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„Acute impact of dairy on plasma protein carbonyls in healthy adult women - a randomized controlled trial"

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Declaration of Authorship

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Abstract

Introduction

Excessive physical exercise can result in oxidative stress and exercise-induced muscle damage. 17-β-Estradiol seems to possess antioxidative properties. Recent data suggest a possible antioxidative effect of dairy. The aim of this master's thesis is to examine an acute effect of 250mL skimmed milk on plasma protein carbonyls in healthy untrained adult women.

Material & methods

To induce oxidative stress in healthy untrained adult women a high-intensity-interval-training was conducted. Subjects consumed water or milk in a crossover design prior to training. 17-β-Estradiol concentration was measured using a standard kit and protein carbonyl content was assessed using an adapted protocol. The statistical analysis was performed with SPSS(V27) & a significance level at $p < 0,05$.

Results

The consumption of 250mL skimmed milk had a significant ($p < 0,05$) overall protective effect of protein carbonyl content generation (-0,052±0,0264, $p = 0,05$). 17-β-Estradiol concentrations showed no significant ($p > 0,05$) additive antioxidative effect with milk consumption. However, subjects with the lowest 17-β-Estradiol concentrations showed significant higher protein oxidation before training than individuals with higher concentrations immediately after training (Q2: -0,073±0,034, $p < 0,05$; Q3: -0,102. ±0,034, $p < 0,05$).

Discussion

Protein carbonyls are very sensitive to autogenous defence mechanisms and should be therefore not analysed as a single parameter. Additionally, the carbonyl bioavailability may extend up to four hours. Considering more parameters as 17-β-Estradiol is essential to assess the impact of menstrual cycle on oxidative stress. Finally, further research should prioritize fermented dairy as it seems to have a greater influence on redox homeostasis.

Key words

Oxidative stress; Menstrual cycle; Dairy

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List of Abbreviations

AA	Arachidonic-acid
AO	Antioxidants
αToc	αTocopherol
BIA	Bioelectrical Impedance Analysis
β-LG	β-lactoglobulin
BMI	Body-Mass-Index
BORG-scale	Borg's subjective rating of perceived exertion
BSA	Bovine serum albumin
CA	Cancer
CAT	Catalase
CU	Copper
CV	Coefficient variation
CVD	Cardiovascular disease
$_{dd}H_2O$	Deionized water
DNPH	2,4-dinitrophenyl-hydrazine
E	Extinction
ε	Extinctioncoefficient
E2	17-β-Estradiol
EDTA	Ethylenediaminetetraacetic acid
EIMD	Exercise-induced muscle damage
EFP	Early-follicular-phase
FFQ	Food-frequency-Questionnaire
FSH	Follicle-stimulating hormone
FP	Follicular phase
GPX	Glutathione Peroxidase
GSH	Glutathione
HCl	Hydrochloric acid
HIIT	High-intensity-interval-training
H_2O_2	Hydrogen peroxide
hsCRP	high-sensitive C-reactive-Protein
L	Wavelenght in cm
LF	Lactoferrin
LGG	Lactobacillus rhamnosus GG
LH	Luteinizing hormone
LP	Luteal phase
MC	Menstrual-cycle
MFP	Mid-follicular-phase
MLP	Mid-Luteal-Phase
MRF	Myogenic regulation factors
mTOR	Mammalian target of rapamycin
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation 1
MyoG	Myogenin
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NO*	Nitrogen monoxide
NOS	Nitric monoxide synthases
NOX	NADPH oxidases
NuTraLab	Nutrition and Training Laboratory
O_2	Oxygen
O_2^1	Singlet molecular oxygen
O_2^{-*}	Superoxide anion radical
OC	Oral contraceptive
OH*	Hydroxyl radical
OS	Oxidative stress
OV	Ovulation phase
P4	Progesterone
PAX7	Paired box protein 7
PC	Protein carbonyl
PE	Physical exercise
PLA2	Phospholipase A2
PRDXs	Peroxiredoxins
RNS	Reactive nitrogen species
RO*	Alkoxyl radical
ROO*	Peroxyl radical
ROS	Reactive oxygen species
rmANOVA	Repeated measures analysis of variance
RT	Room temperature
SD	Standard derivation
SFA	Saturated fatty acids
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TNFα	Tumor-necrosis-factor-α
Trxs	Thioredoxin
UZA2	Department of nutritional sciences
VA	Vitamin A
VC	Vitamin C
VE	Vitamin E
VO2max	Maximal oxygen uptake
XO	Xanthine oxidases
5-RM-test	Repetition maximum strength tests

1. Introduction and Objectives

1.1. Reactive species

Reactive oxygen species (ROS) define a group of free radicals emerging from oxygen (O_2) by aerobic respiration in all aerobic eucaryotes, when reducing O_2 to produce energy (Adenosine triphosphate). (Zuo et al., 2022)

These chemical compounds have been discovered in 1954 by Commoner et al. (1954) and since then where associated with physiological and pathophysiological impact on cells. (Powers et al., 2020)

Free radicals are chemically characterized by one or more unpaired electrons in their atomic orbital, which makes them reactive and unstable. (Bardaweel et al., 2018; Kawamura & Muraoka, 2018)

Next to ROS there exists a group of reactive nitrogen species (RNS). These are summarized as “reactive species” which are able to trigger a chain reaction of free radicals and non-radicals, listed in Table 1. (Kawamura & Muraoka, 2018)

Table 1: Reactive species (adapted according to Sies et al. (2017))

Reactive species	Free Radicals	Non-radicals
ROS	Superoxide anion radical (O_2^{-}*)	Hydrogen peroxide (H_2O_2)
	Hydroxyl radical (OH*)	Orga, nic hydroperoxide (ROOH)
	Peroxyl radical (ROO*)	Singlet molecular oxygen (O_2^1)
	Alkoxyl radical (RO*)	Electronically excited carbonyls (RCO)
		Ozone (O_3)
RNS	Nitrogen monoxide (NO*)	Nitrite (NO_2^-)
		Nitroxyl anion (NO^-)
		Peroxynitrite ($ONOO^-$)
	Nitrogen Dioxide (NO_2*)	Peroxynitrate (O_2NOO^-)
		Nitroperoxycarbonate ($ONOOCO_2^-$)

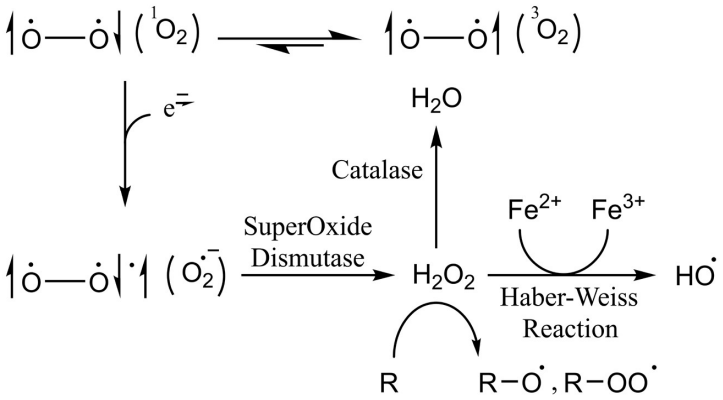
Superoxide anion radical (O_2^{-}*), as the origin for all other ROS, is a comes from various sources including mitochondrial metabolism, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and xanthine oxidases (XO), by the one electron reduction of molecular O_2. The following dismutation of O_2^{-}* on the other hand occurs spontaneously by the enzyme superoxide dismutase (SOD), leading to the production of Hydrogen peroxide (H_2O_2). As O_2^{-}* does not easily cross cell membranes and is short-half-lived, H_2O_2 on the other hand is permeable to membranes and durable, leading with high chronical levels to a cytotoxic effect.

Hydroxyl radical (OH*) is the most damaging ROS because of its strong oxidizing potential. This is a result of reactions which may involve H_2O_2, O_2^{-}* and the reducing body metal iron (so called “Haber-Weiss-Reaction”). OH* typically oxidizes molecules near to their site of production. Other ROS-species, as peroxyl radical (ROO*) and alkoxyl radical (RO*), might be also generated from O_2^{-}*and

seem to be key mediates in lipid peroxide reactions. (Bardaweel et al., 2018; Kehm et al., 2021; Powers et al., 2020; Reid, 2016)

A graphical description of the conversion of O_2 to ROS can be seen in Figure 1.

Figure 1: Conversion of oxygen to ROS (Bardaweel et al., 2018)



RNS are synthesized from L-arginine through nitric monoxide synthases (NOS). Nitrogen monoxide (NO*), as their origin, and its subtypes are among others found in skeletal muscle fibers. Using NADPH, calcium activation and some other cofactors (e.g., 5,6,7,8-tetrahydrobiopterin or iron) NOS converts L-arginine to NO* and L-citrulline. Muscular contraction leads also to an increase in NO*. Besides that, O_2^-* rapidly reacts with NO* to form its subgroups. (Powers et al., 2020; Reid, 2016)

Reactive species are attributed an important role in physiological functions regarding aging process, immune system, cognitive function, and fertility but are also associated with various diseases as cardiovascular disease (CVD) or cancer (CA). (Bardaweel et al., 2018; Zuo et al., 2022)

1.2. Oxidative stress

“Oxidative stress” (OS) is defined as an imbalance between oxidants, as reactive species, and protection mechanisms, so called “antioxidants” (AO), in favour of the oxidants, leading to a disruption of redox signalling, control and molecular damage. (Sies et al., 2017)

1.2.1. Physiology & pathophysiology of oxidative stress

Various physiological and pathophysiological functions in human health and disease are related to OS. On the one hand, reactive species, as being very reactive, are associated with a wide range of degenerative processes and diseases. On the other hand they are essential intracellular signalling molecules and therefore contribute to several body functions, such as immune response or physical exercise (PE). (Bardaweel et al., 2018; Powers et al., 2020)

Initial immune response takes advantages of the oxidative burst, which is produced by phagocytes as one of the first lines of defence mechanisms against pathogens. Phagocytes can accomplish an attack on bacteria by forming a phagosome, and use their intracellular killing mechanism by producing OH* from O_2. Moreover, myeloperoxidase enzymes of neutrophils need H_2O_2 and chlorine to create a highly toxic compound for initial defence. Furthermore, ROS trigger the amplifications of

intracellular signal transduction cascades in T-Lymphocytes and regulate the release of prostaglandins in macrophages, according to their intracellular redox-state. (Bardaweel et al., 2018)

In addition to this, ROS are involved in inflammatory responses by activating inflammasomes. This can lead to a chronic inflammatory state. (Dröge, 2002)

The mitochondrial free radical theory of aging proposes that within aging, ROS induce mitochondrial elements to form even more ROS. This can result in a dysfunction of mitochondria. Besides that, higher oxidated levels of impaired proteins, lipids and DNA are highly correlated to aging. (Muller et al., 2007) Finally, ROS modulate and activate the expression of “mammalian target of rapamycin” (mTOR) and “adenosine monophosphate -activated protein kinase”, both well-known regulators of aging processes. (Bardaweel et al., 2018; Kapahi et al., 2010; Salminen & Kaarniranta, 2012; Zuo et al., 2022)

Furthermore OS is associated with many age related diseases such as CVD or CA. (Chung et al., 2009) These were also the most common death causes in Austria in 2021 (CVD: 34,1 %; CA: 22,5 %). (Statistik Austria, 2021)

ROS derived by NADPH oxidases seem to be one of the main causes of CVD, leading to an increase in oxidated low-density-lipoproteins in macrophages and thus transforming to foam cells. Apart from this, mitochondrial ROS appear to promote a proinflammatory phenotype on arteriosclerosis pathogenesis (Zuo et al., 2022)

In the case of CA genesis ROS signal transduction involves redox-reductions leading to irreversible mutations of protein structures and functions , promoting metastasis. During metastasis, epithelial-mesenchymal-transition may result in circulating tumour cells. This process might be promoted by ROS activating growth factors. Furthermore, resistance of cancer cells against treatment might be triggered by ROS activating growth factors. Also cancer therapeutic resistance might be promoted by OS (Bardaweel et al., 2018; Zuo et al., 2022)

1.3. Physical exercise and oxidative stress

1.3.1. Molecular mechanisms of ROS generation in skeletal muscle cells

ROS are continually produced in skeletal muscle cells during PE and modulate physiological processes including blood flow, muscle force production and muscle adaption to exercise training. Possible origins of exercise induced ROS include the mitochondrial metabolism, phospholipase A2 (PLA2), NADPH oxidases (NOX) and XO. (Powers et al., 2022) A graphical overview of all these mechanisms is shown in Figure 2.

Within oxidative phosphorylation in the mitochondria O_2^{-}* is produced. However, relevant research showed, that during PE O_2^{-}* decreased in muscle mitochondria. (Boveris & Chance, 1973) Therefore the hypothesis was developed, that other molecular mechanisms would produce exercise induced ROS. (Powers et al., 2020)

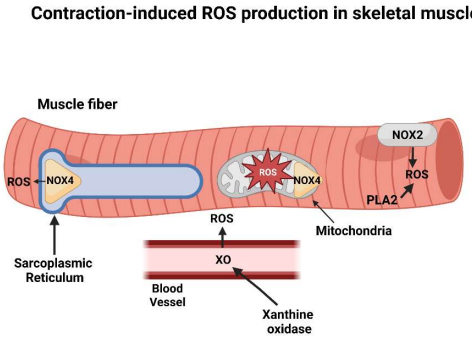
PLA2 is one of the enzymes that can be found in skeletal muscle fibres. It catalyses membrane phospholipids leading to an increase in arachidonic acid (AA). AA is a long-chain fatty acid with pro-inflammatory properties which is the substrate of

several ROS-generating enzyme systems, including the lipoxygenases. (Zuo et al., 2004) The activity of PLA2 can promote ROS production in the skeletal mitochondria and the cytosol. (Powers et al., 2020) Moreover, it seems that PAL2 calcium dependent isoforms lead to physiological ROS generation during resting-periods, the calcium independent isoform, on the other hand, rather promotes ROS production during PE. (Gong et al., 2006)

When PLA2 is activated, it triggers an NOX activation cascade. (Zhao et al., 2002) There are two isoforms of NOX in skeletal muscle cells: NOX2 and NOX4. While NOX4 seems to be continually activated and therefore is responsible for the ROS basal rate in skeletal muscle, NOX2 is activated by specific agonists (such as OS or cytokines) in contracting muscles and sarcoplasmatic rediculum. (Ward et al., 2014) This appears to be the reason why mitochondria are not the primary source of exercise induced ROS. (Powers et al., 2020)

Besides NOX, XO enzymes are also a source of extracellular ROS generation in the endothelium which are claimed to play an important role in contraction-induced release of ROS. (Jackson et al., 2016)

Figure 2: Possible ROS producing mechanisms in contracting skeletal muscle cells (Powers et al., 2022)



1.3.2. Relationship between oxidative stress and physical activity

PE duration, intensity, and muscle temperature influence levels of reactive species generation during contractions. Whereas high levels of reactive species impair muscle force production, physiological levels may lead rather to skeletal muscle adaption. (Powers et al., 2020)

Exercise induced OS decreases as training status increases. (Powers et al., 2020)

This was proved when nine untrained males showed a rise in biomarkers of OS (as protein carbonyls) after performing a high intensity endurance exercise. However, within prolonged training, antioxidative defence mechanisms in trained muscles eliminate exercise induced OS. (Quindry et al., 2003)

In a cell culture model, it was proved that H_2O_2 can activate protein kinase B, leading to an increased protein synthesis by the upregulation of mTOR. (Leslie et al., 2003) In addition to this, in a plantaris muscle model Ito et al. (2013) found mTOR gets activated by NO and O_2^{-}* triggering $ONOO^-$ production. Leading herby to a further activation of contractile proteins gene expression at translational level. (Ito et al., 2013) Putting all this together it seems that contraction- induced reactive

species are the key intracellular signalling molecules inducing muscle hypertrophy by PE. (Powers et al., 2020)

On the other hand, unaccustomed PE may lead to muscle protein degradation, also known as exercise-induced muscle damage (EIMD), due to myofibrillar disruption and membrane damage caused by reactive species in metabolically active tissues. Some of the mostly known consequences from EIMD range from loss of muscle strength, to delayed onset muscle soreness or decrease in the range of motion, as well as increase in limb girth or growth of systemic leakage of muscle enzymes and proteins. (Romero-Parra et al., 2021)

Therefore, following PE, a recovery period is required to manage muscle damage to decrease fatigability and maladaptation to the training load. A wide range of regeneration methods are suggested to improve recovery after PE. They include compressive techniques, electrostimulation, stretching, cold water immersion, active recovery and nutrient intake recommendations. (Dupuy et al., 2018; Moore et al., 2022)

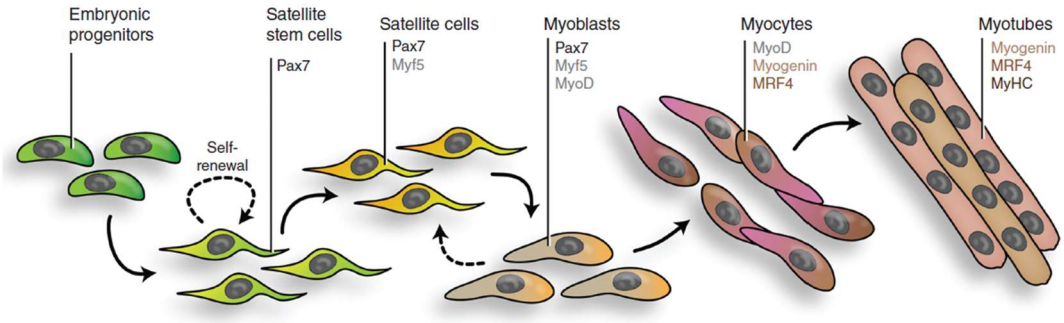
Regeneration of muscle myofibers is based on the myogenic potential of satellite cells. Satellite cells are located between the plasmalemma (fiber membrane) and basal membrane (basement membrane, a layer near to extracellular matrix) of myofibers. (Christov et al., 2007; Dumont et al., 2015)

Satellite cells contribute to muscle cell regeneration through various stages by expressing diverse myogenic regulation factors (MRF). During quiescent stage satellite cells are inactive but constantly express transcription factors, as paired box protein 7 (PAX7) and myogenic factor 5 (Myf5). (Christov et al., 2007; Dumont et al., 2015)

After muscle injury satellite cells are activated and migrate towards the injury site to re-enter the cell cycle for further proliferation. During this stage, next to PAX7 and Myf5, myogenic differentiation 1 (MyoD) is expressed. When the proliferation phase is completed, PAX7 and Myf5 expression decreases in favour of myogenin (MyoG) and MRF-4, leading to myoblasts exiting the cell cycle to differentiate into mature myocytes. Finally, adult myocytes fuse together to build multinucleated myotubes and might fuse to damaged myofibers. During this stage it is important to reduce MyoD levels, because of its critical MRFs balance. (Dumont et al., 2015; Olguin et al., 2007)

A graphical description of this process is shown with Figure 3.

Figure 3: Graphical description of satellite cell differentiation to adult myocytes using different regulation factors (Dumont et al., 2015)



1.4. Methods of assessing oxidative stress

The only way to directly determine reactive oxygen species in biological samples is to use fast response techniques, as electron paramagnetic resonance. However, as these methods are very challenging it is highly recommended to dedicate stable products emerging from OS, as free radicals are very reactive and short lived. (Kehm et al., 2021)

Reactive species are capable of inducing damages in all cellular components, to reach orbital balanced status. It has been shown that proteins scavenge most of them (about 50 to 75%). (Davies et al., 1999)

The higher the reactivity of free radicals the less selective is their way of reaction. Very reactive species tend to damage numerous sites at side-chains and backbones of proteins. (Davies, 2016) These modifications affect protein functions as receptors, enzymes, carriers, or structural proteins, as well as their physical characteristics (e.g., hydrophobicity). (Dröge, 2002) Irreversible alterations tend to be more contributing to pathological conditions than reversible are. (Spickett & Pitt, 2012)

Oxidation of proteins may occur directly within specific amino acids (as sulfur containing or aromatic amino acids), or indirectly through glycoxidation, lipid peroxidation, nitration or carbonylation. (Kehm et al., 2021)

1.4.1. Protein carbonylation

Protein carbonylation is an irreversible and indirect marker of detecting OS in biological samples. Reactive carbonyl groups occur through different mechanisms as direct oxidation of several amino acid residues, protein backbone hydrolysis, lipid peroxidation or glycoxidation reactions. (Colombo et al., 2016; Kehm et al., 2021) Figure 4 shows the summarization of these mechanisms.

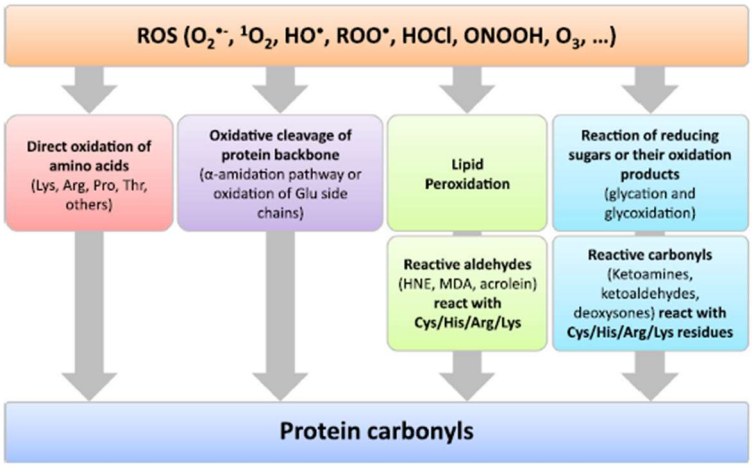
First, aminoadipic - and glutamic semi-aldehyde are products of direct amino acid oxidation. To form aminoadipic semialdehydes the N_6-amino function of lysine metal catalyses oxides H_2O_2 to an imine, which gets further hydrolysed to aminoadipic semialdehyde. To produce glutamic semialdehydes the proton next to the nitrogen of proline, or the proton next to the guanidino function of arginine, is abstracted, oxides H_2O_2 and leading to hydrolysation of the resulting intermediate. (Akagawa et al., 2006) Moreover, OH* induces direct metal catalysed oxidation of proline, arginine, lysine, and threonine too, forming herby aldehydes or ketones. (Requena, 2001)

Second, protein backbone hydrolysis occurs when RO* reacts with the α-carbon next to a peptide bond. Depending on which bond RO* fragments, different carbonyl groups emerge. By using the diamide pathway (carbon-carbon bond) diamide and isocyanate are formed. When the α-amidation pathway (carbon-nitrogen bond) is used, amide and *N*α-ketoacyl derivative are its end products. (Stringfellow et al., 2014) In addition to this, protein backbone hydrolysis can be facilitated by its prolyl, glutamyl, and aspartyl residues in polypeptides. (Stadtman & Levine, 2003)

Third, “Michael addition” occurs when reactive aldehydes react with cysteine, histidine, and lysine residues during lipid peroxidation. (Perluigi et al., 2012; Weber et al., 2015)

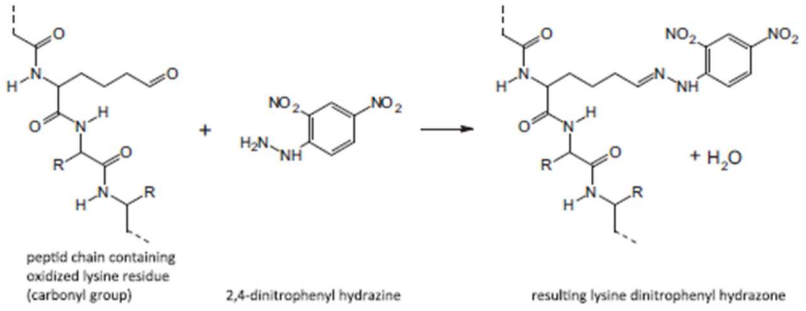
And finally, reducing sugars or their oxidative products react with the amino group of lysine to build carboxymethyl lysine. (Thornalley & Rabbani, 2014)

Figure 4: Selected pathways of protein carbonylation (Weber et al., 2015)



Reactive carbonyl groups or amino acid functional groups on lysine, arginine, serine, threonine, and proline react with 2,4-dinitrophenylhydrazine (DNPH) by derivatization of the carbonyl group. During this condensation reaction 2,4-dinitrophenyl hydrazone is built within the loss of molecular water. (Colombo et al., 2016; Weber et al., 2015) For a graphical description see Figure 5.

Figure 5: Protein carbonyls derivation with DNPH in the example of aminoadipic semialdehyde, as an result of oxidized lysine, resulting in the specific hydrazone as an end product (Weber et al., 2015)



The most widely recognized protocol to detect protein carbonyl (PC)-content using DNPH was implemented by Levine et al. (1990)

At that time, Levine et al. (1990) treated oxidatively modified proteins with 10mM DNPH for one hour. After incubation time the protein precipitated with 20% trichloroacetic acid (TCA), followed by washing steps with ethanol-ethyl-acetate (2:1) and finally dissolved in 6M guanidine buffer. The PC-content was spectrophotometrically measured at 360 to 390 nm to detect built 2,4-dinitrophenyl hydrazone concentrations in nmol. (Colombo et al., 2016; Weber et al., 2015)

Other protocols using DNPH for PC derivation can be applied using high-liquid-chromatography, immunoblotting or enzyme-linked immunosorbent assay (ELISA). (Colombo et al., 2016; Weber et al., 2015)

PC content is mainly expressed as nmol carbonyl per protein concentration in mg/mL. Therefore, a second analysis for protein concentration must be performed (see chapter 2.3.5). (Colombo et al., 2016)

A wide range of spectrometric methods have been established to assess protein concentration of plasma samples. All of them have their pro's and con's. (Okutucu et al., 2007)

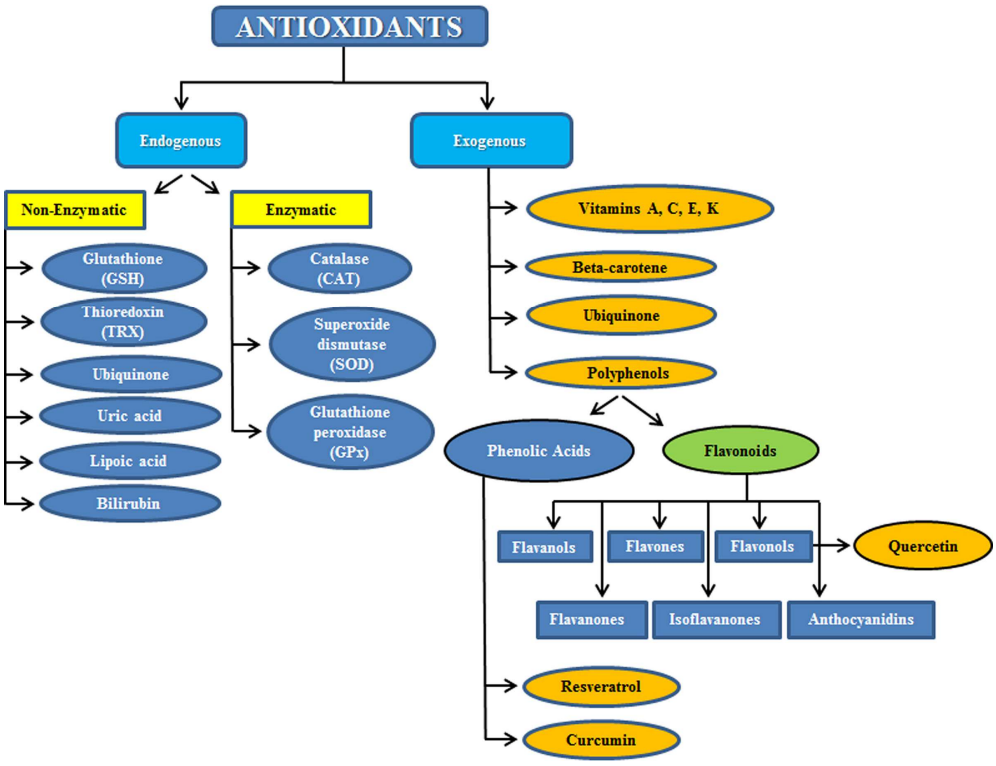
The "Lowry Assay" is a well-implemented protocol in the field of protein carbonylation assessment. (Buonocore et al., 2020; Fayh et al., 2013; Silva et al., 2010) The method was first found as "Biuret-Reaction" by Gornall et al. (1949) but was attributed as being very unpractical due to large volumes of sample required. (Okutucu et al., 2007) Therefore Lowry et al. (1951) optimized the experiment by first reducing Cu^{2+} to Cu^{+} to develop a protein-Cupper-complex. Then Folin–phenol reagent (Folin–Ciocalteau-reagent®) was added to reduce specific amino acids (tyrosine, tryptophan) receiving a blueish solution. Finally, the optical density of this solution was spectrophotometrically measured at 578 nm and protein concentration (mg/mL) calculated using bovine serum albumin (BSA) as a standard.

1.5. Antioxidants

A combination of complex cellular systems is required to degrade reactive species. First, scavenging enzymes can remove free radicals by reducing them. And second, non-enzymatic molecules attempt to prevent or lower the oxidation rate of a substrate. In addition to this, repair and removal systems complete the damage defence. (Kehm et al., 2021; Powers et al., 2022)

Both, enzymatic and non-enzymatic AO, can be produced endogenously by specific triggers. However, non-enzymatic AO may also be supplied by healthy diet. (Powers et al., 2022; Simioni et al., 2018) A graphical description of all AO can be found in Figure 6.

Figure 6: Graphical presentation of endogenous and exogenous antioxidants (Simioni et al., 2018)



1.5.1. Endogenous antioxidants

Endogenous enzymatic AO are distributed in various cellular compartments or extracellular space to facilitate removal of oxidants at the site of production to prevent cellular damage. (Powers et al., 2022) The most relevant ones regarding PE and OS will be discussed in the following part. Furthermore, a graphical description of all possible AO can be found in Figure 6.

SOD primarily reduces O_2^{-}* to H_2O_2. (Bardaweel et al., 2018) Its isoforms usually require metals as cofactors (copper, zinc, and manganese). The resulting H_2O_2 can diffuse long distances and form more reactive species, therefore several antioxidative systems are needed to reduce the possible following oxidation and avoid further cell damage. (Powers et al., 2022)

Glutathione Peroxidase (GPX) depends on selenium as a cofactor and uses glutathione (GSH) protein, as one of the non-enzymatic AO, to reduce H_2O_2 hereby forming water or alcohol. The isoforms of GPX are located in various cell compartments and therefore reduce oxidation in multiple locations. (Powers et al., 2022) Especially GPX-4 plays a major role in ferroptosis and reducing lipid peroxides. (Seibt et al., 2019)

Catalase (CAT) is the oldest known of antioxidative enzymes. (Glorieux & Calderon, 2017) Even though CAT has one of the highest turnover rates, it is not as efficient as other antioxidative enzymes due to its disability of reducing organic H_2O_2. (Powers et al., 2022)

Finally, peroxiredoxins (PRDXs) complete the group of enzymatic AO. (Powers et al., 2022) PRDXs seem to be the dominant enzymatic AO as they are usually expressed in cells at higher levels compared to CAT and GPX, and therefore eliminating > 90% of all reactive species. Furthermore, they do not require cofactors and use cysteine residues for catalysis. (Karplus, 2015) All isoforms reduce H_2O_2 using electrons provided by thioredoxin (Trxs), an inducible antioxidative protein. Moreover, PRDXs might play a major role in cell signalling in skeletal muscle fibers and other cells. (Rhee & Kil, 2017)

Thioredoxin (Trxs) is a non-enzymatic antioxidative protein. It is attributed to reduce cellular antioxidative enzymes resulting in the reduction of the oxidant. Consecutively, Trx is reduced by Trx-reductase using electrons form NADPH. This attributes Trxs as one of the major regulators of the oxidative system. (Balsera & Buchanan, 2019) Furthermore, it supplies electrons to methionine sulfoxide reductase and disulphide reductase. This is important for oxidized proteins to regenerate after reducing radicals. Moreover, it regulates directly antioxidative gene expression by controlling DNA binding to several transcription factors. (Zhang et al., 2021)

Other endogenous antioxidative non-enzymatic-defence systems include among others Coenzym Q10, uric acid, lipoic acid and bilirubin (Simioni et al., 2018) which will not be further discussed in this master's thesis.

To activate endogenous AO systems PE seems to be a good trigger to increase antioxidative capacity which is required to provide protection against several sources of OS. High-intensity-interval-training (HIIT) involves short bouts of vigorous exercise followed by a short recovery period. This leads to an 80% to 150% higher intensity. (Powers et al., 2022)

In rat locomotor muscles HIIT lead to increased GPX activity and SOD in trained muscles. (Cobley et al., 2014) This was already confirmed in human model when eleven men performed a high intense sprint cycling protocol for seven weeks. (Hellsten et al., 1996) Therefore it can be assumed that PE may lead to an increased resistance to oxidative challenges. (Simioni et al., 2018)

1.5.2. 17-β-Estradiol and its potential antioxidant properties

Unfortunately randomized controlled trials dealing with exercise induced OS mainly include male individual at the expense of female subjects. (Costello et al., 2014)

This is clinically relevant since women tend to have significant (sig.) ($p < 0,01$) lower markers of inflammation than men after excessive PE. (Aragón-Vela et al., 2021; Luk et al., 2021)

Moreover, women present fewer anti-inflammatory gene regulation and less fatiguability after isometric muscle contractions than men. (Abbasi et al., 2016; Hunter, 2016)

Furthermore, a meta-analysis published in 2020 found sig. ($p < 0,01$) reduced exercise performance within women in the early follicular phase (EFP) compared to all other phases of their menstrual cycle (MC). (McNulty et al., 2020) This agrees with a recently published survey, where young non oral contraceptives (OC) using female athletes reported a sig. ($p< 0,05$) positive impact of the ovulation phase (OV) on their performance compared to EFP and luteal phase (LP). (Ekenros et al., 2022)

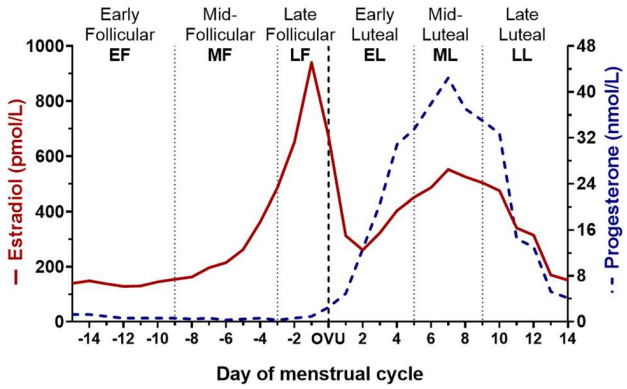
Therefore, an effect of each phase of the MC must be considered as exercise induced OS may leads to EIMD. (Romero-Parra et al., 2021)

17-β-Estradiol (E2) and progesterone (P4), both active steroid hormones of the female MC, have been discussed to have an impact on these observations. (Oosthuyse et al., 2022)

The MC is regulated by feedback loops within hypothalamus-pituitary-ovarian axis. (Hawkins & Matzuk, 2008) The average duration in healthy women persists for a period of 28 days, with an individual variation of approximately 21 to 35 days. (Leburn et al., 1995) Its major purpose is to prepare the female endometrium for a possible implantation of an embryo. Therefore, every 21 to 35 days a follicle is built, ovulation conducted, and eventually endometrium shed (a phenomenon so called “menstruation”), when no fertilisation occurred. This process is controlled by hormones, such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are triggered by the hypothalamus to produce sex steroid hormones (E2 and P4) in the ovaries. (Peinado et al., 2021)

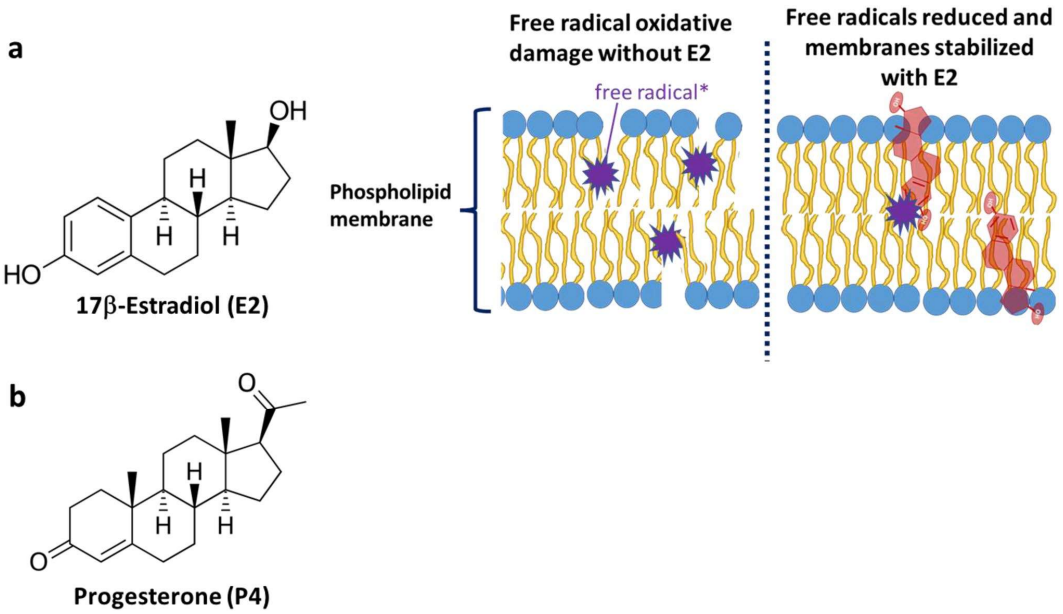
During MC E2 increases flatly in the follicular phase (FP) (day 8 to 10) and peaks as ovulation occurs (day 10-14). Afterwards, it sharply decreases and reaches a low level. When P4 starts to increase, E2 rises with it to finally decrease to a minimum and menstruation starts (day 28). (Stricker et al., 2006) This MC-phase specific mechanism may lead to E2 availability shift depending on each cycle phase. (Oosthuyse et al., 2022) For graphical description see Figure 7.

Figure 7: Menstrual cycle 17-β-Estradiol (pmol/L) and progesterone (nmol/L) concentrations from Stricker et al. (2006) adapted by Oosthuyse et al. (2022)



E2 is attributed an antioxidant potency due to its phenolic structure and therefore might be able to act like tocopherol (see chapter 1.5.3) in free radical quenching. Furthermore, its structure allows it to intercalate in plasma membrane bilayers to improve membrane stability while holding lipid peroxidation chain reaction, as seen in Figure 8. (Kendall & Eston, 2002; Oosthuyse et al., 2022) Furthermore, it stimulates enzymatic antioxidative defence enzymes expression (e.g. SOD) in monocytes (Strehlow et al., 2003)

Figure 8: The chemical structure of 17-β-Estradiol and progesteron and a graphical representation of 17-β-Estradiol leading to membrane stability against OS (Oosthuyse et al., 2022)



During the regeneration of injured muscles after PE, and therefore EIMD prevention, E2 seems to play a major key role, depending on P4 concentration. (Oosthuyse et al., 2022)

Sex steroid hormones are reported being connected with skeletal muscles, which is the largest tissue containing E2 and P4 receptors. (Greising et al., 2009) E2 favours Myo-D expression, which is essential for proliferation-phase of myocytes (see chapter 1.3.2) and therefore might be important for muscle cell recovery. (Thomas et al., 2010)

Because of the interaction of E2 with P4 during the MC it seems quite logical that the influence of E2 in mid follicular phase (MFP) on EIMD is more noticeable, than

in mid luteal phase (MLP). (Haines et al., 2018) Myo-D expression appeared to be sig. ($p < 0,05$) greater in women in MFP than MLP. This effect was not confirmed regarding E2 plasma concentrations, as women in MLP had higher mean concentrations. This seems to occur because of the supressing effect of P4 on E2 promotion, possible by the downregulation of E2-receptor-expression. (Haines et al., 2018)

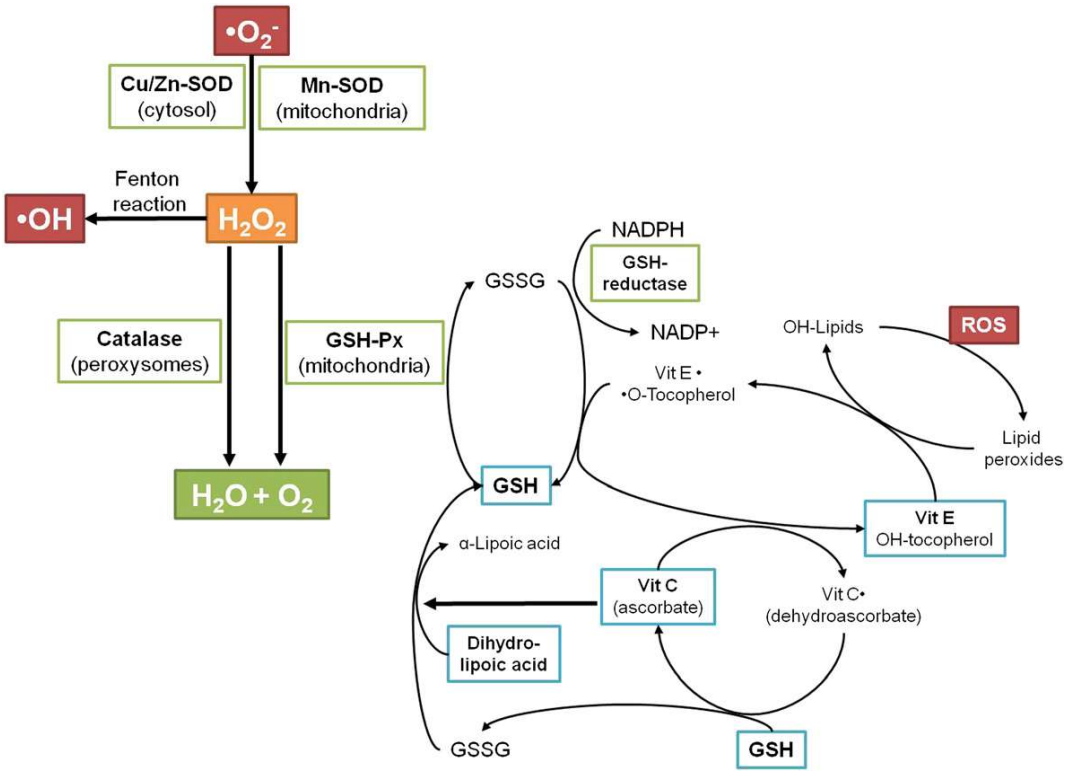
Moreover, it was found that when 20 women trained one leg in FP had greater muscle strength than training the other leg in LP. (Sung et al., 2014) In addition to this, greater strength gain was discovered when 19 non OC using women trained specifically in FP than in LP. (Wikström-Frisén et al., 2017)

However, sex steroid hormones may play a protective role against EIMD, literature provides little evidence, as controlled trials tend to be inhomogeneous in their MC-phase-cut-offs, eligibility criteria and rather include men than women in their study protocols. (Costello et al., 2014; Thompson et al., 2020)

1.5.3. Exogenous antioxidants

Exogenous AO are mainly provided by a healthy diet and tend to act as free radical scavengers in addition to endogenous AO. They possess the ability to neutralize reactive species, repair oxidized membranes, decrease ROS or RNS production and neutralize them via fat metabolism, using short-chain-free-fatty-acids and cholesteryl esters. (Berger, 2005) For a graphical description of these supportive mechanisms see Figure 9.

Figure 9: Antioxidant defence in the organism (Kurutas, 2016)



Vitamin E (VE) defines a group of fat-soluble compounds, including tocopherols and tocotrienols, which contain a chromanol ring, a phytyl-trial and methyl group. (Schmölz et al., 2016) α-tocopherol (α-Toc) has shown to protect effectively against lipid peroxidation. (Azlina et al., 2018) To do so, it traps ROO* and breaks hereby the chain reaction of lipid peroxidation. So, its primary purpose seems to

prevent the formation of secondary radicals rather than avoiding the initial formation. By quickly reacting with ROO* α-Toc forms a relatively stable tocopheroxyl radical which then gets regenerated by endogenous AO, as GPX or urate. Furthermore, Vitamin C (VC) seems to reduce the tocopheroxyl radical, and therefore regenerates α-Toc, in a cyclic type process (see Figure 9). (Wang & Quinn, 2000)

Besides that, VE might also have a structural role in stabilising cell membranes and lipoproteins. (Wang & Quinn, 2000)

The major sources of VE in diet are plant oils, nuts, seeds and avocados. Healthy adult women up to 51 years should consume 12 mg of α-Tocopherol equivalent a day. (DGE et al., 2021)

VC – or L-ascorbic acid- is a strong reducing agent which can neutralize reactive species directly, also in the intracellular space by using glucose transporter after being oxidized of metal ions. It interacts with GSH (see Figure 9) and protects thiol proteins against oxidation. Furthermore, it has a synergistic antioxidative effect with VE and carotenoids. (Kojo, 2004; Peternelj & Coombes, 2011)

Healthy non-smoking adult women up to 51 years should consume 95 mg of VC per day, preferably from sources such as fresh fruits and vegetables, such as green pepper, currants or parsley. (DGE et al., 2021)

Carotenoids, as β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin, can be converted into retinol (VA) and act as AO, anticancerogenic and promote the immune system. (Simioni et al., 2018) Carotenoids are efficient scavengers of singlet molecular oxygen (O_2^1) and trap ROO* in cell membranes and lipoproteins. Therefore, they might be able to make a remarkable contribution in the prevention of lipid peroxidation. (Fiedor & Burda, 2014)

Carotenoids are mainly found in orange, red, and green vegetables, such as carrots, tomatoes, or spinach. Retinol on the other hand is mainly found in liver, eggs, and dairy products. Adult women up to 51 years should consume 700 µg retinol-equivalents per day. (DGE et al., 2021)

However, the influence of plant derived AO is well explored, the impact of animal sources is yet unknown. (Abeyrathne et al., 2022)

1.6. The impact of milk on oxidative stress and inflammation

The Austrian association of nutrition (ÖGE) recommends a daily consumption of three portions of low-fat dairy for adults up to 65 years, for instance: 200mL drinking milk plus 180-250 g yoghurt plus 50-60 g cheese a day, for adults from 19 to 65 years. This should provide high quality protein, calcium, and vitamin B2. Following a balanced healthy diet can reduce the risk of suffering from non-communicable diseases, as CVD or CA. (ÖGE, 2022)

The composition of milk differs within species. Cow's milk contains caseins (β, κ, αs1, αs2) and whey proteins (α-lactalbumin and β-lactoglobulin (β-LG)) as a primary protein source. Furthermore, cow's milk is rich in water, minerals and vitamins. (Samtiya et al., 2022)

Especially the fat-soluble vitamins concentration differs with fat content of milk. (Muskat et al., 2021) The exact composition of cow's whole milk can be found in Table 2.

Table 2: Whole milk composition adapted according to Muskat et al. (2021) & Samtiya et al. (2022)

Cow's whole milk 3,5% (100g)	
Energy (kcal)	64
Fat (g)	3,46
SFA (g)	1,99
Proteins (g)	3,43
Total Casein protein (g)	2,72
αs1 Casein (mg)	10 - 15
αs2 Casein (mg)	3 – 4
κ Casein (mg)	3 – 4
β Casein (mg)	9 – 11
Total Whey protein (g)	0,45
α-Lactalbumin (mg)	1 – 1,5
β-Lactoglobulin (mg)	3,3 - 4
Carbohydrates Lactose (g)	4,71
Water (g)	87,62
Retinol-equivalents (µg)	31
Tocopherol equivalent (mg)	0,1
L-ascorbic acid (mg)	1

In Austria "milk" is defined as the total milk of cows. During the processing, raw milk is centrifuged, standardised, filtration sterilised, pasteurised, and homogenised. Through standardisation the fat content of milk is adjusted. Low-fat milk contains from 1,5 to 1,8% total fat. "Extended-shelf-life milk" is defined as milk with a longer shelf life that has been at least superheated. During superheating, the raw milk is heated for at least a few seconds at a minimum of 85°C, which leads to an extended shelf life of 45 days since superheating. (BMSGPK, 2023)

In addition to species and the heat treated production process, the diet and lactation phase of the animals seem also to have an influence on the composition of milk. (Stobiecka et al., 2022)

However the acute impact of milk on inflammation and oxidated stress remains unknown. (Bordoni et al., 2017; Nieman et al., 2021; Ulven et al., 2019)

The following part will deal with the current state of research of milk and dairy regarding the influence of milk on OS and inflammation.

1.6.1. Pro-inflammatory aspects of milk

On the one hand milk contains extracellular saturated fatty acids (SFA), including palmitic acid, which may activate directly Toll-Like-Receptor-4 signalling by macrophages. This activation can lead to an increased production of proinflammatory cytokines and cellular stress. (Hess et al., 2021)

This was already confirmed in retrospective food-frequency-questionnaire (FFQ)-studies, in which subjects following diets including full-fat dairy, which usually contain high levels of cholesterol, SFA, and trans fatty acids, were associated with higher levels of high-sensitive-C-reactive-Protein (hs-CRP). (Khayyatzadeh et al., 2017) These conditions can lead to an accumulation of cholesterol in immune cells, such as macrophages, and therefore increase inflammatory response, as with producing hs-CRP. (Tall & Yvan-Charvet, 2015)

In addition to this, Esmaillzadeh et al. (2007), Nettleton et al. (2006) and Anderson et al. (2012) found an increase in Interleukin(IL)-6-concentrations in subjects consuming high fat dairy according to FFQ too.

Furthermore, following a diet with three servings of dairy a day (375 mL low-fat milk, 175 g low-fat yogurt, and 30 g regular-fat cheddar cheese) for four weeks led to a sig. ($p < 0,05$) increase in hs-CRP in 112 healthy overweight adults, in comparison to energy-matched crossover. (Labonté et al., 2014)

Cellular stress can also be increased by milk using transcription factor activation, showed by Vázquez-Agell et al. (2013) when 18 healthy subjects consumed cocoa powder with a milk and water mixture, instead of 500mL milk.

Finally, a proinflammatory acute impact of high-fat dairy, was also observed when 48 healthy subjects consumed isocaloric drinks in comparison to water. The SFA -rich-cream-group showed sig. ($p < 0,05$) increases in pro-inflammatory cytokines as well as transcription factors. (Deopurkar et al., 2010)

1.6.2. Antioxidative and anti-inflammatory aspects of milk

On the other hand, antioxidant capacity of milk was found within the presence of endogenous enzymatic antioxidative systems and non-enzymatic components as sulphur amino acids, whey proteins, VA, VE and VC and β-carotene. (Stobiecka et al., 2022)

Milk is high in vitamins and minerals to prevent automatic oxidation. Therefore, it contains VE, VA and β-carotene. These fat-soluble vitamins act as AO by performing direct radical scavenging, inhibit lipid peroxidation and protect DNA-oxidative damage. Moreover, the contained active form of vitamin D3 (1,25-dihydroxycholecalciferol) prevents lipid peroxidation too, next to regulating calcium-phosphate metabolisms and maintaining calcium homeostasis. Finally, L-ascorbic acid completes the non-enzymatic AO in milk. (Stobiecka et al., 2022)

Furthermore, depending on their amino acid composition, and milk processing, milk proteins show also antioxidant capacity. (Stobiecka et al., 2022)

Especially β-LG seems to have the highest antioxidative potential of all milk proteins because of the high content in sulfur amino acids, such as cysteine. Cysteine is an essential amino acid for GSH synthesis and acts therefore as a cofactor for endogenous AO systems (see chapter 1.5.1). (Stobiecka et al., 2022)

Furthermore, lactoferrin (LF), as another antioxidative protein, has been acknowledged for its antioxidative potential since 1939 due to its high affinity for iron. Therefore it inhibits inflammatory response and ROS release in leukocytes. (Moreno-Expósito et al., 2018)

Moreover, it seems that caseins and β-LG have a synergistic effect on the antioxidative capacity of milk. It was found in vivo, that myoblasts differentiation and maturation was enhanced by milk's proteins. In addition to this, cell aging was also reduced. (Kim et al., 2019)

Additionally, milk and dairy products contain bioactive peptides, produced by protein hydrolysis in the gastrointestinal tract by digestive enzymes such as pepsin or pancreatic enzymes. (Stobiecka et al., 2022) These peptides seem to show antioxidative capacities, depending on amino acid sequence, -composition and -length. (Abeyrathne et al., 2022) Bovine milk itself especially contains protease enzymes, which can also break down milk proteins producing water-soluble peptides that show antioxidative potential. (Toldrá et al., 2020)

The antioxidative capacity of bioactive peptides ranges from direct radical scavenging to chelating metal ions and inhibiting lipid peroxidation. (Stobiecka et al., 2022)

This was already discovered in a cell line model, when Tonolo et al. (2022) found in immortalized cell lines of human colorectal adenocarcinoma a sig. ($p < 0,01$) downregulation of proinflammatory cytokines gene, when cells were treated with peptides of milk ("K-8-K" sequence KVLPVPEK and "S-10-S" sequence SLVNNDDRDS). This could mean that OS triggered activation of transcription factors can be reduced by bioactive peptides of milk. (Tonolo et al., 2022)

However, the acute impact of milk on inflammation or OS in human trials is still unknown as human trials usually demonstrate retrospective diet surveys or dietary intervention for a period of time. (Bordoni et al., 2017; Nieman et al., 2021; Ulven et al., 2019)

For example when Zemel et al. (2010) gave 20 overweight subjects three dairy based smoothies a day for 28 days, they showed sig. ($p < 0,01$) lower markers of OS and inflammation in comparison to soy based smoothies. They discussed that the amino acid leucin is one of the major reasons why participants showed reduced inflammation status, as leucin stimulates transcription factors to decrease proinflammatory cytokines gene expression. (Zemel et al., 2010)

Furthermore, Rossi et al. (2015) found sig. ($p < 0,05$) reduced IL-6 contents when 800 mg of calcium were consumed by low fat dairy products for three weeks in comparison to supplements within hypocaloric diets in 40 premenopausal obese women. However, the effect can be explained by the greater weight loss in dairy diets, as IL-6, as one of the proinflammatory cytokines, is mainly produced in adipose tissues. (Rossi et al., 2015)

In addition to this, daily consumption of > 3,5 servings of dairy over a period of twelve weeks showed sig. ($p < 0,05$) decreased markers of inflammation and oxidative stress in 20 overweight adults with metabolic syndrome, compared to subjects consuming < 0,5 daily servings. (Stancliffe et al., 2011) Stancliffe et al. (2011) assumed that milk proteins, especially the whey fraction, may improve insulin resistance and therefore might mitigate the cytokine expression and secretion from adipocytes.

Dugan et al. (2016) found the same sig. ($p < 0,05$) effect when 24 females with metabolic syndrome consumed 300 mL of skimmed milk, 180 ml non-fat yoghurt and 115g of 2% fat cheese daily, for six weeks, compared to a cross over carbohydrate-based-control.

Moreover, Anderson et al. (2012) found increased IL-6 just in people consuming high-fat-diary, in comparison low-fat-dairy using an FFQ.

However, the acute impact of 500mL skimmed milk showed no sig. influence on PC-content in ten female athletes after performing a high intensity cycling protocol (Rankin et al., 2018), Fraschetti et al. (2022) found sig. ($p < 0,05$) reduced proinflammatory cytokines concentration 48h after consuming 1100mL skimmed milk before resistance training in 13 untrained female individuals.

This is clinically relevant as trained athletes seem to adapt their defence mechanisms on OS, induced by eccentric concentration exercise, even after a single bout, according to the "repeated bout effect." (Kawamura & Muraoka, 2018)

1.7. Aim, Objectives & Hypothesis

Due to its high nutrient density in carbohydrates and high-quality proteins milk seems to be an optimal nutritional strategy for enhancing muscle regeneration after PE. (James et al., 2019)

Moreover, regarding the possible anti-inflammatory and antioxidant effects of milk it may have EIMD reducing potential and therefore should be tested in untrained healthy adult women in a high intensity training model. (Khan et al., 2019; Stobiecka et al., 2022) Considering E2 as being a possible additional antioxidative compound, trials wishing to assess influence of OS in females, should include this parameter too. (Oosthuyse et al., 2022) However, women must be from a young age, as neuroendocrine changes occur during menopause. (Burger et al., 2002)

Fraschetti et al. (2022) observed an acute anti-inflammatory effect in 13 untrained females using IL-1β and IL-10 as parameters. However, no studies dealing with PC, as an irreversible and indirect marker of detecting OS in biological samples (Colombo et al., 2016), and the acute impact of milk have been conducted yet.

Therefore, the aim of this master's thesis is to explore the acute impact of skimmed milk on plasma protein carbonyls in healthy adult women, performing a HIIT. Furthermore, the potential antioxidative effect of E2 concentrations will be considered.

2. Material & methods

2.1. Study subjects

For this master's thesis data from a cross-over randomised controlled trial of a multidisciplinary human study project at the university of Vienna, department of sport sciences, division of nutrition, physical activity, and public health was used. The aim of the study was to explore the acute impact of drinks and foods on inflammatory markers and regeneration in untrained healthy adult women.

Possible eligible subjects had to be female, healthy, non-pregnant or lactating, 18 to 40 years old, non-smokers, primarily physically untrained (less than 120 minutes of regular physical activity / week), normal weight, with normal body fat relation and no clinical signs of intolerances or aversions to intervention foods or contraindications to PE.

A summarization of the inclusion criteria can be found in Table 3.

Only female individuals were included in this trial to investigate the impact of E2, a steroid hormone with possible antioxidative properties (Costello et al., 2014; Oosthuyse et al., 2022), on OS.

Furthermore, all participants had to be 18- to 40-year-old as an association between increased mitochondrial ROS production and lifetime was found in several organisms. (Lambert et al., 2007) Additionally, neuroendocrine changes occur during menopause. (Burger et al., 2002)

Moreover, cigarette smoking interference with redox homeostasis too and alters endogenous AO levels. Therefore, eligible subjects were non -smokers. (Chang et al., 2018)

Subjects had to be physically untrained, as training status increases OS tolerance of skeletal muscle cells. (Powers et al., 2020)

All participants had to be normal weight according to the Body-Mass-Index (BMI) and with normal body-fat-relation, using body composition analysis by bioelectric-impedance-analysis (BIA).

The BMI cut-off value for healthy adults up to 65 years of age is 18,5 – 24,9 kg/m². (WHO, 2000) It was found in overweight (Manna & Jain, 2015) as well as in anorexic patients (Solmi et al., 2016) that levels of OS tend to be elevated. Furthermore, low body fat relation in athletes seems to improve body tolerance to OS. (Aguiar et al., 2021)

In addition to this, eligible subjects had to have no intolerances or aversions to intervention drinks and foods or contraindications to PE, as study protocol required specific food and drinks consumption as well as performing a HIIT (see Figure 10).

Because of the association of elevated markers in OS and chronic diseases, patients who suffer from diseases regarding the metabolism, cardiovascular -, pulmonal - or immune system had to be excluded from the trial. (Chung et al., 2009)

At study intervention days subjects had to be free from any kind of acute illness such as upper respiratory tract infections. Acute infections may impact redox homeostasis due to ROS being the key molecule of initial immune response.

(Bardaweel et al., 2018) Therefore, also all vaccinations had to be at least seven days apart from study interventions. Additionally, subjects were not allowed to donate blood at least seven days before study interventions, as giving blood usually increases SOD-activity. (Yunce et al., 2016) Moreover, subjects had to avoid a positive alcohol intake anamnesis (> 7 drinks / week) or drink alcohol one day before study intervention at all, as alcohol intake normally increases OS. (Das & Vasudevan, 2007) And finally, taking specific medicines or supplements which influence the antioxidative capacity were forbidden. (Kawamura & Muraoka, 2018)

The effect size was determined using 80% G-Power analysis, based on the data of Zeng et al. (2020). According to this calculation, 20 participants should complete the study to receive sig. results. Considering a safety margin of four, 24 female individuals should be included in the study.

Table 3: Inclusion criteria for study trial and of study interventions (own illustration, 2023)

Inclusion criteria to study trial	Female sex Age: 18 to 40 years Non-smokers Physically untrained (<= 120 Min./week of endurance, <1x/week resistance training for the past six months) No intolerances or aversions to intervention foods & drinks BMI 18.5 – 24.9 kg/m² Body fat percentage > 20 % No contraindications to PE Not suffering from any chronical diseases Not pregnant Non lactating No chronical alcohol intake
Inclusion criteria of study intervention	Overnight fasting (12 hours) No physical exercise in the last 24 hours No alcohol intake in the last 24 hours No vaccination past the last 7 days No blood donation past the last 7 days No resistance training in the last seven days Max. 120 Min. endurance training in the last seven days No history of medicine or supplement intake, which influence redox homeostasis

If subjects did not comply with the inclusion criteria (see Table 3), this led to an immediate drop out during screening procedures.

If participants violated the inclusion criteria (see Table 3) on study intervention days, procedures had to be postponed to another day.

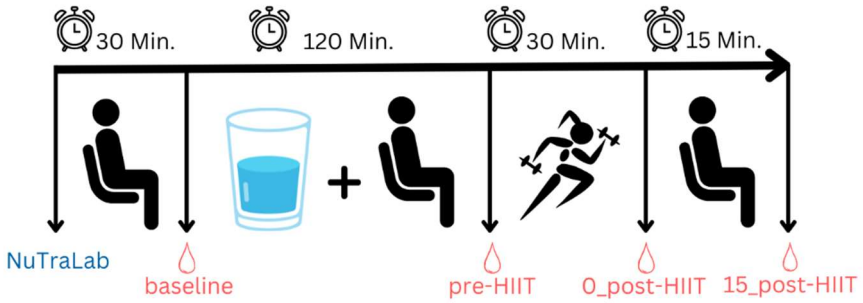
The study aim, interventions, experimental procedures, and potential risks were explained to each possible eligible subject before trial enrolment. After obtaining informed consent and giving handwritten consent, subjects were enrolled in the

trial by starting the screening process. The study consent was able to be discontinued at every time without stating any reasons.

The research project was approved by the local ethics committee of the university of Vienna at 1st December, 2021 according to the latest revision of the Declaration of Helsinki. (EK-Nr.: 00743)

2.2. Study design

Figure 10: Graphical abstract of a study cycle (own illustration using Canva®, 2023)



The present study trial was designed as repeated-measures, cross-over, randomized, controlled trial conducted at the University of Vienna in Vienna, Austria. Study interventions took place at the Nutrition and Training Laboratory (NuTraLab), at the department of nutritional and sports sciences, division of nutrition, physical activity, and public health. Laboratory measurements were conducted at the department of nutritional sciences (UZA2). Furthermore, for specific parameters an external laboratory was involved.

A flow chart of the applied study design can be found in Table 4.

Possible eligible subjects were recruited using social media platforms. Interested female individuals got invited to an information appointment at the NuTraLab for trial explanation, descripted in chapter 2.1.

Table 4: Flow-chart of study design (own illustration, 2023)

Inclusion criteria	Informed consent	Screening	Study intervention
• female, 18-40 years, BMI= 18,5 - 24,9 kg/m², BF > 20%, non pregnant, non lactating, non-smoking, untrained	• Version 2, 04.10.2021 • handwritten consent	• Fasted venous blood samples • ECG • BIA • 5-RM test	• 7 study cycles • 7 randomly assigned study drinks (water = control) • HIIT (BORG 15-19) • 4 venous blood samples (baseline, pre-HIIT, 0_post-HIIT, 15_post-HIIT)

After signing informed consent, the subjects were enrolled by study director through coding them (subject-ID) and started study screening process immediately. In case of the subject-ID all investigators were blinded except for the study director.

During screening a fasted blood sample was taken, including all safety parameters regarding the cardiovascular -, metabolic-, or immune system. Additionally, an electrocardiogram, using CARDIOVIT CS-104® (DACHCOM.CH AG, Switzerland), was written. Moreover, demographic, and anthropometric data were collected and body composition was measured using seca mBCA 515® (seca gmbh & co. kg, Germany). For BIA subject preparation, conduction and interpretation, specific guidelines according to Kyle et al. (2004) were followed.

Finally, eligible subjects were instructed to perform a technical resistance training, supervised by sports science professionals, as a familiarization procedure to the HIIT intervention (see chapter 2.3.1).

After positive screening, subjects were enrolled by the study medical director in the present study and had to perform seven study cycles to complete the study protocol.

At every study cycle the participants received one of the intervention foods or beverages, which were randomly assigned (see chapter 2.3.2). For randomization software Microsoft Excel® (Microsoft Corporation, USA) was used.

After the women arrived at the study centre, fasting for at least over twelve hours, they had to rest for 30 minutes. Afterwards, the first blood sample was drawn (baseline) and randomly assigned study beverage was consumed by the participants. Then subjects were instructed to only consume water till study procedures were finished (min. 5-10 mL per body weight (Mosler et al., 2019)). After 120 minutes of resting time another blood sample was drawn (pre-HIIT). Following that, the participants had to perform the HIIT immediately according to protocol, followed by a post-exercise blood sample (0_post-HIIT). Finally, after another 15 minutes of resting the last blood sample (15_post-HIIT) was drawn.

A graphical abstract of the study intervention during the study cycles can be found in Figure 10.

In total seven study cycles, with seven different beverages, had to be completed, which led to 28 blood samples in total per subject and 672 samples scheduled in total per study cycle “beverage”.

2.3. Study Interventions

2.3.1. High intensity interval training

To induce OS in all study participants, a training protocol recently evaluated by Zeng et al. (2020) as well as Bizheh and Jaafari (2011) was applied.

Subjects had to perform a HIIT, using rowing, chest press, leg curl, lat pull down and leg press, with preferably 20 repeats within 40 seconds and ten seconds of resting, three repeats and one minute of resting in between. Before every training session a standardized warm-up-procedure, including body mobilisation and HIIT-warm-up, was completed.

To assess training intensity Borg's subjective rating of perceived exertion (BORG-scale) was used. This is a subjective scale of intensity and is highly correlated with heart rate, and therefore validated for training intensity. (Borg, 1982) During study interventions BORG-scale was assessed at every blood sampling (baseline, pre-HIIT, 0_post-HIIT, 15_post-HIIT).

To set the maximum weight, all female individuals performed multiple repetition maximum strength tests (5-RM-test), using five exercises, preferably on an empty stomach. (Gail et al., 2015) Applying 50% of maximum weight leads to optimal training intensity, similar to the training protocol from Bizheh and Jaafari (2011) at study intervention. The exercise weight had to be elevated by 5 % during study cycles if subjects were able to perform over 3x 20 repetitions of an exercise in a cycle.

2.3.2. Study intervention beverages

From April to November 2022 participants received 250 mL of filtered coffee, orange juice, pomegranate juice, matcha green tea, sugar sweetened beverage and skimmed milk according to protocol. Tap water was used as a control.

This master's thesis will focus on all aspects regarding skimmed milk and water as a control.

To explore the acute impact of skimmed milk on OS in untrained healthy adult women an Austrian low fat (1,5%) extended-shelf-life organic cow's milk was chosen for study intervention. The composition of Bio-Bergbauern-Milch 1,5% (Fettarme Bio Milch mit 1,5% Fett, hocherhitzt, länger haltbar, homogenisiert, SalzburgMilch GmbH, Salzburg, Austria) can be found in Table 5.

Table 5: Composition of testing skimmed milk
according to SalzburgMilch GmbH (2023) & Muskat et al. (2021)

Testing skimmed milch, 1,5%		
Portion size	**100mL**	**250mL**
Energy (kcal)	47	117,5
Fat (g)	1,5	3,75
SFA (g)	0,9	2,25
Proteins (g)	3,4	8,5
KH/ Lactose (g)	4,9	12,25
Water (g)	89,3	223,25
Retinol-equivalents (µg)	13	32,5
Tocopherol equivalent (mg)	Only traces	Only traces
L-ascorbic acid (mg)	1	2,5

The fat content was selected to be low (1,5%), as high fat dairy was associated with elevated levels of inflammation. (Anderson et al., 2012; Esmaillzadeh et al., 2007; Nettleton et al., 2006) Moreover, ÖGE (2022) recommends one portion a day of low fat dairy such as 200mL drinking milk, when following a healthy diet. However, portion size was chosen to be 250mL, to be equivalent to all other study interventional beverages (see chapter 2.3.2).

In addition to this, skimmed milk had to be organic, as organic feeding influences the composition of milk significantly. (Stobiecka et al., 2022).

2.3.3. Sample collection

Venous blood samples were drawn four times at each study cycle in a laying position (see Figure 10).

This master's thesis will further focus on venous blood samples, for protein carbonylation and E2 analysis.

At baseline two vacutainer tubes of venous blood (Greiner Bio-One GmbH, Austria) were taken. One to detect fasting concentration of E2 at baseline (see chapter 2.3.4) and another one for obtaining plasma samples, for further analyzation. To receive sufficient blood plasma samples, adding anticoagulant, such as ethylenediaminetetraacetic acid (EDTA), is required. (Hallbach, 2011) Therefore, for all four blood plasma analysations (baseline, pre-HIIT, 0_post-HIIT, 15_post-HIIT) EDTA-tubes were used.

Freshly received venous EDTA blood samples needed to be immediately shaken and centrifuged (21°C, 15 minutes, 3500 RPM). E2 samples were stored at room temperature (RT) and further analysed by an external laboratory (see chapter 2.3.4).

Centrifuged EDTA-plasma-samples had to be aliquoted into 200µL in 0,5 mL tubes manually, using a standard pipette (Eppendorf SE, Germany), and stored at -80°C for further analyzation.

2.3.4. 17-β-Estradiol concentrations

To detect E2-content an external laboratory was involved.

For E2 analyzation serum blood samples are commonly required. (Gronowoski A. M., 2008) At blood sampling tubes without any anticoagulant additives were used, as blood serum normally does not contain any anticoagulants. (Hallbach, 2011)

Obtained E2 concentrations were expressed in pg/mL.

2.3.5. Protein carbonyl contents

Between November 2022 to February 2023 PC-contents of EDTA-plasma samples were analysed using an adapted protocol from Levine et al. (1990).

Protein carbonyl content protocol

Originally Levine et al. (1990) treated oxidatively modified proteins with 10mM DNPH in 2M hydrochloric acid (HCl), for one hour at RT. After incubation time the protein precipitated with 20% TCA in) in deionized water ($_{dd}H_2O$) solution, followed by washing steps with ethanol:ethylacetate (1:2) and finally dissolved in 6M guanidine buffer. The PC-content was spectrophotometrically measured at 360 to 390 nm to detect built 2,4-dinitrophenyl hydrazone absorption.

Obtained absorption was further used for concentration-calculation using Beer-Lambert-Law (Colombo et al., 2016; Weber et al., 2015)

Furthermore, total protein concentration was also spectrophotometrically determined using generated PC-guanidine solution, only treated with 2M HCl in $_{dd}H_2O$ solution as a so called “blank”, at 276 nm, using BSA as a standard. (Levine et al., 1990)

Finally, PC-content was calculated when nmol were related to protein concentration (mg/mL), and expressed in PC content in nmol/mg (see Equation 1). (Colombo et al., 2016)

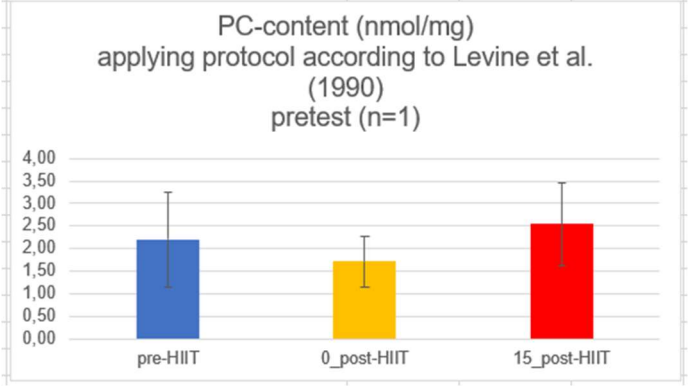
Equation 1: Protein carbonyl content (nmol/mg) calculation
(own illustration according to Colombo et al., 2016)

$$Protein\ carbonyle\ content\left(\frac{nmol}{\mathrm{mg}}\right) = \frac{Protein\ carbonyle\ \left(\frac{nmol}{\mathrm{mL}}\right)}{Total\ protein\ \frac{mg}{mL}}$$

Due to high coefficient of variation (CV) % values (see Figure 11), following this protocol in a pretest (n = 1), the procedures had to be adapted as descripted in the following part.

Figure 11: Pretest PC-content (nmol/mg)
according to Levine et al. (1990) (own illustration using Microsoft Excel®, 2023)

PC-content (nmol/mg)	Mean	SD	CV %
pre-HIIT	2,20	1,0476971	47,58%
0_post-HIIT	1,71	0,5476681	32,00%
15_post-HIIT	2,54	0,9193428	36,20%



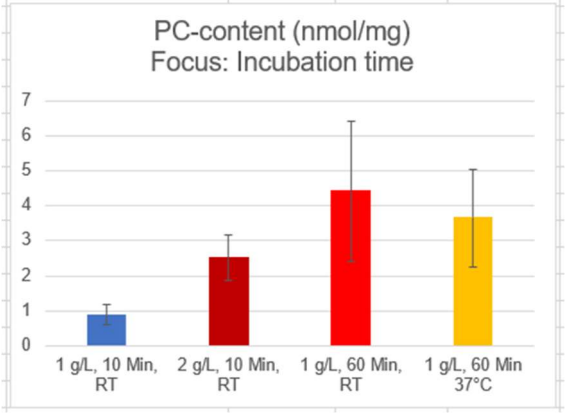
Protocol adaptions

First of all, sample-DNPH-incubation time was focused, as there were differences noted in papers applying the protocol by Levine et al. (1990).

Originally Levine et al. (1990) poses one hour at RT to be the optimal incubation time. In addition to this, Colombo et al. (2016) agrees on that. Moreover, Berzosa et al. (2011) performed an incubation step at 37 °C (60 minutes). Finally, Wehr and Levine (2013) recommended ten minutes at RT to be optimal for detecting protein carbonylation.

Figure 12: Analysing pretest pre-HIIT EDTA-plasma-sample
with different incubation time approaches (own illustration using Microsoft Excel®, 2023)

PC-content (nmol/mg)			
pre-HIIT	**mean**	**SD**	**CV %**
1 g/L, 10 Min, RT	0,897686	0,3039098	33,85%
2 g/L, 10 Min, RT	2,5125964	0,6450208	25,67%
1 g/L, 60 Min, RT	4,4134003	1,9911488	45,12%
1 g/L, 60 Min 37°C	3,6457732	1,4093384	38,66%



The PC-content increased after adapted incubation step (duration = 60 minutes, see Figure 12). However, CV% was the lowest when samples incubated for ten minutes at RT.

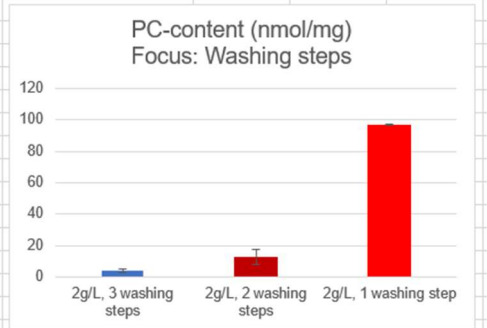
Furthermore, a higher protein concentration (2 g/L) in samples led to clearer results as CV% was lower, compared to samples with 1 g/L concentration (see Figure 12).

Additionally, variable CV% were found when washing was reduced (see Figure 13).

This seemed relevant as two different people performed washing procedures with possible personal fluctuations. CV% was the lowest when washing was performed just one time (see Figure 13). On the other hand, PC-content was unusually high in potentially unoxidized baseline samples (3,9 vs. 96,7 nmol/mg/mL). (Weber et al., 2015) This agrees with Colombo et al. (2016), which pose to take care of removing every unbind DNPH from sample, as there could be interference, leading to a possible overestimation of PC content.

Figure 13: Comparing washing steps in higher concentrated (2g/L) samples
(own illustration using Microsoft Excel®, 2023)

PC-content (nmol/mg)			
pre-HIIT	**mean**	**SD**	**CV %**
2g/L, 3 washing steps	3,9414042	1,1703733	29,69%
2g/L, 2 washing steps	12,766535	4,522288	35,42%
2g/L, 1 washing step	96,715003	0,5550668	0,57%

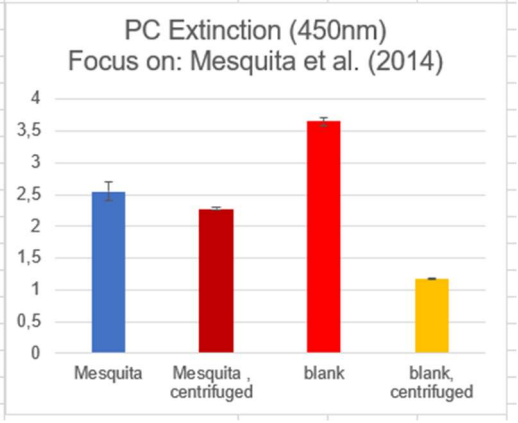


Therefore, another protocol was performed, which does not require any sample washing. (Mesquita et al., 2014)

In this protocol Mesquita et al. (2014) added 6M sodium hydroxide (NaOH) in $_{dd}H_2O$ solution to DNPH-protein-solution, causing hereby an absorbance shift from 370 to 450nm. Thus, avoiding unbind DNPH interference. Unfortunately, samples and blanks ($_{dd}H_2O$) extinction (E) was unusually high (over 0,5), which could lead to distorted results according to Beer-Lambert-Law. (Oshina & Spigulis, 2021) Centrifugation (3500 rpm, 2 minutes, RT) did not solve that problem (see Figure 14).

Figure 14: Extinction (450nm) of generated samples using protocol according to Mesquita et al. (2014) (own illustration using Microsoft Excel®, 2023)

PC Extinction (450nm)			
pre-HIIT	**mean**	**SD**	**CV %**
Mesquita	2,54867	0,14672	5,76%
Mesquita , centrifuged	2,28083	0,02125	0,93%
blank	3,653	0,05604	1,53%
blank, centrifuged	1,176	0,01023	0,87%



Moreover, the protein concentration of these samples should not had been higher than 1g/L, as obtained protein pellets can be difficult to resuspend during the experiment. (Colombo et al., 2016) Therefore, for further adaption, sample protein concentration of 1g/L was used.

With additional protocol adaptions acetone was rather applied than ethanol:ethylacetate (1:2) for washing procedures. Acetones have been observed to show good ability to react with unbind DNPH in acid samples. During condensation reaction, under acidic conditions, a yellow-coloured layer precipitates when unbind DNPH reacts with acetone. (Wang & Powell, 2010)

This precipitate is more easily to identify as supernatant and therefore washing could be performed more precisely. This was important as final received pellets should be clean and free from yellow unbended DNPH to avoid possible overestimation of PC content. (Colombo et al., 2016)

Furthermore, TCA was added ice cold. (Berzosa et al., 2011) And the concentration of TCA was elevated according to a commercial PC-kit (Proteincarbonyl-Assay-Kit ®, Merck KGaA, Germany).

Finally, the obtained total protein concentration using protocol according to Levine et al. (1990) was unusually high (greater to 90 mg/mL), as physiological plasma protein concentrations range from 60 to 80 g/L. (Leeman et al., 2018) Therefore,

an additional protocol for total protein concentration analysis in plasma samples was implemented.

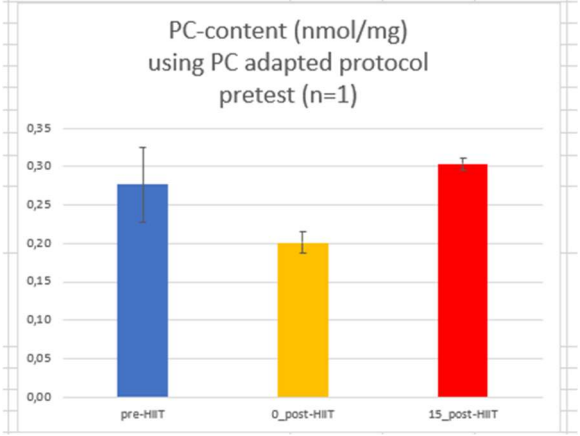
A wide range of spectrophotometric methods had been established to assess protein concentration of plasma samples. (Okutucu et al., 2007) After performing Biuret-, Bradford- and Lowry-Assay, it was decided to apply Lowry-Assay, as it is a well-implemented protocol in the field of protein carbonylation assessment. (Buonocore et al., 2020; Fayh et al., 2013; Silva et al., 2010).

The protocol according to Lowry et al. (1951) was also adapted to measure samples in microplate rather than macrocuvette, to improve planed 672 sample analysations efficiency. To do so, EDTA-plasma samples were diluted (1:40) and directly aliquoted in 1,5 mL tubes, Lowry reaction was finished according to protocol and obtained blueish solution was added directly on microplate using “reverse pipetting”. Absorbance was measured spectrophotometrically at 578 nm. (Lowry et al., 1951)

Performing the final adapted protocol CV% had been reduced and obtained results in pretest seemed physiological (see Figure 15).

Figure 15: Results of the pretest according to final adapted PC-content-protocol (own illustration using Microsoft Excel®, 2023)

PC-content (nmol/mg)	mean	SD	CV %
pre-HIIT	0,28	0,0486136	17,59%
0_post-HIIT	0,20	0,0140547	6,98%
15_post-HIIT	0,30	0,0075404	2,48%



The following part deals with final adapted protocol for detecting plasma PC-content.

Finished adapted protein carbonyl content protocol

A detailed description of the finished adapted protocol, including all reagent preparations, can be found in the Appendix (Protein carbonyl protocol) written in German.

Reagents, instruments, and technical equipment for lab procedures

For PC concentration (nmol/mL) the following laboratory instruments, technical equipment and reagents had been used (see Table 6 to Table 7):

Table 6: Instruments for lab procedures for PC concentration analysis (own illustration, 2023)

Instruments	Company	Product number
Centrifuge 5427 R	Eppendorf SE, Germany	EP022620701
Greiner-UV-Star®-96-Well-plates, COC, F-Bottom	Greiner Bio-One GmbH, Germany	655801
LaboStar pure water system UV	Evoqua Water Technologies, USA	EVO_21004
Palssonic Eco – Ultrasound cleaner UD10	Allpax Palssonic GmbH & Co. KG	10011281
Thermoshaker - Mixer HC	Starlab International GmbH, Germany	S8012-0000
826 pH mobile / 827 pH lab	Metrohm AG, Swizzerland	2.827.0214
Eppendorf Xplorer®	Eppendorf SE, Germany	4861000023
Multipette® E3	Eppendorf SE, Germany	4987000010
CLARIOstar® Plus	BMG Labtech GmbH, Germany	N.A.

Table 7: Reagents for lab procedures for PC concentration analysis (own illustration, 2023)

Reagents	Chemical formula	Company	Product number
2,4-Dinitrophenylhydrazine (DNPH)	$C_6H_6N_4O_4$	Fluka Chemie, Switzerland	42210
Guanidine hydrochloride	$CH_5N_3 \bullet HCl$	Sigma Aldrich, Germany	102438591
Hydrochloric acid	HCl	Merck KGaA, Germany	1.00316.2511
Potassium phosphate monobasic	KH_2PO_4	Sigma Aldrich, Germany	1003064796
Trichloroacetic acid (TCA)	$C_2HCl_3O_2$	Sigma Aldrich, Germany	102425168
Acetone ≥99%	CH_3COCH_3	VWR International, USA	20063.467

Additionally, for total protein concentration (mg/mL) the following laboratory instruments, technical equipment and reagents had been used (see Table 8 Table 9).

Table 8: Instruments for lab procedures for Lowry assay (own illustration, 2023)

Instruments	Company	Product number
BRANDplates® microtitration plate, 96-well, F-Botto	BRAND GMBH + CO KG, Germany	781614
LaboStar pure water system UV	Evoqua Water Technologies, USA	354900
Multipette® E3	Eppendorf SE, Germany	4987000010
Eppendorf Xplorer®	Eppendorf SE, Germany	4861000023
CLARIOstar® Plus	BMG Labtech GmbH, Germany	N.A.

Table 9: Reagents for lab procedures for Lowry assay (own illustration, 2023)

Reagents	Chemical formula	Company	Product number
Sodium carbonate	Na_2CO_3	Sigma Aldrich, Germany	S7795
Sodium hydroxide	NaOH	Sigma Aldrich, Germany	06203
Cupric sulfate Pentahydrate	$CuSO_4 \bullet 5H_2O$	Fluka Chemie, Switzerland	61245
Sodium potassium tartrate	$C_4H_4KNaO_6 \bullet 4H_2O$	Sigma Aldrich, Germany	S2377
Folin & Ciocalteu's phenol reagent	$C_{10}H_5NaO_5S$	Sigma Aldrich, Germany	F9252
Bovin Serum Albumin	-	Sigma Aldrich, Germany	A7030

Additional used technical equipment:

- Laboratory glassware (breakers, volumetric flask + stopper, graduated cylinder, funnel, watch glass, clear & brown laboratory bottles)
- Laboratory tubes from 1,5 up to 10 mL
- Magnetic stir bar and magnetic rod
- Disposable transfer pipets from 5 to 15 mL
- Micropipettes from up 100 to 1000 µL
- Combitips advanced from 1 to 50 mL (Eppendorf SE, Germany)
- Wash bottle with deionized Water
- Magnetic stirrer with heating
- Vortexer
- -20°C & -80°C freezer
- Scales and scaling supplies
- Aluminium foil
- Safety googles & gloves

Protein carbonyl content analysis procedure

For PC concentration (nmol/mL) EDTA-plasma samples were taken from -80°C storage, thawed and till further processing kept on ice. Reagents were prepared as instructed (Appendix see Protein carbonyl protocol) and pH-value of 6M guanidine buffer was checked once a week. All reagents, except of 87%TCA and acetone, should be on RT prior to usage.

First, 10µL of EDTA-plasma and 90 µL of $_{dd}H_2O$ were added in 1,5 mL tubes. Afterwards, 100µL of 0,01M DNPH in 2M HCl solution was added under dark conditions and samples were vortexed properly. After ten minutes of incubation (RT, dark) 30µL of ice-cold 87% TCA was added and samples were vortexed. After five minutes of incubation on ice, samples were centrifuged (13000g, 2 minutes, 4°C). Following that, supernatant was carefully manually removed, paying attention on received pellet, and washing steps were initiated. Briefly, the following washing step: 500µL of ice-cold acetone was added and samples were dissolved using ultra-sound-bath (RT, 30 seconds). Afterwards, samples had to incubate at -20°C, followed by discharging supernatant, paying attention on the pellet. Washing steps were completed two times. Finally, clear and dry DNPH-free protein pellets had been dissolved with 400µL 6M guanidine in 0,5M potassium phosphate solution buffer using ultra sound bath (RT, 30 seconds). When dissolving was not successful, tubes had to incubate at 60°C for another 15 minutes. Afterwards, all PC-guanidine buffer solutions should be clear.

Finally, 100µL of PC-guanidine buffer solutions were directly applied as a triplet on the Greiner-UV-Star®-96-Well-plates, using “reverse pipetting” to ensure clear solutions in each well. Absorbance was measured spectrophotometrically at 375 nm and obtained absorbance was used for further calculation. $_{dd}H_2O$ was measured as a blank according to pipetting scheme illustrated in Figure 16.

Figure 16: Applied pipetting scheme for protein carbonyl measurement (own illustration using Microsoft Word®, 2023)

PL												
	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	H_2O	H_2O	Plasma 8	Plasma 8	Plasma 8	Plasma 16	Plasma 16	Plasma 16	Plasma 24	Plasma 24	Plasma 24
B	Plasma 1	Plasma 1	Plasma 1	Plasma 9	Plasma 9	Plasma 9	Plasma 17	Plasma 17	Plasma 17	Plasma 25	Plasma 25	Plasma 25
C	Plasma 2	Plasma 2	Plasma 2	Plasma 10	Plasma 10	Plasma 10	Plasma 18	Plasma 18	Plasma 18	Plasma 26	Plasma 26	Plasma 26
D	Plasma 3	Plasma 3	Plasma 3	Plasma 11	Plasma 11	Plasma 11	Plasma 19	Plasma 19	Plasma 19	Plasma 27	Plasma 27	Plasma 27
E	Plasma 4	Plasma 4	Plasma 4	Plasma 12	Plasma 12	Plasma 12	Plasma 20	Plasma 20	Plasma 20	Plasma 28	Plasma 28	Plasma 28
F	Plasma 5	Plasma 5	Plasma 5	Plasma 13	Plasma 13	Plasma 13	Plasma 21	Plasma 21	Plasma 21	Plasma 29	Plasma 29	Plasma 29
G	Plasma 6	Plasma 6	Plasma 6	Plasma 14	Plasma 14	Plasma 14	Plasma 22	Plasma 22	Plasma 22	Plasma 30	Plasma 30	Plasma 30
H	Plasma 7	Plasma 7	Plasma 7	Plasma 15	Plasma 15	Plasma 15	Plasma 23	Plasma 23	Plasma 23	Plasma 31	Plasma 31	Plasma 31

When total protein concentration (mg/mL) was determined, EDTA-plasma samples were again taken from -80°C storage, thawed and kept on ice till further processing. Reagents and BSA-standards were prepared as instructed (Appendix see Lowry Assay protocol).

EDTA-plasma samples had to be diluted (1:40) before processing, therefore 10µL of plasma and 390 µL of $_{dd}H_2O$ were added in 1,5 mL tubes. Afterwards 40µL of sample, standard or $_{dd}H_2O$ were aliquoted in 1,5 mL tubes. Then 1000µL of freshly prepared Lowry-usage-solution was added, samples were vortexed and incubated ten minutes (RT, dark). Following that, 100µL of 1:2 diluted Folin–Ciocalteau-reagent® was added, samples were vortexed and incubated for 35 minutes (RT, dark).

Finally, 200µL of received blueish solution were directly applied as a doublet on BRANDplates® microtitration plate, using “reverse pipetting” for clean solutions. Samples, BSA standards, and blank were spectrophotometrically measured at 578 nm and received absorbance used for further calculation.

Protein carbonylation content calculation

PC concentration in nmol/mL was calculated using Beer-Lambert-Law (see Equation 2).

Equation 2: Beer-Lambert-Law (own illustration, 2023)

$$Extinction\ (E) = \varepsilon \left(\frac{L}{\text{nmol} \times cm}\right) \times L(cm) \times c(\frac{nmol}{\text{L}})$$

For extinction coefficient (ε) literature poses 22,000 $M^{-1}x\ cm^{-1}$ to be specific for DNPH absorbance. (Colombo et al., 2016)

Wavelenght in cm (L) for Greiner-UV-Star® was calculated using Equation 3, as Greiner-wells had diameters of 0,9 cm and volumes of 0,1cm³, when 100µL of PC-guanidine buffer solution was added (see Finished adapted protein carbonyl content protocol).

Finally, for final calculation following Equation 3 (dilution factor of sample = 1:10) was used:

Equation 3: PC-concentration in nmol/L (own illustration, 2023)

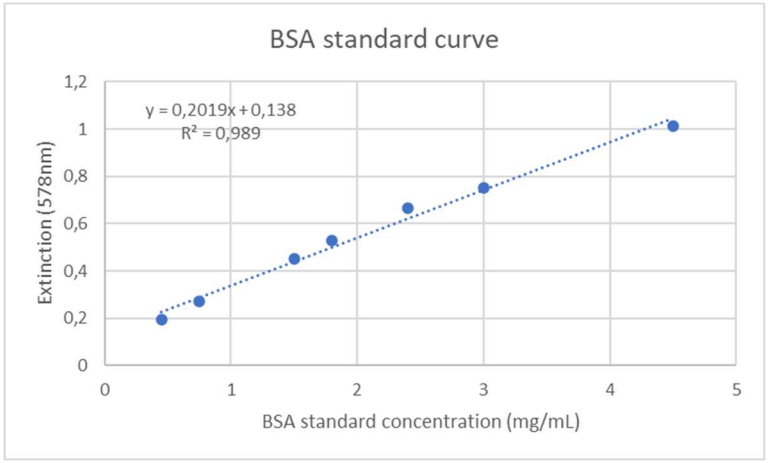
$$c\left(\frac{nmol}{\mathrm{L}}\right) = \frac{E\ (A_{375})}{\varepsilon\ \left(\frac{L}{\mathrm{nmol} \times cm}\right) \times L\ (cm) = 22 \times (\pi \times 0{,}45^2) = 6{,}9168} \times 10$$

Total protein concentration was measured at 578 nm and calculated from a mean BSA standard regression curve ranging from 0,45 mg/mL to 4,5 mg/mL concentration (see Figure 17) using the following Equation 4.

Equation 4: Total protein concentration in mg/mL (own illustration, 2023)

$$c\ \left(\frac{mg}{mL}\right) = \frac{E\ (A_{578}) - d}{k} \times 40$$

Figure 17: Applied mean BSA standard curve (own illustration using Microsoft Excel®, 2023)



Finally, PC-concentration was related to total protein concentration (see Equation 1, 1:40 dilution factor) and all calculated data was expressed in PC-content (nmol/mg) and used for further statistical analysis.

2.4. Statistical analysis

For statistical analysis IBM® SPSS® Statistics 27 (IBM Deutschland GmbH, Germany) was used. Alpha level was set at $p < 0{,}05$ and confidence interval was 95%.

First, the corresponding morning baseline was subtracted from each measurement point in order to capture just the beverage effect.

Afterwards, all generated data was tested for normal-distribution by using Shapiro-Wilk test. Following that, a general linear model was applied using PC-content-Baseline-corrected as dependent variable, testing all possible interactions among variables. Furthermore, interaction effects were tested with repeated measures analysis of variance (rmANOVA). And finally, independent t-test were performed for the examination of significant differences.

All data are presented as the mean including standard derivation (SD) and CV%.

Equation 5: CV% calculation (own illustration, 2023)

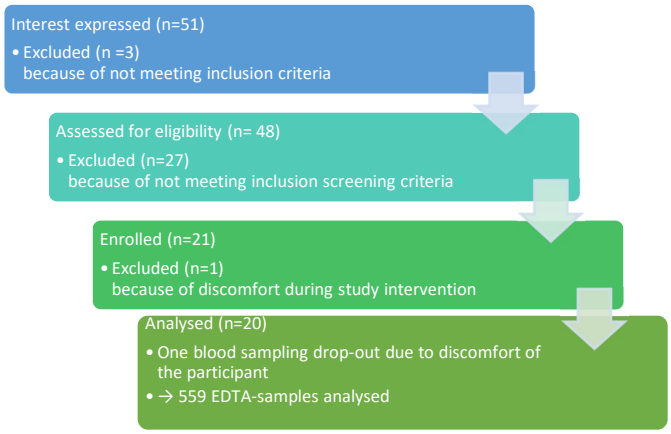
$$CV\% = \frac{SD}{mean}$$

3. Results

3.1. Subjects

From December 2021 to August 2022, 51 female individuals expressed their interest in participating in the study, 48 subjects started with the screening process, 21 participants were enrolled in the project and 20 women completed their study participation successfully. A flow chart of the recruitment, screening, enrolment, and study process can be found in Figure 18.

Figure 18: Flow chart of study enrolment (own illustration, 2023)



Starting April 2022 and finishing in November 2022 all 20 participants completed the trial without any adverse events noted. Six of them used OC. The characteristics of the study subjects is listed in Table 10.

Table 10: Demographic and anthropometric data of study subjects (own illustration, 2023)

Variable	Mean±SD	Min	Max
Age (years)	26,5±4,1	20,0	37,0
Weight (kg)	59,0±6,8	46,3	70,8
Height (m)	1,7±0,1	1,6	1,8
BMI (kg/m²)	21,0±1,9	18,8	25,0
Body-fat (%)	27,5±3,9	21,8	34,6

3.2. 17-β-Estradiol concentrations

E2 serum samples were successfully collected at baseline from all study subjects. In total 560 samples had been analysed and expressed in pg/mL.

E2 concentrations were not normally distributed (see Figure 19), as assessed by the Shapiro-Wilk test ($p < 0,001$).

Unfortunately, when E2 cut - offs for midcycle according to Roberts et al. (2008) had been applied, E2-groups-frequencies were not equal (see Figure 20). Therefore, it was decided to define own E2-groups by using study quarters based on IBM® SPSS® Statistics 27 calculation (see Figure 21).

Based on this categorisation, the statistical analyses could be continued.

Figure 19: Normal distribution of 17-β-Estradiol concentrations using Histogram and Boxplot
(own illustration using IBM® SPSS® Statistics 27, 2023)

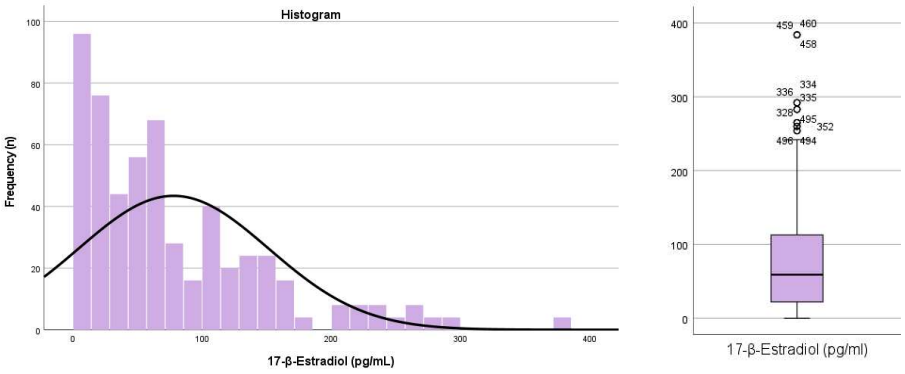


Figure 20: 17-β-Estradiol concentrations cut-offs according to Roberts et al. (2008)
low < 150 pg/mL; high > = 150 pg/mL
(own illustration using IBM® SPSS® Statistics 27, 2023)

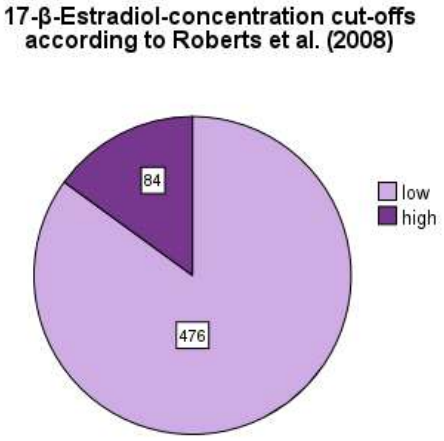
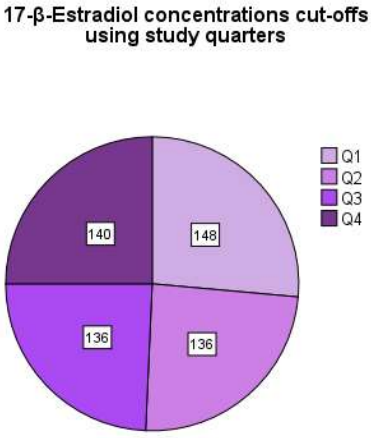


Figure 21: 17-β-Estradiol concentrations-cut off using study quarters;
Q1 = < 22 pg/mL; Q2 = 22-59 pg/mL; Q3 = 59 – 112,75 pg/mL; Q4 >= 112,75 pg/mL
(own illustration using IBM® SPSS® Statistics 27, 2023)

n	560
Mean±SD	77,97±73,46
Min	0
Max	384
25th percentile	22
50th percentile	59
75th percentile	112,75



3.3. Protein carbonyl contents

3.3.1. Sample collection

From April to November 2022 all 20 women completed the study protocol successfully. Unfortunately, one blood sampling (GS_3.3.4) could not be finished, as blood drawing was not possible due to discomfort of the participant.

Furthermore, 14 blood samples showed haemolytic status, but had been analysed anyway.

3.3.2. Quality assessment of laboratory procedures

To ensure the highest quality during PC concentration analysis from November 2022 to February 2023, an additional analysation of a known concentration EDTA-control-plasma-sample was performed at every measurement. In addition to this, 6M guanidine buffer was added at every microplate as well, to assure measurements strength against day-to-day variances.

After 20 well plates measurements were performed CV% of buffer was < 5% and of EDTA-control-plasma was < 15% (see Table 11). Therefore, it was ensured that the spectrophotometer was calibrated well and robust against day-to-day variances, as well as possible personal fluctuations while sample preparation had been avoided.

Table 11: Mean±SD Absorbance (375nm) of EDTA-control-plasma-sample and 6M guanidine buffer (own illustration, 2023)

Absorbance (375nm)	Mean	±SD	CV%
EDTA-control-plasma	0,272	0,037	13,52%
6M guanidine buffer	0,164	0,007	4,13%

In case of applied Lowry assay, the BSA-standards and a known concentration EDTA-control-plasma-sample had been analysed at every measurement from February to March 2023. To calculate total protein concentration a mean BSA-standards regression curve (CV < 5%; see Table 12) was used (see Figure 17):

After nine measurements were performed CV% of EDTA-control-plasma was < 10% and blank was < 5% (see Table 12).

Table 12: Mean±SD Absorbance (578nm) of EDTA-control-plasma-sample, blank and BSA-Standards (own illustration, 2023)

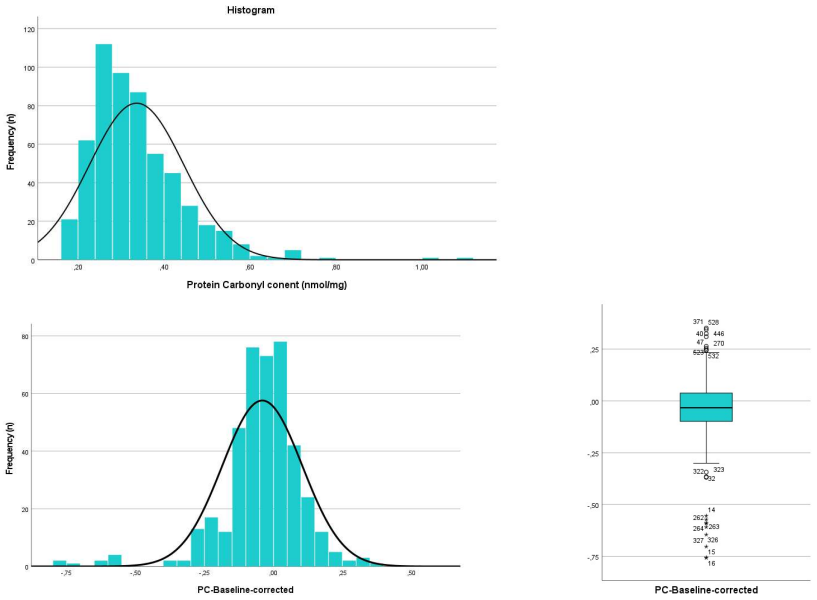
	c (mg/mL)	c solved for x (mg/mL)	Absorbance (578nm)		
			Mean	±SD	CV%
EDTA-control-plasma	-	68,7	0,48	0,03	6,77%
Blank	-	-	0,0395	0,0016	4,01%
Standard 1	0,45	0,28	0,19	0,01	3,33%
Standard 2	0,75	0,67	0,27	0,01	4,18%
Standard 3	1,5	1,55	0,45	0,02	4,55%
Standard 4	1,8	1,94	0,53	0,02	3,45%
Standard 5	2,4	2,61	0,66	0,02	3,70%
Standard 6	3	3,03	0,75	0,02	3,18%
Standard 7	4,5	4,33	1,01	0,04	3,60%

After all obtained data was checked, ten samples showed unphysiological low total protein concentration according to Leeman et al. (2018). Therefore, measurements had to be repeated and physiological results were obtained. Finally, all generated PC-contents (nmol/mg) could be successfully calculated as described in chapter 2.3.5.

3.4. Shapiro-Wilk test

PC-contents (nmol/mg) and PC-contents-Baseline-corrected (nmol/mg) were not normally distributed (see Figure 22), as assessed by the Shapiro-Wilk test ($p < 0{,}001$).

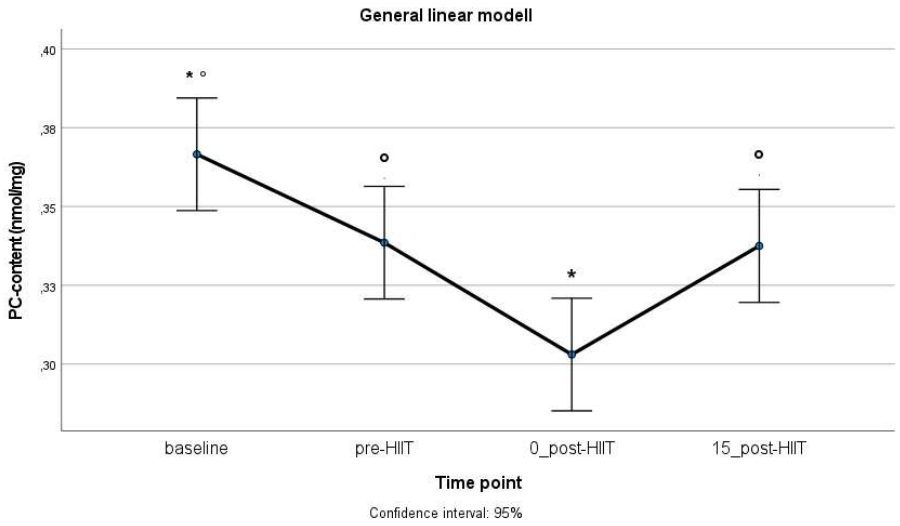
Figure 22: Normal distribution of PC-content (nmol/mg) using Histogram and Boxplot (own illustration using IBM® SPSS® Statistics 27, 2023)



3.5. General linear model

3.5.1. Time point effect

Figure 23: Time point differences tested with a general linear model (° = p<0,05; * = p <0,01) (own illustration using IBM® SPSS® Statistics 27, 2023)



Performing a HIIT in untrained adult healthy women showed a sig. reducing effect on plasma PC-contents (nmol/mg) directly ($p < 0,01$) and 15 minutes ($p < 0,05$) after finished training compared to baseline, in a general linear model calculation. There was also a sig. decrease after two hours of resting ($p < 0,05$) in comparison to baseline (see Figure 23 & Table 13).

Table 13: Timepoint effect on PC-content (nmol/mg) tested with a general linear model (own illustration, 2023)

	PC-content (nmol/mg)	p-value
Baseline	Reference	-
pre-HIIT	-0,028±0,0129	p = 0,03
0_post-HIIT	-0,064±0,0129	p < 0,01
15_post-HIIT	-0,029±0,0129	p = 0,024

3.5.2. 17-β-Estradiol effect

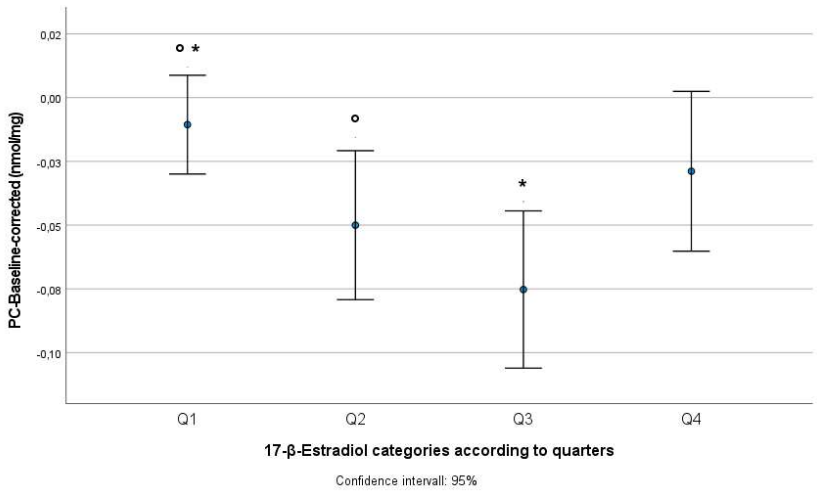
After the E2-catergories had been set as described in chapter 3.2, E2 concentrations showed a sig. influence on PC-contents-Baseline-corrected (nmol/mg).

Tested with a general linear model, subjects in the lowest E2 category had sig. lower PC-contents-Baseline-corrected reductions ($p < 0,05$), as subjects in higher categories (see Table 14 & Figure 24).

Table 14: Effects of E2 categories on PC-contents-Baseline-corrected (nmol/mg) (own illustration, 2023)

	E2-conc. (pg/mL)	PC-content (nmol/mg)	p-value
Q1	< 22	Reference	-
Q2	22-59	-0,039±0,0197	p = 0,046
Q3	59 – 112,75	-0,065±0,0197	p < 0,001
Q4	>= 112,75	0,182±0,0196	p = 0,351 (n.s.)

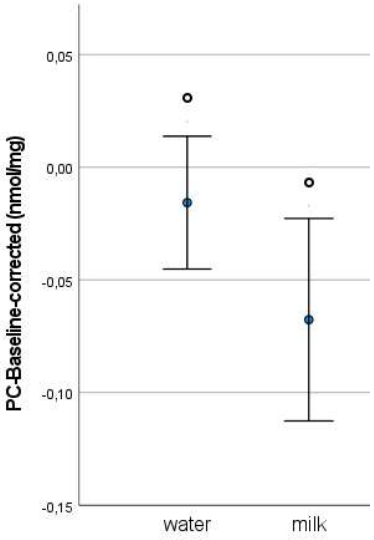
Figure 24: E2 categories differences in PC-contents-Baseline-corrected (° = p<0,05; * = p <0,01) (own illustration using IBM® SPSS® Statistics 27, 2023)



3.5.3. Beverage effect

After a general linear model was calculated, subjects in the milk group tended to have overall higher PC-content-reductions (nmol/mg) to baseline (-0,052±0,0264, p = 0,05) than the water group. This effect was considered being statistically sig. (p = 0,05) (see Figure 25).

Figure 25: PC-contents-Baseline-corrected consuming milk in comparison to water (° = p = 0,05) (own illustration using IBM® SPSS® Statistics 27, 2023)



3.6. Interactions in a general linear model

The milk group showed lower PC-contents-Baseline-corrected (nmol/mg) compared to the water group at every point of time, unfortunately none were statistically sig. in a general linear model (0_post-HIIT -0,056±0,143, p = 0,697; 15_post-HIIT -0,128±0,143, p = 0,372).

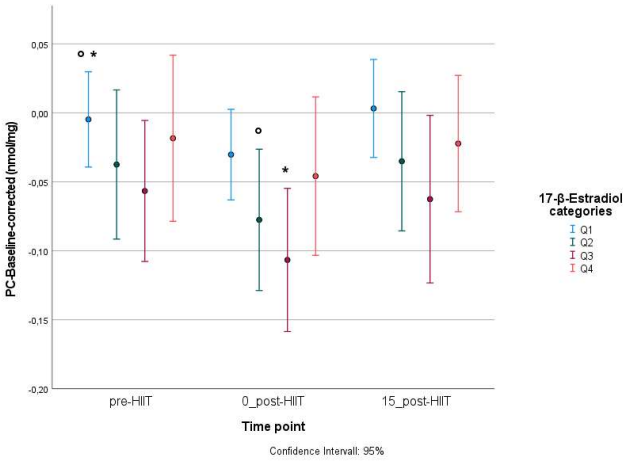
Only an almost sig difference between water_pre-HIIT and milk_0_post-HIIT (-0,077±0,046, p = 0,094) and as well as milk_15_post-HIIT (-0,08±0,046, p = 0,082) could be documented (see Figure 27). However, performing a rmANOVA found also no sig. effect (see chapter 3.7).

Adding E2 categories to the general linear model calculation did not change that (p > 0,05).

Therefore, an additional protective effect of E2 to milk consumption on PC-contents generation in a HIIT model could not be confirmed.

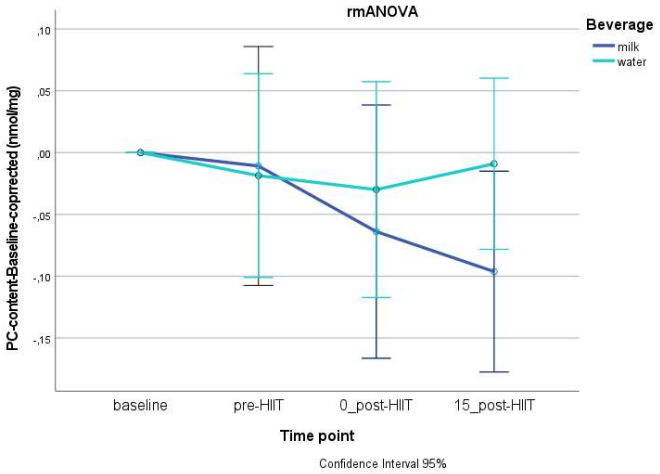
However, when E2 categories interactions were tested without the acute impact of the beverages, the lowest group (Q1) showed sig. higher PC-contents at pre-HIIT as Q2 at 0_post-HIIT (+0,073±0,034, p < 0,05) and Q3 at 0_post-HIIT (+0,102±0,034, p < 0,01) in a general linear model (see Figure 26). Therefore, an additive protective mechanism of E2 on PC generation in this HIIT model could be assumed.

Figure 26: PC-Baseline-corrected interactions: timepoint x E2quarters (° = p<0,05; * = p <0,01)
(own illustration using IBM® SPSS® Statistics 27, 2023)



3.7. Repeated measurements ANOVA

Figure 27: rmANOVA PC-contents-Baseline-corrected
(own illustration using IBM® SPSS® Statistics 27, 2023)



When rmANOVA was calculated, no sig. (p > 0,05) effect on PC-contents-Baseline-corrected (nmol/mg), of the milk group in comparison to the water group, at different time points was found (see Figure 27).

The mean difference of PC-contents-Baseline-corrected from milk to water was at pre-HIIT -0,011 nmol/mg (±0,046; p = 0,83), at 0_post-HIIT -0,034 nmol/mg (±0,045; p = 0,448) and finally at 15_post-HIIT -0,087 nmol/mg (±0,046; p = 0,065). However, all these negative tendencies were not statistically sig.

Especially 15_post-HIIT seemed to be close to a sig. result, therefore an independent paired t-test was performed to test this hypothesis.

Unfortunately, performed independent paired t-test did not detect statistical significance, as mean difference from milk to water group was -0,087 ±0,21 with a p-value of 0,082.

Taking this together is must be assumed that, the protective effect of acute milk consumption in comparison to water in a HIIT model in untrained adult and healthy women could not be confirmed.

4. Discussion

4.1. Protein carbonyl content assessment

After 30 minutes of HIIT, the PC-contents (nmol/mg) were sig. reduced immediately ($p < 0,01$) and after 15 minutes ($p < 0,05$) of resting, compared to baseline, in 20 healthy untrained adult women (see Figure 23).

Normally, intense PE leads to an increase in PC-contents. (Wadley et al., 2016) However, a decrease in plasma carbonyls after following a training protocol may occur because of suboptimal training intensity and duration, training adaption of study subjects, deficient plasma carbonyls bioavailability and endogenous defence mechanisms. (Wadley et al., 2016)

Optimal BORG-scale in applied HIIT model to adjust the trainings intensity was from 15 to 19 according to Zeng et al. (2020) and Bizheh and Jaafari (2011) (see chapter 2.3.1).

A BORG-scale from 15 to 19 is approximately 80 to 100% on an effort scale (Conconi test). (Guidi et al., 2011) Unfortunately, Gjestvang et al. (2017) found an overestimation in subjectively estimated maximal oxygen uptake (VO_{2max}) when BORG-scale was used in 125 healthy untrained subjects. This seems relevant, as VO_{2max} is the most common and valid measurement of cardiorespiratory fitness. (Evans et al., 2015)

However, Norton et al. (2010) defined a BORG-scale from14 to 16 to be equivalent to 60-85% VO_{2max} and therefore can be classified as “vigorous training intensity”. Additionally, BORG-scale greater than 17 is related to >85% VO_{2max} and therefore associated with “high intense” PE. (Norton et al., 2010)

Taking this together, it seems that using BORG-scale as a single parameter is probably not precise enough for the exercise intensity assessment and therefore could have led to non-sufficient plasma carbonyls generation in applied training protocol.

Additionally, training duration seemed to have an influence on plasma carbonyls secretion too.

Bloomer et al. (2007) found greater PC-contents when eight men performed a cycling protocol for 120 than 60 minutes. However, 30 minutes of resistance training led to sig. ($p < 0,05$) rises in PC-contents (µmol/µg) immediately after training and after 60 minutes of resting in seven resistance trained men. (Goldfarb et al., 2008) Therefore, 30 minutes of HIIT should be adequate to generate sufficient PC levels.

Moreover, when the same HIIT was performed seven times, plus two technical training sessions (see chapter 2.3.1), this may had led to subject training adaption in applied protocol. (Wadley et al., 2016) However, the trainings intensity was increased by 5% each time a subject completed at least 3x 20 repetitions of an exercise to avoid training adaptation as much as possible (see chapter 2.3.1).

To generate sufficient PC-contents, training stimulus must be novel to study subjects, as presented by Bloomer et al. (2007). When Bloomer et al. (2005) performed a cycling protocol there was a sig. ($p <0,05$) increase in PC-contents in ten cross-trained men, but in 2007th the same men had been more resistant.

However, the applied inclusion criteria for this trial focused on untrained female individuals (see Table 3), with minimum seven days of resting (wash-out phase) between study cycles. Hereby trying to avoid a training-resistance-effect, as muscle hypertrophy occurs, depending on the muscle type, with minimum two repetitions a week. (Wernbom et al., 2007) Anyway, it seems unclear, but very likely, that subjects did not adapt to study protocol over all seven study cycles.

Furthermore, the time point of blood sampling can be also relevant to discover successful PC-increase. (Wadley et al., 2016)

When Wadley et al. (2014) performed a high intense (70%) 30 to 40 minutes cycling training in twelve untrained patients with rheumatoid arthritis, they discovered a peak 30 minutes after training and not immediately after completed exercise.

Additionally, Michailidis et al. (2007) found a PC-content-peak after four hours of resting, when in eleven untrained males followed a 45 minutes intense (70%) treadmill exercise.

In Figure 27 a higher increase in PC-contents after 15 minutes of resting of the water group can be assumed. This effect was not statistically sig. (+0,087±0,21, p=0,082) applying an independent t-test. However, it would be therefore interesting to extend the study design up to four hours (0, 15, 30, 60, 120, 240 minutes post-HIIT) in order to be able to detect all the possible carbonyls formed.

However, this would lead to increased blood sampling and might increase the risk of dropouts if more than one or two study days are performed.

Besides that, considering endogenous defence mechanisms leading to PC clearance may be also helpful to interpret a decrease in PC-contents after PE. (Wadley et al., 2016)

The proteasome system is a collection of proteins located in all cell types to degrade irreversibly modified proteins, as plasma carbonyls. The ubiquitin-independent 20S proteasome and the 11S proteasome facilities seem to be the primary systems to degrade oxidatively damaged proteins. (Jung & Grune, 2008; Jung et al., 2014) They are found in plasma and are enzymatically functional, which poses that they cleave PC-groups in plasma directly. (Egerer et al., 2002; Hoffmann et al., 2011; Wada et al., 1993; Zoeger et al., 2006)

After resistance training the activity of ubiquitin-independent 20S proteasome gene expression is increased. (Nedergaard et al., 2007) The expression may be decreased within training duration, leading therefore to higher PC-formation, due to ROS-induced inactivation. (Radak et al., 2005) The mechanism behind an inactivation of protease activities by ROS is yet unknown (Wadley et al., 2016), however, looking over 11S and 20S proteosome gene expression in applied study design may explain received PC-content reducing effect.

In addition to this, exercise-induced shifts in plasma protein concentrations must be taken into account at every blood sampling time point, as some proteins (especially serotransferrin and fibrinogen) are more susceptible to exercise-induced OS than others. (Guidi et al., 2011) This influence was considered, as protein concentration (mg/mL) was measured at every time point as descripted in chapter 2.3.5.

Nevertheless, the most abundant plasma protein, albumin, had shown to exhibit a high concentration of PC-groups. (Madian & Regnier, 2010) Lamprecht et al. (2008) pose that, at training intensities of 80% (approximately at BORG-scale about 16 (Norton et al., 2010) or higher are needed to generate successful carbonylation of albumin. Moreover, exercise-induced proteinuria may also affect the amount of present protein to be oxidized. (Junglee et al., 2012) Therefore, trails aiming to assess the effect of OS due to PE in the future should also measure proteinuria at every blood sampling.

Finally, the uptake and turnover of proteins in muscle cells during resistance training (especially albumin and fibrinogen) may also lead to a protein availability shift for oxidation. (Sheffield-Moore et al., 2005) This is an explanation of a possible protecting effect of carbonylation in the milk group (see Figure 27), as milk is rich in proteins (Muskat et al., 2021), leading to lower endogenous protein oxidation during resistance training than water. (Sheffield-Moore et al., 2005) However, this effect was not statistically sig. in this trial applying an independent t-test 15 minutes after resting (PC-contents-Baseline-corrected -0,087 ±0,21 nmol/mg, p=0,082).

In conclusion the spectrophotometrically assessing protocol by Levine et al. (1990) is attributed being very cost efficient but on the other hand work-intensive, time-consuming and unusual for high throughput measurements. (Kehm et al., 2021)

Besides that, acid-soluble proteins may get lost during washing steps, therefore about 10% to 15% must be reconsidered during PC-content calculation. (Colombo et al., 2016)

Moreover, additional carbonyl groups may occur because of acid conditions during the protocol. (Kehm et al., 2021) Additionally, DNPH may be trapped in the protein pellet, hence resolubilization may be incomplete, leading to falsified results. (Rogowska-Wrzesinska et al., 2014)

Finally, since there is no validated standard for plasma carbonyls, it is difficult to compare generated data with results obtained in other laboratories. (Weber et al., 2015)

The most accurate and informative technique to assess protein characteristics, as carbonylation status, seem to be mass spectrometric methods. (Kehm et al., 2021) Besides of being the gold standard, Kehm et al. (2021) pose that, in the future new oxidation products in the field may be discovered using mass spectrometric methods.

Moreover, trials with the purpose to assess the oxidative response of PE should focus, besides PC-contents, also on other biomarkers of OS, as cellular markers of OS (e.g. plasma lipid hydroperoxides and total antioxidant capacity) can be elevated and PC-contents are still decreased as descripted. (Wadley et al., 2015)

4.2. 17-β-Estradiol categories

Applied E2 categories showed a sig. influence of PC-content-reduction compared to baseline, independent of the time of blood sampling (see Figure 24).

Subjects in the lowest category (Q1, serum E2 < 22pg/mL) showed sig. less PC-content reduction than females in category 2 (Q2, serum E2 22-59 pg/mL, $p < 0,01$) and category 3 (Q3, serum E2 50-112,75 pg/mL, $p < 0,05$). Category 4 (Q4, serum E2 > 112,75 pg/mL, $p = 351$) showed no sig. difference from Q1 (see Figure 24).

This may occur because of E2 availability shift during each MC-phase, as P4 expression supresses E2 promotion. (Haines et al., 2018)

Therefore, studies considering the possible antioxidative effect of E2 in female subjects should include more parameters regarding MC-phase-verification.

As seen in Figure 7, during the MC steroid hormones as E2 and P4 rise, peak, and drop depending on each cycle phase. However, to verify MC-phases by biomarkers it is essential to include the retrospective monitoring of cycle length, as physiological MC tend to last from 21 to 35 days (Leburn et al., 1995), the urine LH concentration, to confirm ovulation (Peinado et al., 2021) and the highest rise of E2 in blood serum.

The peak of E2 is considered to start at 150 pg/mL. (Roberts et al., 2008) Unfortunately, included study subjects did mainly have E2 concentrations lower to 150 pg/mL (see Figure 20). Therefore, study quarters were used for E2 categorisation (see Figure 21).

A reconsideration of MC-phase was also not possible, as LP can only be confirmed with at least 5 ng/mL of P4. (Stricker et al., 2006) Furthermore, the average individual cycle length was not known at screening. Additionally, OV, as a phase characterized with peaking E2, could only be approved using LH as biomarker. Therefore, for future investigations individual average MC length at screening and P4 and LH must be analysed next to E2 at every study cycle. (Stricker et al., 2006)

Considering the MC-phase by using valid parameters and biomarkers may be relevant in applied protocol.

Mackay et al. (2019) found a faster recovery of high intense (90%) 30 min. cycling in ten non OC using females in their FP than OV. On the other hand, Sipavičienė et al. (2013) discovered an opposite effect in 18 trained women, as subjects in OV returned faster to baseline parameters after stretch-shortening exercise than individuals in FP. Surprisingly McKinley-Barnard et al. (2018) found increased SOD as well as tumor necrosis factor α (TNFα) concentrations at MLP in 22 physically active women, after an eccentric resistance model.

Taking this together, it seems clear that for future research MC-phases need to be considered in trials wishing to asses OS response of PE in females.

E2 content variation during MC-phases is clinically relevant as women using OC have higher levels of OS and inflammatory markers when performing PE than women who are OC-non-users. (Oosthuyse et al., 2022) An exclusion or separate statistical calculation of OC users seem therefore to be logical.

Considering E2-concentrations interactions with time point of blood sampling, showed also sig. results (see Figure 26). Subjects in the lowest category had sig.

higher PC-contents-corrected to baseline before (pre-HIIT) than individuals in Q2 (+0,073±0,034, $p < 0,05$) and Q3 (+0,102±0,034, $p < 0,01$) immediately after HIIT (0_post-HIIT). This could occur because of the ability of E2 to quench directly generated ROS (Kendall & Eston, 2002; Oosthuyse et al., 2022) and leading herby to greater endogenous defence mechanisms, as protease system activation. (Radak et al., 2005)

The acute consumption of 250mL skimmed milk in comparison to water, however, had no sig. ($p > 0,05$) influence on PC-content-Baseline-corrected (nmol/mg) at every point of time. Considering an E2 category cut-off in the calculation did not change that ($p > 0,05$). Therefore, an additive antioxidative effect of E2 could not be confirmed in this trial.

Nevertheless, circulating steroid hormones are linked to a higher risk of breast, endometrial, and ovarian cancers in women (Chuffa et al., 2017), suggesting herby a preferable reducing effect through dietary interventions. (Chuffa et al., 2017; Nimptsch & Pischon, 2016)

In a retrospective cohort study, using data from “Nurses’ Health Study” and “Health Professionals Follow-Up Study” (Colditz et al., 1997; Hunter et al., 1997; Rimm et al., 1991), Hang et al. (2019) found a sig. ($p < 0,01$) reduction of E2, CRP and IL-6 concentrations when subjects consumed four or more cups of coffee a day. Hang et al. (2019) discussed that coffee is rich in chlorogenic acids, herby reducing inflammatory mediators’ production. (Grosso et al., 2017)

However, Décarie-Spain et al. (2021) found in female mice (C57Bl6N) after a 24-week high fat diet, primarily consisting of palm oil , which is rich in SFA, sig. ($p < 0,05$) higher concentrations of E2. Décarie-Spain et al. (2021) pose that E2 protected the neuroimmune system of the mice by dampen pro-inflammatory signalling in astrocytes and microglia and by anti-inflammatory-cytokine-secretion in T-cell-lymphocytes. (Décarie-Spain et al., 2021; Straub, 2007)

In addition to this, McKinley-Barnard et al. (2018) tested the impact of 6g fish oil per day for 21 days, in an eccentric exercise resistance training model in 22 physically active females, considering their MC-phases. Surprisingly subjects in MFP had sig. ($p < 0,01$) lower E2 contents, but higher SOD and TNF-α concentrations than individuals in MLP. Interestingly the fish oil group had higher TNFα levels in MF-phase than fish oil group in ML-phase, suggesting that E2 attenuates inflammatory response. (Strehlow et al., 2003) However, E2 showed no additive anti-inflammatory effect with fish oil supplementation. (McKinley-Barnard et al., 2018)

Taking this together it seems clear, that more trials which aim at explaining the impact of specific nutrients on OS and the possible additive antioxidative effect of MC-phases and E2 need to be conducted in the future to get a better insight of possible mechanisms explaining these results.

4.3. Impact of dairy on oxidative stress and inflammation

The acute impact of 250mL skimmed milk showed no sig. time specific effect on PC-content generation (pre-HIIT -0,011 ±0,046, p = 0,83; 0_post-HIIT -0,034±0,045, p = 0,448; 15_post-HIIT -0,087±0,046, p = 0,065) in comparison to water as a control after 30 minutes of HIIT in untrained healthy adult women.

Rankin et al. (2018) found the same not changing effect in PC-content when ten trained women consumed 500mL skimmed milk in a muscle function and cycling protocol, in comparison to an isocaloric carbohydrate control.

However, the acute milk consumption decreased PC-contents independent of time points (see Figure 25) in this trial, suggesting hereby an autogenous protection of protein oxidation during HIIT (Sheffield-Moore et al., 2005).

Nevertheless, many bioactive peptides from animal sources emerge during fermentation. Therefore it seems not surprising that cheese or yoghurt indeed showed antioxidative capacities on OS. (Toldrá et al., 2020)

Huma et al. (2018) found sig. ($p < 0,05$) decreased intracellular ROS production in vivo in immortalized cell lines of human colorectal adenocarcinoma after incubation with water-soluble peptides from cheddar cheese.

In addition to this, Aloğlu and Oner (2011) showed sig. ($p < 0,05$) increased antioxidative capacity of isolated peptides form yoghurt in vitro too, using 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid and 2,2-diphenyl-1-picrylhydrazyl methods.

Moreover, when Barboza et al. (2018) applicated kefir (0,3mL per 100g of body weight) in male swiss mice, they had sig. ($p < 0,01$) lower indomethacin induced gastric ulcer attenuation. The authors discussed that, produced NO, by enhanced NOS expression, leads to increased blood flow in the mucosa, herby regulating mucus production and inhibition of neutrophil attachment to endothelial cells. This may also lead to ROS reduction. (Barboza et al., 2018)

Additionally reduced ROS generation and endogenous antioxidative systems improvement (such as SOD, GPX and CAT), may be enhanced by kefir beverage and its isolated microorganisms. (Tang et al., 2018; Zhang et al., 2017)

Especially PC-contents (nmol/mg) had been sig. ($p < 0,01$) reduced when El Golli-Bennour et al. (2019) applicated kefir (25mg/kg bodyweight) in male Wister Albino rats prior or with deltamethrin intoxication. Furthermore, other markers of OS, such as malondialdehyde, CAT and SOD had been sig. (< 0,05) positively influenced with kefir application. Suggesting herby another antioxidative effect of kefir, as a fermented beverage product which is high in symbiotic microbes with antioxidant compounds. (El Golli-Bennour et al., 2019)

For example, contained Lactobacillus fermentum ME-3 can reduce oxidative damage by using its own SOD. (Kullisaar et al., 2002; Songisepp et al., 2004) Additionally, ROS can also be scavenged by other strains form lactobacilli and bifidobacteria. (Savini et al., 2010; Wang et al., 2006) Finally, contained AO, as VE, VC and VA, apply a synergistic effect on these antioxidative mechanisms. (Satir & Guzel-Seydim, 2016; Yilmaz-Ersan et al., 2018)

This anti-inflammatory properties were already confirmed in human trials, when O'Brien et al. (2015) tested a long term (15 weeks) consumption of a 454g fermented-milk (kefir)-beverage in comparison to milk in an endurance training model in 67 healthy individuals. O'Brien et al. (2015) found a sig. ($p < 0,05$) decrease in CRP and pose that kefir may have a greater impact on gastrointestinal health maintenance, reduced susceptibility of illness (Gleeson et al., 2011), enhanced resistance to upper-respiratory-tract-infections (Cox et al., 2010) and reduced gut permeability (Lamprecht et al., 2012).

Furthermore, Pei et al. (2017) found in 120 normal weight and obese females a sig. ($p < 0,05$) greater reduction in IL-6 and TNFα after nine weeks of 339g low-fat yogurt (Lactobacillus bulgaricus, Streptococcus thermophilus) in comparison to nine weeks of soy-pudding consumption. Additionally, Immunoglobulin M endotoxin-core antibody, as a protective marker of intestinal barrier and endotoxemia (Poxton, 1995), was sig. ($p < 0,01$) increased after yoghurt consumption, suggesting hereby decreased endotoxin levels.

Moreover, Burton et al. (2017) found a sig. ($p < 0,01$) decrease in IL-6 and TNFα in 14 healthy men after two weeks of 400g per day of probiotic yoghurt (Lactobacillus rhamnosus GG (LGG), Lactobacillus delbrueckii spp.bulgaricus and Streptococcus thermophilus) consumption compared to baseline in a cross over study with a high fat meal test. These findings agree with Kekkonen et al. (2008), as a three week consumption of fermented milk with LGG, showed a sig. ($p < 0,01$) higher reduction in hs-CRP in 62 healthy volunteers. Both authors pose that, LGG has a role in modulating cytokine expression and response and therefore an anti-inflammatory potential. (Burton et al., 2017; Kekkonen et al., 2008)

The acute impact of 1100mL skimmed milk compared to an isocaloric carbohydrate control on inflammation in 13 healthy untrained women was already tested by Fraschetti et al. (2022) using a high-intensity exercise protocol, consisting of plyometric and resistance exercise. The authors found a sig. reducing effect of IL-6 ($p < 0,05$) at every time point and IL-10 ($p < 0,05$) 15 minutes post-exercise. Fraschetti et al. (2022) discussed that beneficial amino-acids, as cysteine, histidine and glycine, may reduce TNFα production in monocytes and therefore act anti-inflammatory. (Hasegawa et al., 2011)

Finally, diets high in dairy (over three servings a day) seem to have an anti-inflammatory effect in twelve to 24 weeks observational studies in obese subjects. (Zemel, Richards, Mathis et al., 2005; Zemel, Richards, Milstead, & Campbell, 2005; Zemel & Sun, 2008)

A 2020 published meta-analysis agreed on these anti-inflammatory properties of dairy in adults (CRP, TNF-α, IL-6). (Moosavian et al., 2020)

Moosavian et al. (2020) pose that this may occur due to the beneficial effect of calcium on body fat mass, as adipose tissue is an active endocrine organ which secrets adipokines and proinflammatory cytokines (Elwood et al., 2004; Ridker et al., 2000) and Monocyte chemotactic protein-1 suppression. (Sun & Zemel, 2007; van Meijl & Mensink, 2010)

Additionally, leucin, as amino-acid rich in dairy (Moosavian et al., 2020), increases anti-inflammatory adiponectin expression and decreases proinflammatory cytokines. (Zhang et al., 2020) Furthermore, leucin seems to reduce OS by stimulation of O_2 consumption in mitochondria, fatty acids oxidation in adipocytes

and skeletal muscle cells and suppression of protein degeneration. (Stipanuk, 2007; Stobiecka et al., 2022; Sun & Zemel, 2009)

In sports nutrition milk might be the optimal sports drink for exercise regeneration, as its supports muscle protein synthesis, rehydration and glycogen resynthesis, therefore leading to possible reduced muscle damage. (James et al., 2019) Kefir seems to be even more beneficial as it is easier to digest, due to its lower content of osmotic lactose. (O'Brien et al. 2015) Thomas et al. (2016) recommends 15 to 25g of protein immediately or two hours after PE to improve muscle protein synthesis. This effect may be improved by E2 as it may supports Myo-D expression, leading to adult myocyte differentiation while muscle regeneration (see chapter 1.3.21.3).

However, this trial did not find a supportive effect of PC-content generation by E2 concentration when subjects drank milk in comparison to water (see chapter 3.6).

In conclusion, fermented dairy seems to have a higher impact on anti-inflammatory and antioxidant properties and future investigations wishing to explore the acute effect of dairy on OS in PE should therefore preferably use kefir or yoghurt as interventional food instead of milk in comparison to an isocaloric carbohydrate control.

4.4. Future aspects

To the best of the authors knowledge, this is the first cross-over randomised controlled trial to investigate the acute impact of 250mL skimmed milk on PC generation performing a HIIT-model in healthy untrained adult women, considering E2 concentrations.

The trials strengths lay in the cross over design, as subjects drank every beverage and therefore act as their very own control.

Additionally, study design was conducted with multidisciplinary health professionals, assuring the highest quality of intervention implementation.

However, to explore PC-contents a mass spectrometry seems to be the gold standard, as it is not as susceptible to interference as the spectrophotometric assay. (Kehm et al., 2021)

Moreover, trials aiming to assess MC with valid biomarkers should include more parameters than E2 as a single. (Romero-Parra et al., 2021)

Furthermore, subjects need to be distinguished in OC- or no-OC-users. (Oosthuyse et al., 2022)

Finally, homiletical blood samples should better not be further processed, as this could lead to falsified results. (Wan Azman et al., 2019)

5. Conclusion

This master thesis aimed at investigating the acute impact of skimmed milk on plasma carbonyls and the possible additive antioxidative effect of E2 in healthy untrained adult women performing a HIIT.

250mL of skimmed milk had a sig. ($p < 0,05$) overall protective effect of PC-content generation, independent of the time, in 20 healthy untrained adult women after a HIIT. E2 concentrations showed no sig. ($p > 0,05$) additive antioxidative effect with milk consumption on PC-generation, however, subjects with the lowest E2 concentrations showed sig. ($p < 0,05$) higher protein oxidation before HIIT than individuals with higher concentrations immediately after HIIT, independent on beverage consumption.

Plasma carbonyls are irreversible biomarkers of OS. (Colombo et al., 2016; Kehm et al., 2021) In order to be able to make a clear statement on PC-contents variations, plasma carbonyls need to be related to protein concentration. (Colombo et al., 2016; Guidi et al., 2011) Additionally, PC generation can occur or peak after a specific time of resting too, therefore blood sampling should include as least four hours: 0, 15, 30, 60, 120, 240 minutes post-exercise (Michailidis et al., 2007). Leading herby to possible more study drop outs, due to the high number of blood sampling required. Furthermore, proteinuria should be assessed as well, as autooxidation of proteins occur when subjects perform PE fasting. (Wadley et al., 2016) In addition to this, ubiquitin-independent 20S proteasome and the 11S proteasome facilities expression need to be investigated additionally. (Nedergaard et al., 2007; Wadley et al., 2016) To get a clearer insight of redox homeostasis in study subjects' other biomarkers of OS should be explored also. (Wadley et al., 2016) Finally, to assess sufficient HIIT intensity VO_{2max} should be included next to BORG-scale. (Evans et al., 2015; Gjestvang et al., 2017; Norton et al., 2010)

E2 has a possible antioxidative effect (Kendall & Eston, 2002; Oosthuyse et al., 2022), nevertheless higher concentrations are associated with cancer. (Chuffa et al., 2017; Nimptsch & Pischon, 2016) In this trial E2 showed a PC-content-reducing effect, however MC-phase validation was not possible. To interpret study subjects' individual MC-phases in the future, retrospective monitoring of cycle length, urine LH and serum E2 and P4 should be assessed at every study cycle. (Romero-Parra et al., 2021)

And finally, there exists emerging evidence that fermented dairy seems to have a strong influence on inflammation and redox homeostasis. (Burton et al., 2017; El Golli-Bennour et al., 2019; O'Brien et al., 2015; Pei et al., 2017; Toldrá et al., 2020)

Further investigations should focus on dairy, preferably fermented with LGG (Burton et al., 2017; Kekkonen et al., 2008), and its acute impact on OS.

In this trial an acute reducing impact of 250mL skimmed milk of PC-content-generation in healthy untrained adult women performing a HIIT was not found immediately and 15 minutes after training in comparison to water. However literature shows that 339 to 1100mL (Burton et al., 2017; Fraschetti et al., 2022; O'Brien et al., 2015; Pei et al., 2017) of kefir (Toldrá et al., 2020) compared to an isocaloric carbohydrate control (Fraschetti et al., 2022; Rankin et al., 2018) would be therefore a more promising approach for this research question.

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Appendix

Abstract written in German (“Zusammenfassung”)

Einleitung

Zu intensives Training kann zu oxidativem Stress und Muskelschädigungen führen. 17-β-Estradiol scheint einen potentiellen antioxidativen Effekt zu haben. Aktuelle Daten schlagen außerdem den antioxidativen Effekt von Milchprodukten vor. Das Ziel dieser Masterarbeit ist es daher, den akuten Einfluss von 250mL fettarmer Milch auf die Bildung von Proteincarbonylen bei untrainierten jungen gesunden Frauen zu untersuchen.

Methodik

Um eine akute Stressreaktion bei untrainierten jungen gesunden Frauen auszulösen, sollten die Probandinnen ein hoch-intensives-Intervall-Training durchführen. Zwei Stunden vor Training wurde ihnen Wasser oder Milch, im cross-over-Design, gegeben. 17-β-Estradiol wurde mit einem Standard-Kit untersucht und Proteincarbonyle mit einer adaptieren Spektralphotometer-Methode gemessen. Die statistische Auswertung wurde mit SPSS(V27) durchgeführt und $p < 0,05$ als statistische Signifikanz erachtet.

Ergebnisse

Der Konsum von 250mL fettarmer Milch führte zu einem signifikanten ($p < 0,05$) schützenden Effekt vor Proteincarbonylbildung (-0,052±0,0264, $p = 0,05$). 17-β-Estradiol hatte keinen zusätzlichen Effekt. Allerdings zeigte sich eine höhere Proteinoxidation bei Frauen mit niedrigen 17-β-Estradiol im Vergleich zu jenem mit höherer Konzentration (Q2: -0,073±0,034, $p < 0,05$; Q3: -0,102. ±0,034, $p < 0,05$).

Diskussion

Proteincarbonyle sind sehr anfällig für autogene Schutzmechanismen und sollten daher nicht als Einzelparameter gemessen werden. Außerdem, scheint die Bioverfügbarkeit von Carbonylen bis zu vier Stunden anzudauern. Zusätzliche Parameter zu 17-β-Estradiol sind außerdem notwendig um eine Aussage über den Einfluss des Menstruationszyklus zu machen. Für zukünftige Forschungsarbeiten erscheinen fermentierte Milchprodukte vielversprechender, da sie einen stärkeren Effekt auf die Redox-Homöostase haben.

Schlüsselwörter

Oxidativer Stress; Menstruationszyklus; Milchprodukte

Protein carbonyl protocol

Protein Carbonyle (nach Levine et al. 1990, adaptiert von SigmaAldrich®)

Reagenzien

- 10 mM DNPH (2,4-Dinitrophenyl Hydrazin) in 2M HCl Lösung
- 87% TCA, auf Eis
- 6M-Guanidin-in 0,5M Kalium-Phosphat Lösung, RT
- Aceton, -20°C
- 96-Well-Clear-Plate (UV)

- Proteinkonzentration mit extra Methode (z.B. Lowry Methode nach Lowry et al. 1951)

1. **87% TCA:**

   8,7g TCA (s) in 10ml H_2O
   mind. 1 Monat haltbar bei Raumtemperatur (RT)

- bereitstellen: 10ml Messkolben, 1 Stopfen, 1 Trichter, 1 mittleres Becherglas
- Menge in Becherglas einwiegen und beschriften
- Becherglas + TCA mit etwa 2 mL Aqua dest. auffüllen und mit Magnetstab (etwa 500 T/Min.) rühren lassen
  Magnetstab rausgeben und über Becherglas mit Aq.dest abspülen
  Achtung: TCA gewinnt an Volumen nach Lösvorgang
- Lösung in den 10mL Kolben leeren (Becherglas mit Spritzflache ausspülen)
  mit Aqua dest. auffüllen und beschriften
  Beschriftung: 20% TCA + Kürzel + Datum
  in Kühlschrank lagern

2. **2M-HCl:**

   72,92ml in 1000 mL (100%ige)
   197,08ml in 1000 mL (37%ige) H_2O
   19,7mL (37%ige) in 100 mL H_2O
   3 Monate haltbar bei RT

- bereitstellen: 100 mL Messkolben, 1 Stopfen, 1 Trichter, 50ml Messzylinder
- H_2O im Messkolben vorlegen (etwa 60mL)
- HCl in Messzylinder abmessen (doppeltbeschichtete Handschuhe + im Abzug!)
- in Messkolben überführen und mit Aqua dest. auf 100 mL auffüllen
- schwenken (bis Lösung klar ist)
- Beschriften: 2MHCL + Kürzel + Datum (+ evtl. MHD)
- in Kühlschrank geben (Achtung: Säure verliert bei Kälte Volumen, nicht nachadjustieren wenn 1L abgemessen worden ist!)

3. **2,4-Dinitrophenyl Hydrazin (in 2M-HCl):**
   2g DNPH in 1L 2M-HCl
   0,4 g DNPH in 100mL 2M-HCl
   0,2 g DNPH in 50mL 2M-HCl
   7 Tage haltbar im Dunkeln und RT; Nicht einfrieren! Nicht kühlen! (Auskristallisationsgefahr!)

- bereitstellen: Schraubglas(dunkel), 100ml Messzylinder, Alufolie
- 0,4g DNPH direkt in Schraubglas einwiegen
  (VORSICHTIG im Abzug arbeiten! Kein Licht einschalten!)
  Glas beschriften (Etikettiergerät: DNPH in 2M-HCl + Kürzel + Datum (+ evtl. MHD))
- Schraubglas + DNPH mit 100ml 2 M HCl auffüllen
  mit Magnetstab im Abzug rühren lassen
  (~2-3 Stunden, besser über Nacht im Dunkeln (Alufolie))
- Magnetstab rausgeben (NICHT abspülen)
- Im Säure-Kasten mit Alufolie ummantelt lagern

4. **6M-Guanidin in 0,5M - KH_2PO_4 (pH= 2,5) / Puffer:**

   0,5M-KH_2PO_4, 500 mL (pH 3,5):

   34,02g in 500ml H_2O

- bereitstellen: 500 ml Becherglas, Messkolben, Messzylinder
- 34,02g KH_2PO_4 (s) auf großer Waage ins Becherglas einwiegen
- In ~ 450 ml Aqua dest. Lösen mit Magnetrührer
- pH messen mit ph-Meter (CAVE: davor Kalibrieren!)
- eventuell auf pH 3,5 einstellen mit 2M HCl (Minipasteurpipette)
- In Messkolben überführen und auf 500 ml mit Aqua dest. Auffüllen
- Im Becherglas überführen noch einmal auf pH 3,5 überprüfen
  ggf. mit 2M HCl einstellen
- Beschriften: KH_2PO_4 + Kürzel + Datum + pH 3,5
- Jedes Mal vor Verwendung pH überprüfen

   6M-Guanidin:

   171,95g in 300ml 0,5M-KH_2PO_4

- bereitstellen: 500ml Schraubglas, 500ml Becherglas, 500ml Messzylinder
- 171,95g GndHCl (s) auf der großen Waage ins Becherglas einwiegen und beschriften
- Mit ~30 ml 0,5M KH_2PO_4 auffüllen und mit Magnetstab rühren lassen, bis sich alles gut löst und die Lösung klar ist (etwa 30 Minuten, Abhängig von KH2PO4 Menge, ggf. nachtitrieren)
- In großen Messzylinder überführen und mit 0,5M KH_2PO_4 auf ~290 ml auffüllen
- Im Messzylinder pH überprüfen
  Ggf. auf 2,5 einstellen mit 2M HCl (Minipasteurpipette)
- Auf 300 ml mit 0,5M KH_2PO_4 auffüllen
- Im Messzylinder pH überprüfen
  Ggf. auf 2,5 einstellen mit 2M HCl (Minipasteurpipette)
- Ins Schraubglas leeren, beschriften
  (6M-Guanidin in 0,5M- KH2PO4+ Kürzel + Datum + pH 2,5) und in Kühlschrank geben
- Puffer jede Woche auf Ziel-pH: 2,5 kontrollieren!

Durchführung für eine Platte

- Laborjounal: Datum, Mitarbeiter, Labortemperatur
- Aceton ca. 50mL in -20°C Freezer lagern, 1mL 87% TCA auf Eis geben
- Eisbox mit Eis auffüllen und Plasma hineinstellen
  Proben in der Hand erwärmen + kurz vortexen
  1,5mL Eppis vorbereiten und beschriften
- Plasma mit Aq. Dest. Verdünnen (blauer Combitip)
  (max. 0,5-2mg Protein/Tube, etwa 10g/L)
  1:10 Verdünnung: 10 µL Plasma + 90 µL Aq. Dest
- 100 µL DNPH zu jeder Probe im Abzug im Dunkeln zugeben, vortexen
- 10 Minuten bei RT im Dunkeln inkubieren
- 30µL der eiskalten 87% TCA zugeben, vortexen
- 5 Minuten auf Eis inkubieren
- Zentrifugieren I: 13000 g für 2 Minuten 4°C
- Supernatant vorsichtig mit 200µL Kolbenhubpipette entfernen
- 500µL eiskaltes Aceton zugeben (roter Combitip)
- Für 30 Sekunden in ein Ultraschallbad geben
- 5 Minuten bei -20°C inkubieren
- Zentrifugieren II: 13000 g für 2 Minuten 4°C
- Supernatant vorsichtig mit 500µL + 100µL Kolbenhubpipette entfernen
- 500µL eiskaltes Aceton zugeben
- Für 30 Sekunden in ein Ultraschallbad geben
- 5 Minuten bei -20°C inkubieren
- Zentrifugieren III: 13000 g für 2 Minuten 4°C
- Supernatant vorsichtig mit 500µL + 100µL Kolbenhubpipette entfernen
  Pellets sollten trocken sein
- 400µL 6M Guanidin zugeben
- Lösungsvorgang im Ultraschallbad vollenden
  CAVE: Wenn Pellets nicht gelöst sind, nochmals für 5 Sekunden im US-Bad beschallen.
  Sonst samples 15-30 Minuten bei 60°C inkubieren
- 100µL der Guandin-Pellet-Lösung vortexen und als Dreifachansatz mit „reverse pipetting" in well pipettieren
- 100µL Aq.dest. in extra-Well
  Pipettiervorlage:

PL.												
	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	H_2O	H_2O	Plasma 8	Plasma 8	Plasma 8	Plasma 16	Plasma 16	Plasma 16	Plasma 24	Plasma 24	Plasma 24
B	Plasma 1	Plasma 1	Plasma 1	Plasma 9	Plasma 9	Plasma 9	Plasma 17	Plasma 17	Plasma 17	Plasma 25	Plasma 25	Plasma 25
C	Plasma 2	Plasma 2	Plasma 2	Plasma 10	Plasma 10	Plasma 10	Plasma 18	Plasma 18	Plasma 18	Plasma 26	Plasma 26	Plasma 26
D	Plasma 3	Plasma 3	Plasma 3	Plasma 11	Plasma 11	Plasma 11	Plasma 19	Plasma 19	Plasma 19	Plasma 27	Plasma 27	Plasma 27
E	Plasma 4	Plasma 4	Plasma 4	Plasma 12	Plasma 12	Plasma 12	Plasma 20	Plasma 20	Plasma 20	Plasma 28	Plasma 28	Plasma 28
F	Plasma 5	Plasma 5	Plasma 5	Plasma 13	Plasma 13	Plasma 13	Plasma 21	Plasma 21	Plasma 21	Plasma 29	Plasma 29	Plasma 29
G	Plasma 6	Plasma 6	Plasma 6	Plasma 14	Plasma 14	Plasma 14	Plasma 22	Plasma 22	Plasma 22	Plasma 30	Plasma 30	Plasma 30
H	Plasma 7	Plasma 7	Plasma 7	Plasma 15	Plasma 15	Plasma 15	Plasma 23	Plasma 23	Plasma 23	Plasma 31	Plasma 31	Plasma 31

- Extinktionen bei 375nm messen

Berechnung

1. Blank = H_2O (A_{450}) von allen Extinktionen abziehen
2. Protein-Carbonyl-content berechnen:

$$V\,(cm^3) = A\,(cm^2) \times L\,(cm)$$

$$L = \frac{V(cm^3)}{A\,(\pi \times r^2)}$$

$$r = \frac{d}{2}$$

Durchmesser UV-Mikrowell-Platte (Greiner): 0,9 cm

Volumen UV-Mikrowell-Platte (Greiner): 100µL = 0,1 cm3

Unsere optische Wellenlänge daher:

$$L = \frac{V(cm^2)}{A\,(\pi \times r^2)} = \frac{0{,}1}{(\pi \times 0{,}45^{\,2})} = 0{,}3144\ \text{cm}$$

Lambeert'sches Gesetz:

$$E = \varepsilon\left(\frac{L}{\text{nmol} \times cm}\right) \times L\,(cm) \times c\left(\frac{nmol}{\text{L}}\right)$$

Unsere Formel für PC-content in nmol:

$$c\left(\frac{nmol}{\text{L}}\right) = \frac{E\ lt.\ ClarioStar}{\varepsilon\left(\frac{L}{\text{nmol} \times cm}\right) \times L\,(cm) = 22 \times 0{,}3144 = 6{,}9168}$$

Unsere Formel für PC-content in nmol/mg:

$$PC\left(\frac{nmol}{\text{mg}}\right) = c\left(\frac{nmol}{\text{L}}\right) + Protein\ nach\ LOWRY\left(\frac{mg}{mL}\right)$$

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Lowry Assay protocol

Lowry-Methode

Reagenzien

- Na_2CO_3
- NaOH
- $CuSO_4$
- $C_4H_2KNaO_6x4H_2O$
- Folin & Ciocalteu's phenol reagent
- BSA: Bovin Serum Albumin

1. Natriumcarbonat-Lösung (0,189 M in 0,1 M NaOH) herstellen

   Pro Liter: 20g Na_2CO_3 (molare Masse: 105,9888g/mol)
   4g NaOH (molare Masse: 39,997g/mol)

   Pro 100 mL 2g Na_2CO_3 (molare Masse: 105,9888g/mol)
   0,4g NaOH (molare Masse: 39,997g/mol)

   Pro 50mL 1g Na_2CO_3 (molare Masse: 105,9888g/mol)
   0,2g NaOH (molare Masse: 39,997g/mol)

   Herstellung: Einwaage in Wiegeschiffchen
   Schiffchen mit Aqua dest. Abspülen (~10mL)
   Na_2CO_3unter schwenken lösen
   NaOH separat in etwa 10mL lösen, Achtung: erwärmt sich und verliert an Volumen, zuerst auskühlen lassen bevor weitergearbeitet wird.
   Lösungen in 50mL Messkolben überführen und mit Aqua dest. Auffüllen.

2. Kupfersulfatlösung (0,04M) herstellen

   Pro 100mL: 0,65g $CuSO_4$ x$5H_2O$ (molare Masse: 249,68g/mol)
   Pro 50 mL: 0,325g $CuSO_4$ x$5H_2O$
   Pro 5mL: 0,0325g $CuSO_4$ x$5H_2O$

3. Kalium-Natrium-Tartrat-Lösung (0,07 M) herstellen

   Pro 100mL: 1,975g $C_4H_4KNaO_6x4H_2O$ (molare Masse: 282,23g/mol)
   Pro 50 mL: 0,9875g $C_4H_4KNaO_6x4H_2O$
   Pro 5mL: 0,09875g $C_4H_4KNaO_6x4H_2O$

   Herstellung K-Na-Tartrat und $CuSO_4$-Lösung:
   - Einwaage in Wiegeschiffchen
   - Überführen in Becherglas/Epi
   - Wiegeschiffchen mit 5mL Aqua dest. abspülen und unter ständigem vortexen lösen

4. Folinreagenz frisch herstellen (1:2)
   300mL Folin + 300mL Aq. dest, evtl. Bedarf berechnen
   Vorsicht: Vor UV-Licht unbedingt schützen, dunkle Gefäße oder Alufolie ggf. verwenden!

5. Proteinstandard (60 g/L) herstellen (= Stocklösung = SL)

- 6g Albumin werden in ein 100mL Becherglas überführt und in ca. 90mL Aqua dest. mit Hilfe eines Magnetrührers gelöst.
  Vorsicht: BSA darf nicht zu schnell geschwenkt werden. Denaturierungsgefahr! Max. 250/T empfehlenswert.
- Gelöstes BSA wird auf 100mL mit Aqua dest .im Messkolben aufgefüllt.
  Tipp: Mit Pasteurpipette arbeiten. Sonst Blasenbildung.
  Vorsicht: Albumin schäumt und ist verderblich, d.h. max. 2 Wochen gekühlt (-30°C) lagern.
  Idee: 250 mL herstellen und im -80°C Freezer lagern
  Aufgetaute SL nicht vortexen!

Verdünnungsreihe herstellen (Spitze spülen) + vortexen

Standard Nr.	c (mg/mL)	SL (µl)	H2O (µL)	Gesamtvolumen(µl)
SL	60			
7	4,5	75	925	1000
6	3	50	950	1000
5	2,4	40	960	1000
4	1,8	30	970	1000
3	1,5	25	975	1000
2	0,75	12,5	987,5	1000
1	0,45	7,5	992,5	1000

6. Gebrauchslösung herstellen
   - 100 mL Natriumcarbonat-Lösung
   - 1 mL Kupfersulfatlösung
   - 1 mL Kalium-Natrium-Tartrat-Lösung
   - Gut mischen und zur Seite stellen
     250 µL für 10 µL Probe verdünnt/Standard, Bedarf daher:
     1 Platte mit 96 wells => 24 mL pro Platte
     Beschriften: Gebrauchslösung Lowry, Kürzel, Datum + MHD
     jedes Mal frisch herstellen empfehlenswert

Herstellung 51mL Gebrauchslösung für zwei Platten:

- 500µL Kupfersulfatlösung
- 500µL Kalium-Natrium-Tartrat-Lösung
- In 50mL Messkolben von Natriumcarbonat-Lösung geben
- WICHITG: es sind jetzt mehr als 50mL in Messkolben!

Haltbarkeit Gebrauchslösung:
Im verschlossenen Gefäß bei RT 7 Tage, bei 4°C 1 Monat und bei -20°C 1 Jahr (Thomas, 2020).

7. Plasmaproben verdünnen (auf 400 µL):

1:40 Verdünnung 10 µL Plasma in 390 µL Aq.dest

Plasma vorlegen (gelbe Multipette), Wasser hinzu (blauer Combitip)

Durchführung

1. Plasmaproben in der Hand auftauen und auf Eis lagern
2. Plasma in 1,5 mL Epi verdünnen

   Plasma vorlegen (gelbe Multipette), Wasser hinzu (blauer Combitip)

   Gut vortexen
3. Verdünnungsreihe herstellen
4. Im 1,5 mL Epi Pipettierschema befolgen:

	STANDARD 1-6	PROBE	BLANK
STANDARD 1-6	40 µL		
PROBE		40 µL	
AQ. DEST			40 µL
GEBRAUCHSLÖSUNG MULTIPETTE, ROT	1000µL	1000µL	1000µL
INKUBATION		10 Min. bei RT im Dunkeln	
FOLIN 1:2 MULTIPETTE, BLAU	100µL	100µL	100µL
INKUBATION		35 in. bei RT im Dunkeln	

5. Mikroplatte (BRANDplates®) mit 200µL befüllen (Doppelansatz), reverse pipetting
6. Extinktionen bei 578 nm messen

Berechnung

- Linearer Regression der Standardgerade durchführen (R^2 beachten!)
- Blank bei A abziehen
- Konzentration (g/L) berechnen:

$$y\,(E) = k \times x\,\left(\frac{g}{L}\right) + d$$

$$x\,\left(\frac{g}{L}\right) = \frac{y\,(E) - d}{k}$$

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