-MH ratio from both MPH and placebo drink.
*Measured in urine samples after intake of either MPH or placebo post exhaustion endurance cycling collected over a period of 9hours in each participant.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
MPH	0,54			0,13	0,13	0,08	1,47	0,25	0,05	0,37	0,05	0,04		
PLACEBO		1,45	1,10	0,03	0,06	0,18	0,25	0,20	0,10	0,59	0,31	0,03		0,03
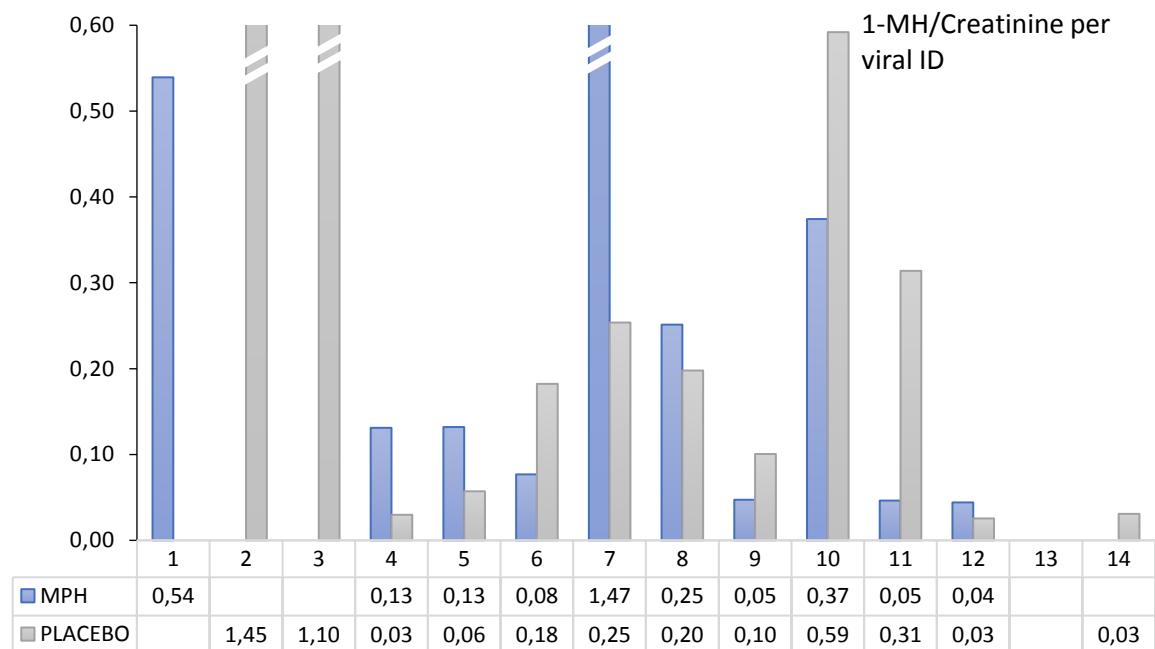
3-MH/ Creatinine ratio from both MPH and placebo drink.
*Measured in urine samples after intake of either MPH or placebo post exhaustion endurance cycling collected over a period of 9hours in each participant. 3-MH used is the true value representing the 1-MH/ 3-MH ratio.*

APPENDIX 6. CORRELATION ANALYSIS.

Correlations between nitrogen in urine and the other biomarkers in plasma/ serum following MPH.

MPH	Nitrogen [1]	3-MH[2]/ Creatinine	CK	RBP4
Nitrogen [1]				
Coefficient	X	-0.103	0.248	0.405
P-value	X	0.777	0.489	0.320
3-MH[2]/ Creatinine				
Coefficient	-0.103	X	-0.382	-0.333
P-value	0.777	X	0.276	0.420
CK				
Coefficient	0.248	-0.382	X	0.548
P-value	0.489	0.276	X	0.160
RBP4				
Coefficient	0.405	-0.333	0.548	X
P-value	0.320	0.420	0.160	X

Statistical significance was evaluated using two-sided pair wise t-test accepting P < 0.05 as significant.
[1] Nitrogen metabolized.
[2] 1-MH/ 3-MH

Correlations between nitrogen in urine and the other biomarkers in plasma/ serum following placebo.

Placebo	Nitrogen [1]	3-MH[2]/ Creatinine	CK	RBP4
Nitrogen [1]				
Coefficient	X	-0.203	-0.042	-0.345
P-value	X	0.527	0.897	0.298
3-MH[2]/ Creatinine				
Coefficient	-0.203	X	-0.021	0.164
P-value	0.527	X	0.948	0.631
CK				
Coefficient	-0.042	-0.021	X	-0.028
P-value	0.897	0.948	X	0.931
RBP4				
Coefficient	-0.345	0.164	-0.028	X
P-value	0.298	0.631	0.931	X

Statistical significance was evaluated using two-sided pair wise t-test accepting P < 0.05 as significant.
[1] Nitrogen metabolized.
[2] 1-MH/ 3-MH

APPENDIX 7. MICROTITER PLATE LAYOUT FOR RBP4 ELISA.

RBP4 06.12.2018

1 STD1	9 STD5	17 1A0	25 3A	33 4C3	41 5A0	49 7A	57 8C3	65 9AC	73 11A	81 12C3	89 13A0
2 STD1	10 STD5	18 1A3	26 3C0	34 4D	42 5F3	50 7A0	58 8D	66 9F3	74 11C0	82 12D	90 13F5
3 STD2	11 Kontroll 1 2A	19 2A	27 3C3	35 4A0	43 6A	51 7C3	59 8A0	67 1CA	75 11C3	83 RAC	91 A4
4 STD2 Kontroll 2	12 2C0	20 2C0	28 3D	36 4F3	44 6C0	52 7D	60 8F3	68 1CC0	76 11D	84 12F3	92 14C0
5 STD3	13 1A	21 1C3	29 3F0	37 5A	45 6C3	53 7F0	61 9A	69 1CC3	77 11F0	85 12F3	93 14C3
6 STD3	14 1C0	22 2D	30 3F3	38 5C0	46 6D	54 7F3	62 9C0	70 1CD	78 11F3	86 13A	94 14D
7 STD3	15 1C3	23 2F0	31 4A	39 5C3	47 6F0	55 8A	63 9C3	71 1CF0	79 12A	87 13C0	95 14F0
8 STD4	16 1D	24 2F3	32 4C0	40 5D	48 6F3	56 8A0	64 9D	72 1CF3	80 12C0	88 13C3	96 14F3

APPENDIX 8. KANE SYMPTOM QUESTIONNAIRE.

Kvantisering av symptomer etter Kane

Angis på en skala fra 0 til 10 der 0 = ingen symptomer og 10 = alvorlige symptomer.

Spørsmål	Svar
Kvalme	
Oppblåsthet	
Magesmerter	
Forstoppelse	
Diaré	
Sulten/Mett (sulten = 0 mett = 10)	

APPENDIX 9. VAS OF GASTRO INTESTINAL SYMPTOMS.

Dato:	Nr:	Tilskudd:

Gradering av eventuelle magesymptomer. Sett en strek på linjen som beskriver hvordan du føler deg.

FØR INNTAK AV Drikke

1. Har du smerter i øvre midtre del av magen?

Ingen smerter		Meget ubehagelig

2. Er du kvalm?

Ikke kvalm		Meget kvalm

3. Føler du deg oppfylt i magen?

Ikke i det hele tatt		Meget oppfylt

4. Totalt ubehag i øvre del av magen?

Ikke i det hele tatt		Meget stort ubehag

5. Er du mett?

Meget sulten		Helt mett

Rett ETTER INNTAK AV drikke

1. Har du smerter i øvre midtre del av magen?

Ingen smerter		Meget ubehagelig

2. Er du kvalm?

Ikke kvalm		Meget kvalm

3. Føler du deg oppfylt i magen?

Ikke i det hele tatt		Meget oppfylt

4. Totalt ubehag i øvre del av magen?

Ikke i det hele tatt		Meget stort ubehag

5. Er du mett?

Meget sulten		Helt mett